British Journal of Pharmacology

December 1993

Volume 110 Number 4 pages 1273–1644

Br. J. Pharmacol. (1993), **110**, 1275–1277 © Macmillan Press Ltd, 1993

OBITUARY

Hugh Blaschko, FRS (1900–1993)

Hugh Blaschko was, without doubt, one of the great pharmacologists of his time but he was also one of the best loved of men, who bore his greatness lightly. In giving a brief survey of Hugh's life in science I have selected just one of the many topics he studied and reported in several hundred articles: his studies on adrenaline. Hugh discovered how adrenaline is made in the body, how it is stored in cells and how it is destroyed in the body so that it no longer has its potent actions. Hugh's work on adrenaline has had far reaching implications for human society, something he could never have dreamt of and something he never boasted about.

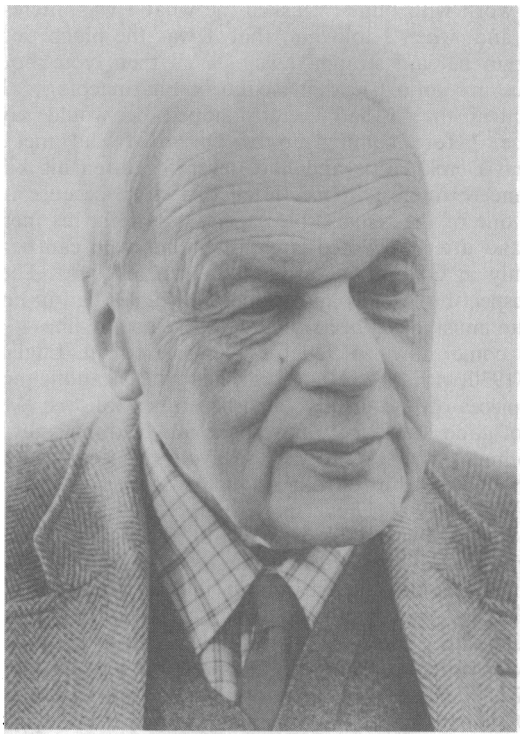
Hermann Karl Felix Blaschko, who died on 18 April 1993, was born on January 4th 1900, the son of a leading dermatologist, Alfred Blaschko, who practised all his life in the working-class districts and who was deeply concerned about social issues in medicine. Perhaps Hugh learnt his natural way with people from his father; one thing we all know is that Hugh cared equally for everyone he came across, irrespective of class, race or religion.

Hugh's father was also physician to some of Berlin's famous politicians, scholars and scientists – then, of course, Berlin was the capital of Germany. Amongst the scientists were Alfred Einstein, Enrico Fermi and Max Born, whose son Gustav was later to work with Hugh in Oxford. It must have been a wonderful cultural, social and intellectual climate for the young Hermann and it left a strong impression upon him, since he often used to recount experiences from those days. He recalled darker events as well, like standing in a huge crowd outside the Imperial Palace in Berlin to hear the Kaiser declare war in 1914. Ever since then, he hated mass crowds and the jingoism that they evoke.

However, his abiding memories of those early days were of visits to the countryside around Berlin with his father, a keen naturalist, who had retained his interest in natural science and who was ever ready to answer the questions of his inquisitive children. While a teenager, Hugh met Alfred Berliner, the founder of *Naturwissenschaften*, a periodical that Hugh read avidly and which was responsible for much of his early knowledge of science. Clearly, he displayed very early in his life that crucial quality of a scientist: curiosity – and it was never to leave him, since right to the end of his life he would come regularly to Departmental seminars to listen to the latest research findings. Indeed, he last attended a seminar only three weeks before he died, when he heard two research students present their work. Afterwards, he said to me 'I didn't understand everything about the ion channels but I like the project – and didn't the girl do her's well.'

Hugh's own introduction to laboratory work came in his teens, as, owing to the 1st World War, students were encouraged to take their school-leaving examination early in order to do work that was of national importance. Fortunately, he avoided working in a munitions factory and went instead to the Institute of Animal Physiology of the Agricultural Academy in Berlin soon after his 17th birthday. Here he learnt simple laboratory procedures that included the estimation of nitrogen in foodstuffs by the Kjeldahl procedure, a method that he set me to use when I joined his laboratory in Oxford in 1962.

Coming from a family of doctors, it was inevitable that he would study medicine, which he began in the autumn of 1917 in Berlin. He used to tell us how privileged the German students were, something we forget today when we think our students have a lot of freedom, because they could choose which lectures to attend and indeed could move from University to University. In his case that meant missing his anatomy lectures in order to hear a series by Einstein and moving to Freiburg University where the professors were much more interesting than in Berlin.



Dr Hugh Blaschko

After his final examinations in 1922 he spent a year working in a hospital as an intern in order to gain experience of practical medicine and complete his qualification. Upon Max Born's advice, he went to Göttingen, where he was lucky to be taught by Eric Meyer, whose scientific approach to medicine left a deep impression upon him. While in Göttingen, in 1924, he was asked by Richard Courant, the professor of mathematics, 'What are your plans for the future?' This seemingly innocent question was to determine the rest of his life's work, for, in response Hugh said, hesitatingly, that he wanted to do research at the interface between physiology and biochemistry. Courant replied: 'Have you ever heard of Otto Meyerhof? – he might be the right man for you'. Meyerhof had just won the Nobel prize jointly with A.V. Hill and he was then in the famous Kaiser Wilhelm Institute for Biology in Dahlem. Eventually, Hugh began work on 1 January 1925 with Meyerhof and so began a time which Hugh wrote was 'one of the great formative periods of my life'. This time was interrupted twice by periods of ill health since Hugh contracted tuberculosis, a disease which kept recurring until finally he was cured when he spent several months in the 1940's in the Osler Clinic, which was situated in Headington, where the John Radcliffe Hospital now lies.

We can well imagine how important this period with Meyerhof was for, not only did he have daily contact with Meyerhof himself but the laboratory was a magnet for many others who were to make their mark on 20th century biochemistry, physiology and pharmacology: just some of the names are David Nachmanson, Ralph Gerard, Frank

Schmitt, Karl Lohmann (the discoverer of ATP), Severo Ochoa, and Fritz Lipmann. In the same Institute was Otto Warburg and with him a friend that Hugh had made when they were medical students together in Freiburg, Hans Krebs. Friendships with all these were to last all Hugh's life. It was through Hans Krebs that I myself came to work with Hugh. I was in my last year at Oxford reading Biochemistry and I wanted to stay on to do research, so I went to ask Krebs if I could work with him. He asked me what I was interested in doing and, when I told him that it was the biochemistry of the brain he said straight away: 'Well then, you should go and see my good friend Blaschko in Pharmacology.' I have to confess that I had secretly hoped he would say that because, before coming up to Oxford I had met Hugh through Derek Hope and had never forgotten his kindness and sincere interest in me. I tell this story because it illustrates one of the remarkable aspects of Hugh: his incredibly wide and utterly devoted circle of friends who can be found not only in Oxford but around the whole world. The trust and respect his friends and former colleagues had in him and for him must have been of great comfort to him.

We come now to Hugh's first period in England in 1929–1930 with A.V. Hill. Hugh has written about the three great pieces of luck in his scientific life, as follows: 'My first piece of good luck was to have been raised in the right family environment, the second was to have been accepted by Otto Meyerhof. The third stroke of luck was to work with A.V. Hill.' Hill was at University College, London and it was while he was with Hill (actually at the Marine Biological Laboratory in Plymouth) that Hugh carried out his first truly independent piece of research. It may surprise some of us that it was not until he was 29 that Hugh worked independently, but it seems that the British tradition even then was different from the German tradition: here, young scientists were given more independence and did not have to follow strictly the plan of work laid down by the professor.

The visit to Hill was to be of crucial importance for another reason. After his return to Germany Hugh suffered several recurrals of tuberculosis and one of these was in 1933, when the advent of the Nazi regime made him realise that he had no future in Germany. He was in hospital in Freiburg, being attended by no other than Hans Krebs as physician; while convalescing he worked in Krebs' laboratory to improve his skills in manometry – a technique that was to be crucial to several of his later discoveries. While still in hospital, a letter arrived from Hill inviting him to return to University College, London. He did not hesitate to accept for, as he wrote later, his return 'felt more like a homecoming'. Two other factors must have influenced him: first, he felt ready to begin truly independent research and, second, he had relatives in London who could give him a room. This reminds me of another remarkable feature of Hugh: he seemed to have relatives not only in every country but in every major city in each country! How often he told us, when we were about to embark on a trip abroad, 'Oh, yes, I have a cousin in . . . wherever it is we are going . . . who is professor of this, or director of that.'

However, as so often in life, fate determined differently: instead of beginning independent research in Hill's laboratory, Hugh had to continue his convalescence and so spent most of his time helping Hill with the society now called the Society for the Protection of Science and Learning. This society had been founded by Hill, Archbishop Temple and Sir William Beveridge to help refugee scholars. Hugh's main job was to interview scholars from Germany and advise Hill about their needs. He has told us that it was then he learnt from Hill that a scientist's duty does not end in the laboratory but extends to the support and care of the human being, something that his whole life since has so wonderfully demonstrated.

We now come to one of the most productive periods of Hugh's scientific life, the move to Cambridge in 1934; upon the advice of Hill and Krebs he went to Barcroft's Department of Physiology with a two year grant from the Society for the Protection of Science and Learning. Cambridge then was a great centre of scientific culture, not only in Physics but also in Physiology, where Adrian, Roughton, Matthews, Rushton and Willmer worked. Nearby was the leading Department of Biochemistry in the world under Gowland Hopkins, and the Molteno Institute, headed by David Keilin, who befriended Hugh.

But it was to be Barcroft who set Hugh on the track that led to one of his most important discoveries, that of the enzyme that destroys adrenaline. Hugh tells of how his laboratory was next door to Barcroft's and of how he used to enjoy the discussions at the end of the day when they would show each other their results. One day, Barcroft asked Hugh: 'How is adrenaline destroyed?' Hugh admits to knowing nothing about adrenaline at the time, but he promised to find the answer – thinking that for such an important compound it must be known already. But he could not find it in the library. So he collaborated with the pharmacologist Schlossmann, who was an expert in the biological assay of adrenaline and within a few weeks they had shown that the oxidation of adrenaline by tissues was very different from the chemical autoxidation that occurred in the air. They had discovered an enzyme that they called amine oxidase, later monoamine oxidase. When Derek Richter joined them they soon showed that the enzyme also acted on two other compounds, dopamine and noradrenaline, that were at the time just curiosities. The discovery, in 1937, came at an opportune time as the idea that nerves influence other tissues by the release of powerful chemicals was beginning to gain ground and Hugh's work on the inactivation of adrenaline soon became widely known.

What could not have been foretold was that drugs would be discovered 15 years later that inhibited the action of monoamine oxidase and that these drugs would revolutionize the treatment of depression. The monoamine oxidase inhibitors began the modern era of the drug therapy of mental illness. After a while, these drugs were replaced by others with a different mode of action, but Hugh was delighted earlier this year when a completely new type of monoamine oxidase inhibitor was introduced for the treatment of depression, moclobemide, that is reversible in action and appears to have none of the side effects of the older drugs; it may herald a new and safer form of treatment.

Hugh's grant lasted only two years and so he had to support himself by teaching at Cambridge, a somewhat precarious business since he never knew until the beginning of each term whether or not he would have any students. But what students he had! He often used to recount the future careers of his Cambridge pupils. He told these stories at times when his young colleagues, and I was one, became despondent about the heavy teaching load they had to bear: 'Never mind, you never know whether one of your pupils will be another Sanger' he used to say. Fred Sanger, of course, won the Nobel Prize twice and was one of Hugh's pupils in Cambridge.

So, Hugh's first great discovery was of the enzyme that destroys adrenaline in the body. Within another two years he had proposed the sequence of chemical reactions in the body that lead to the formation of adrenaline. The way in which he came upon this synthetic pathway was not so much from his own experiments as by the brilliant analysis of the apparently disconnected findings of others. It was not until 10 years, in 1949, that convincing experimental evidence was found that showed that Hugh was right. The understanding of how adrenaline is made in the body opened up the possibility of designing drugs to block the synthesis of this potent compound and such drugs (e.g. α-methyldopa) are still in use today for the treatment of high blood pressure.

Finally we come to the last great flowering of Hugh's scientific life, when he moved to Oxford in 1944 in response to an invitation from J.H. Burn to join the Department of Pharmacology. Hugh had the great fortune to move from a

department that was the mecca for physiologists to one that rapidly became the mecca for pharmacologists. Burn's department became renowned throughout the world and no small part of that renown was due to Hugh. People flocked to work with him from all over the world. He very quickly realised that the days when he did his own experiments alone had gone; no longer did he spend hours washing up his manometer pots in the lab: instead he recruited a remarkable band of technicians to help in the lab. The way in which he cared for his technicians was a joy to see: each and every one was important to Hugh. If any of them showed the slightest sparkle, they were encouraged to study for a degree and several of them went on to do doctorates and to become independent scientists of some renown. At least one of them married one of the visiting scientists in Hugh's lab: Jean Himms, who married Paul Hagen and became a leading light in Canadian biological science.

Which brings me to what must have been the most important event in Hugh's life: his marriage in 1944 to Mary Black soon after he arrived in Oxford. With Mary at his side Hugh was a man at peace and we also know how their home in Part Town became a second home to so many visitors from around the world.

One of the visitors was Arnold Welch, who came from the United States in 1952. He and Hugh looked at the question of how enormously high concentrations of a very potent substance, adrenaline, were stored in the adrenal gland. They used the recently introduced method of centrifugation and found, to their surprise, that most of the adrenaline could be sedimented from an homogenate and so must be present within a particle inside the cell. This work led to the discovery of the way in which not only adrenaline but almost all hormones and chemical mediators in the nervous system are stored inside cells. The importance of this discovery is such that its implications are still being worked out today. It also led to an understanding of how several important drugs produce their actions, drugs like reserpine that have been used for the treatment of high blood pressure and of schizophrenia.

Another of these visitors was Oleh Hornykiewicz, who came from Vienna in 1956 with a British Council scholarship and who had written to Krebs to see if he would accept him. Krebs took one look at his CV and sent it across to Hugh. So begins a chapter in Hugh's scientific life that has directly benefited hundreds of thousands of people over the world; these are people with Parkinson's disease. This disease was not treatable before 1961 and caused a great deal of suffering. What happened when Hornykiewicz came to Oxford was that Hugh set him to work on dopamine, that was considered by many to be just a precursor to adrenaline in the body. Hugh, on the other hand, thought that it might have a biological role in its own right. While he was in Hugh's laboratory, Hornykiewicz did experiments that supported this idea and, when he left to return to Vienna, Hugh told him: 'Oleh, you should stick to dopamine – dopamine has a bright future.' As Hornykiewicz has written 'I took Blaschko's advice, treasured it in my heart and, back in Vienna, switched from dopamine's cardiovascular effects to the central nervous system.' What Hornykiewicz discovered was that the brains of people who had died from Parkinson's disease had hardly any dopamine in them in contrast to the normal brain. He reasoned that, if you could replace the dopamine then the patients might feel better. But dopamine itself does not pass from the blood to the brain, so in 1961 he gave the precursor of dopamine, L-DOPA, to 4 patients with Parkinson's disease and found a dramatic improvement in their symptoms. So, the modern therapy for this disease was born. The drug that is used, L-DOPA, was first suggested by Hugh to be the natural precursor of dopamine in 1939.

I have had to leave out a great deal from my brief survey of Hugh Blaschko's life in science, for Hugh's scientific interests were very wide. He received many honours around the world for his work, including honorary degrees at Yale, Bergen and Heidelberg, Fellowship of the Royal Society in 1962, the Schmiedeberg Plakette of the German Pharmacological Society, the Thudicum Medal of the Biochemical Society and the Wellcome Gold Medal of the British Pharmacological Society. He generously donated the Wellcome prize money to help young pharmacologists from abroad to come and do research in Oxford.

Hugh seemed to me to be a very contented man in the 35 years I had the privilege of knowing him. In his scientific life he was content because he had managed to satisfy his curiosity many times and had revealed nature's secrets; I think he was also content because throughout his life he had been able to help his fellow human beings in many ways. Probably what gave him the greatest joy were the successes of his scientific family, but the world will remember him as one of the scientists whose work made possible the healing of those with diseases such as high blood pressure, depression, schizophrenia and Parkinson's disease.

From a tribute delivered at the Friends Meeting House, Oxford on 22 May 1993

A. David Smith
University Department of Pharmacology, Oxford.

Br. J. Pharmacol. (1993), **110**, 1278–1279

SPECIAL REPORT

© Macmillan Press Ltd, 1993

Thrombolytic effect of a plasminogen-plasminogen activator chimera in a photochemically induced thrombosis (PIT) model

[1]Hiroyuki Matsuno, Toshihiko Uematsu & Mitsuyoshi Nakashima

Department of Pharmacology, Hamamatsu University School of Medicine, Japan

The thrombolytic effects of the plasminogen/plasminogen activator chimera (SUN9216), comprising the fibrin-binding kringle 1 domain of plasminogen and two kringle and the serine protease domain of the wild-type tissue plasminogen activator (t-PA) including a modification of the mannose glycosylation on the kringle 1 of t-PA (PK1ΔFE1X), was compared with that of t-PA by use of a photochemically induced thrombus (PIT) in the rat femoral artery. When SUN9216 was administered either as an i.v. infusion (1.0 mg kg^{-1}) or as a single bolus i.v. injection (1.0 mg kg^{-1}), all parameters were markedly improved compared to t-PA administered as an i.v. infusion (3.0 mg kg^{-1}). A higher concentration of plasminogen activator (PA) activity in plasma was observed after administration of SUN9216 which persisted for longer than that after t-PA. It is concluded that the thrombolytic effect of SUN9216 is markedly greater than that of t-PA.

Keywords: A novel t-PA (SUN9216); photochemically induced thrombus (PIT); arterial thrombolysis; prevention of reocclusion

Introduction Intravenous infusion of tissue plasminogen activator (t-PA) results in early coronary artery patency in approximately 75% of patients (Collen *et al.*, 1988); however, the initial half-life of t-PA is a few minutes and so it may be unsuitable for the maintenance of a therapeutic plasma level (Clozel *et al.*, 1989). An important improvement would be the prolongation of half-life (Suzuki *et al.*, 1991) resulting in derivatives with slower clearance. In the present study, we have evaluated the effects of SUN9216 (t-PA-ΔFE1X), which has a long half-life, on thrombolysis and the prevention of reocclusion and compared its properties with those of t-PA, in the photochemically induced thrombus (PIT) model in the rat (Matsuno *et al.*, 1991).

Methods Male Wistar rats weighing 240–260 g were anaesthetized by intraperitoneal injection of sodium pentobarbitone 50 mg kg^{-1}.

Production of thrombus The experimental procedure to induce a thrombus in the rat femoral artery was as described in detail previously (Matsuno *et al.*, 1991). A part of the right femoral artery was isolated and a pulsed dopplar flow probe (Model PDV-20 Crystal Biotech, U.S.A.) was placed on the vessel. The irradiation of Xenon light proximal to the flow probe was started, and then a photosensitive dye Rose Bengal (Sigma) was injected (10 mg kg^{-1} in saline). The thrombus was judged to be established when the blood flow fell to zero.

Experimental protocol SUN9216 or t-PA was given through a catheter introduced into the left femoral vein as a bolus injection of 0.2 ml, followed by infusion of 1.8 ml over 30 min with a constant-infusion pump (Harvard model 22, U.S.A.). The effectiveness of each drug was evaluated by the following four criteria: (1) the time to reperfusion (TR), (2) blood flow after reperfusion (RF), (3) reperfusion rate (RR) and (4) reocclusion rate (OR). TR and RF were measured by the dopplar flow velocity meter. RR and OR were calculated according to the following formulae: RR(%) = [(number of recanalized animals at 90 min after the start of administration of drugs)/number of animals in each experiment)] × 100, OR(%) = [(number of reoccluded animals at 90 min after the reperfusion)/(number of recanalized animals) × 100. Reocclusion was defined as when the blood flow was less than 25% of the flow before thrombosis.

Measurement of plasminogen activator (PA) activity Blood samples were collected in sodium citrate (final concentrations 0.011 M) at 5, 15, 30, 45, 60 and 90 min after the end of infusion or bolus injections of either SUN9216 or rtPA. PA activity in the Euglobulin fraction of plasma was determined indirectly by use of the plasmin chromogenic substrate H-D-Val-Leu-Lys-p-nitroanilide (S-2251, Daiichi Pure Chemical Co. Ltd., Japan).

Results *Thrombolytic effects of SUN9216 on the PIT model* Table 1 shows TR, RR and OR. All parameters were significantly improved by SUN9216 1.0 mg kg^{-1} compared with the t-PA-treated group. When t-PA was administered as a bolus, reperfusion did not occur. Figure 1 shows the effects of SUN9216 and t-PA on alterations of blood flow (a) and the levels of PA activity with time (b) SUN9216 showed a slower disappearance rate in comparison with that of t-PA.

Discussion The present study demonstrated the thrombolytic efficacy of SUN9216 on the PIT model in the rat femoral artery. SUN9216 has a longer plasma half-life compared with that of t-PA and the modified t-PA, like SUN9216, showed a more advantageous reperfusion rate (Suzuki *et al.*, 1991). Reocclusion after reperfusion has been

Table 1 The time to reperfusion (TR), reperfusion rate (RR) and reocclusion rate (OR) after reperfusion in a photochemically induced thrombosis in rat femoral artery

Agent	*Route* (i.v.)	*Dose* (mg kg^{-1})	*TR*	*RR*	*OR*
t-PA	Infusion	3.0	70.5 ± 6.7	6/14	3/6
	Bolus	3.0	–	0/6	–
SUN9216	Infusion	0.3	72.5 ± 6.3	7/11	1/7
		1.0	57.7 ± 7.9*	10/11*	0/10
	Bolus	1.0	60.3 ± 7.2*	9/11*	1/9

*$P < 0.05$ versus t-PA treated group.

[1] Author for correspondence at Center for Molecular & Vascular Biology, University of Leuven, Herestraat 49, 3000 Leuven, Belgium.

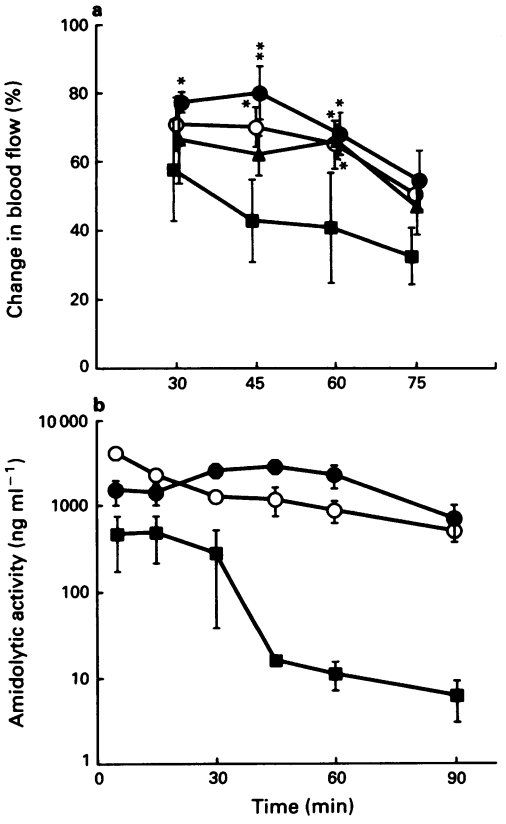


Figure 1 (a) Alteration of blood flow after reperfusion in a rat femoral artery model of thrombus formation. Flow is expressed as % change in flow 10 min before initiation of the induction of the thrombus. The effects of an infusion of SUN9216 (0.3 mg kg^{-1} (▲) and 1.0 mg kg^{-1} (●)), and t-PA (3.0 mg kg^{-1} (■)) and a bolus injection of SUN9216 (1.0 mg kg^{-1} (○)) are shown. (b) Changes in plasma amidolytic activity after the administration of SUN9216 (1 mg kg^{-1} as an infusion (●) and bolus (○)) and t-PA (3 mg kg^{-1} (■)) as in (a). All results are expressed as mean and vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$ vs t-PA treated group.

demonstrated to be an important cause of mortality and loss of ventricular function. Therefore we investigated not only the thrombolytic effect of SUN9216 but also its ability to prevent reocclusion in the artery by use of the photochemical reaction in the platelet-rich thrombus model (Matsuno *et al.*, 1991).

Gold & Leinbach (1987) found that a single injection of t-PA did not produce a significant or sustained reperfusion, and combination with anti-platelet drugs was necessary. Our previous study (Matsuno *et al.*, 1992) also demonstrated that t-PA combined with a thromboxane A_2 receptor antagonist (Vapiprost) was more effective at thrombolysis than t-PA alone. In these studies, both an infusion and a bolus injection of SUN9216 provided a larger plasma concentration of functionally active plasminogen activator and produced more rapid reperfusion than an infusion of tPA. Functional roles for an Asn-linked oligosaccharide in natural and variant forms of t-PA in fibrin binding and fibrinolytic activities has been observed previously (Hansen *et al.*, 1988). Prevention of glycosylation of the t-PA variant was carried out in an attempt to enhance its fibrinolytic activity. Glycosylation was prevented by changing Asn to Gln; Asn-117 to produce ΔFE1X t-PA (SUN9216). The results obtained support the potent effect of SUN9216 on thrombus formation. Additionally, if the plasma level of t-PA decreased rapidly after termination of administration then acute rethrombosis would be formed at the same place. This condition may easily cause reocclusion and decreased blood flow. Therefore if an effective plasma level could be continued longer, the incidence of reocclusion may be reduced compared to that after t-PA treatment alone. In fact, our data showed that the blood flow after reperfusion was maintained and reocclusion incidence decreased in the SUN9216-treated groups. In conclusion, the present study shows that SUN9216 is an effective drug for arterial thrombolysis and prevention of reocclusion.

The authors are grateful to Mr J.M. Stassen (Center for Molecular & Vascular Biology) for helpful discussion.

References

CLOZEL, J.P., TSCHOPP, T., LUEDIN, E. & HOLVOET, P. (1989). Time course of thrombolysis induced by intravenous bolus or infusion of tissue plasminogen activator in a rabbit jugular vein thrombi model. *Circulation*, **79**, 125–133.

COLLEN, D., STASSEN, J.M. & LARSEN, G. (1988). Pharmacokinetics and thrombolytic properties of deletion mutants of human tissue-type plasminogen activator in rabbits. *Blood*, **71**, 216–219.

GOLD, H.K. & LEINBACH, R.C. (1987). Prevention of acute reocculsion after thrombolytsis with intravenous recombinant tissue plasminogen activator. In *Tissue Plasminogen Activator in Thrombolytic Therapy*. ed. Sobel, B.E., Collen, D. & Grossbard, E.B. pp. 115–130. New York: Marcel Dekker.

HANSEN, L., BLUE, Y., BARONE, K., COLLEN, D. & LARSEN, G.R. (1988). Functional effects of Asparagine-linked oligosaccharide on natural and variant human tissue-type plasminogen activator. *J. Biol. Chem.*, **263**, 15713–15719.

MATSUNO, H., UEMATSU, T., NAGASHIMA, S. & NAKASHIMA, M. (1991). Photochemically induced thrombosis in rat femoral artery and evaluation of effects of heparin and tissue-type plasminogen activator with use of this model. *J. Pharmacol. Methods*, **25**, 303–318.

MATSUNO, H., UEMATSU, T., UMENURA, K., TAKIGUCHI, Y., WADA, K. & NAKASHIMA, M. (1992). Effect of vapiprost, a novel thromboxane receptor antagonist, on a thrombus formation and the vascular patency after thrombolysis by tissue-type plasminogen activator. *Br. J. Pharmacol.*, **106**, 533–542.

SUZUKI, S., SAITO, M., SUZUKI, H., NAGAOKA, N., YOSHITAKE, S., MIZUNO, H., YUZURIHA, T., YUI, Y. & KAWAI, C. (1991). Thrombolytic properties of a novel modified human tissue-type plasminogen activator (E6010): A bolus injection of E6010 has equivalent potency of lysing young and aged canine coronary thrombi. *J. Cardior. Pharmacol.*, **17**, 738–746.

(Received June 28, 1993
Accepted September 2, 1993)

Br. J. Pharmacol. (1993), **110**, 1280–1281

SPECIAL REPORT

© Macmillan Press Ltd, 1993

Induction of a glibenclamide-sensitive K-current by modification of a delayed rectifier channel in rat portal vein and insulinoma cells

[1]G. Edwards & A.H. Weston

Smooth Muscle Pharmacology Group, Department of Physiological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT

In insulinoma cells (RINm5F), the glibenclamide-sensitive K-current ($I_{K(ATP)}$) which developed spontaneously or after exposure to levcromakalim or to butanedione monoxime was always accompanied by a reduction in the delayed rectifier current ($I_{K(V)}$). At potentials over which $I_{K(V)}$ was fully activated, the total outward current remained constant. In rat portal vein, the delayed rectifier channel inhibitor, margatoxin, reduced the combined induction of $I_{K(ATP)}$ and inhibition of $I_{K(V)}$ by levcromakalim. These data suggest that the ATP-sensitive K-channel, $K_{(ATP)}$, is a voltage-insensitive state of the delayed rectifier, K_V.

Keywords: Levcromakalim; RINm5F; K_V; K_{ATP}; K-channel; portal vein

Introduction In rat portal vein, we have demonstrated that K-channel openers or procedures designed to reduce channel phosphorylation stimulate a non-inactivating K-current ($I_{K(ATP)}$) and simultaneously inhibit a delayed rectifier K-current ($I_{K(V)}$). We thus concluded that the effects of levcromakalim were consistent with an action on K_V via removal of its voltage-sensitivity (Edwards *et al.*, 1993).

We have now studied the possible conversion of $I_{K(V)}$ into $I_{K(ATP)}$ in insulinoma cells and extended our observations on portal vein to include margatoxin, a potent inhibitor of K_V (Garcia-Calvo *et al.*, 1993).

Methods Single rat portal vein cells were isolated and recordings made as previously described (Ibbotson *et al.*, 1993). RINm5F cells were maintained in culture. The composition of the solutions used was (mM): pipette – NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, glucose 11, HEPES 10, EGTA 1.2, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, pH 7.3; bath (oxygenated) – NaCl 125, KCl 4.8, $MgCl_2$ 3.7, K_2HPO_4 1.2, glucose 11, HEPES 10, EGTA 1, pH 7.3. All experiments were performed at room temperature (22–25°C).

Results When RINm5F cells were voltage-clamped at −90 mV and stepped in 10 mV increments to test potentials more positive than −40 mV, a delayed rectifier K-current ($I_{K(V)}$) was observed. At potentials more negative than −40 mV only a non-inactivating cation current (I_{NI}) was present. When cells were clamped for 1 min at −10 mV, $I_{K(V)}$ inactivated, leaving only I_{NI}. With time, an additional non-inactivating K-current developed (peak approximately 12 min) and simultaneously $I_{K(V)}$ declined (Figure 1a). The non-inactivating K-current ($I_{K(ATP)}$) was totally inhibited by 10 μM glibenclamide (data not shown). $I_{K(V)}$ was obtained by digital subtraction of the non-inactivating currents ((I_{NI} or $I_{NI} + I_{K(ATP)}$) from currents obtained at similar test potentials after stepping from −90 mV. At the more positive test potentials, total current remained constant with time (Figure 1b).

In some cells under conditions in which the extracellular glucose concentration was increased to 22 mM, $I_{K(ATP)}$ did not develop. Application of levcromakalim (30 μM) or the 'chemical phosphatase', butanedione monoxime (BDM, 5 mM), induced $I_{K(ATP)}$ and inhibited $I_{K(V)}$ (Figure 1c,d).

Applying the same voltage-step protocols to rat portal vein cells, only $I_{K(V)}$ and I_{NI} were observed under control conditions. Levcromakalim (1 μM) induced $I_{K(ATP)}$ and simultaneously inhibited $I_{K(V)}$. Margatoxin (100 nM) had no effect on $I_{K(V)}$. However, after pre-exposure to margatoxin and in its continued presence, the ability of levcromakalim to induce $I_{K(ATP)}$ and to inhibit $I_{K(V)}$ were both markedly reduced (Figure 2).

Discussion Recent experiments on rat portal vein cells have suggested that K_{ATP} is a partially-dephosphorylated state of

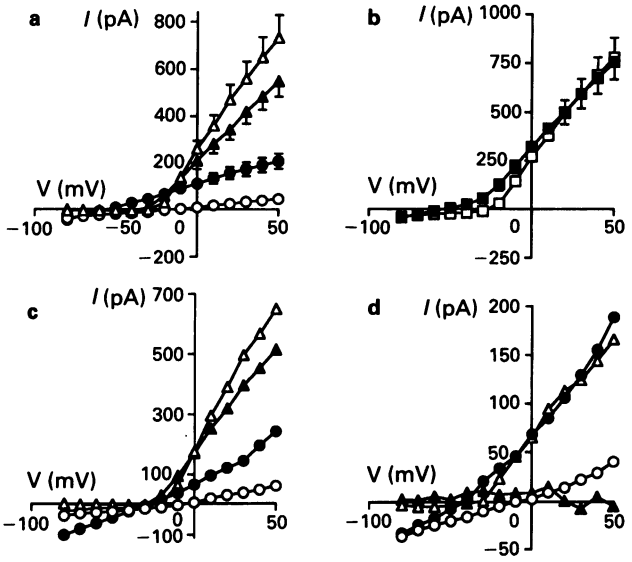


Figure 1 Effect of time on whole-cell currents in RINm5F cells: (a) 14 min after formation of the whole-cell recording configuration a non-inactivating current ($I_{K(ATP)}$, ●) had developed and $I_{K(V)}$ (▲) was reduced. However, the total current at this time (■) was similar to the initial total current (□) (b). Levcromakalim (10 μM, c) and butanedione monoxime (5 mM, d) similarly induced $I_{K(ATP)}$ (●) and inhibited $I_{K(V)}$ (▲); (△) and (○) indicate control $I_{K(V)}$ and non-inactivating currents, respectively. In (a) and (b), each point represents the mean ± s.e.mean, $n = 3$. (c) and (d) each represent the result from a single cell.

[1] Author for correspondence.

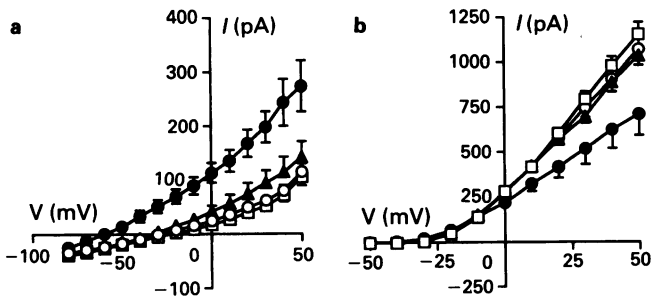


Figure 2 Effect of margatoxin on response to levcromakalim in rat portal vein cells. Levcromakalim (1 μM, ●) induced $I_{K(ATP)}$ (a) and inhibited $I_{K(V)}$ (b). Margatoxin alone (100 nM, □) had no effect but inhibited the effects of levcromakalim (1 μM, ▲); (○) indicates control currents. Each point represents the mean ± s.e.mean, $n = 5$.

K_V, consistent with findings that inhibitors of $I_{K(V)}$ also inhibit $I_{K(ATP)}$ (see Edwards *et al.*, 1993). A key physiological role of K_{ATP} is the regulation of insulin secretion in pancreatic β-cells. We therefore examined the relationship between $I_{K(V)}$ and $I_{K(ATP)}$ in an insulinoma cell (RINm5F). In this cell, as in the β-cell, K_{ATP} is considered to be almost maximally inhibited by physiological concentrations of intracellular ATP ($[ATP]_i$), and only to open as $[ATP]_i$ falls (Harding *et al.*, 1993).

Immediately after cell breakthrough only $I_{K(V)}$ and I_{NI} were detected in RINm5F cells, with no evidence of glibenclamide-sensitive currents. However, the magnitude of $I_{K(V)}$ declined with time and simultaneously a glibenclamide-sensitive K-current ($I_{K(ATP)}$) developed. In the potential range + 30 to + 50 mV, at which $I_{K(V)}$ was probably fully activated, the total outward current after development of $I_{K(ATP)}$ (i.e. $I_{K(V)} + I_{NI} + I_{K(ATP)}$) was essentially identical to that immediately after breakthrough ($I_{K(V)} + I_{NI}$). Furthermore, in RINm5F cells in which the spontaneous induction of $I_{K(ATP)}$ was suppressed by additional glucose, levcromakalim and a 'chemical phosphatase', BDM, each induced a glibenclamide-sensitive K-current and simultaneously inhibited $I_{K(V)}$. Again, the total outward current at the most positive test potentials remained constant. In rat portal vein cells, margatoxin, a potent inhibitor of voltage-dependent K-channels ($K_{V1.3}$; Garcia-Calvo *et al.*, 1993), surprisingly had no effect on $I_{K(V)}$. Nevertheless, this agent antagonized the combined induction of $I_{K(ATP)}$ and the inhibition of $I_{K(V)}$ by levcromakalim.

We cannot yet totally exclude the possibility that K_V and K_{ATP} are two independent K-channel types. However, in both RINm5F cells and in rat portal vein cells, evidence is accumulating which suggests that K_{ATP} and K_V are not separate entities. $K_{(V)}$ and $K_{(ATP)}$ possess almost identical conductances and, in general, share a common pharmacology (see Edwards *et al.*, 1993). Although glibenclamide is a reasonably selective inhibitor of K_{ATP}, high concentrations of this agent do inhibit K_V (Beech *et al.*, 1993). Furthermore, glibenclamide binding is inhibited under phosphorylating conditions (Schwanstecher *et al.*, 1991).

We thus conclude that modification of K_V (probably by partial dephosphorylation as $[ATP]_i$ falls or by levcromakalim or BDM) removes its voltage-sensitivity and simultaneously imparts increased glibenclamide-sensitivity. It is thus possible that insulin secretion in β-cells is not regulated by the voltage-insensitive channel, K_{ATP}, but rather by a modification of the voltage-sensitivity of K_V in response to fluctuating concentrations of $[ATP]_i$. Such a modification can be effected by a metabolic change or by agents such as levcromakalim or BDM which probably also reduce the degree of phosphorylation of K_V.

The authors thank Drs G.J. Kaczorowski and L. Best for kind gifts of margotoxin and RINm5F cells, respectively. G.E. was supported by Pfizer Central Research.

References

BEECH, D.J., ZHANG, H., NAKAO, K. & BOLTON, T.B. (1993). Single channel and whole-cell K-currents evoked by levcromakalim in smooth muscle cells from the rabbit portal vein. *Br. J. Pharmacol.*, **110**, 583–590.

EDWARDS, G., IBBOTSON, T. & WESTON, A.H. (1993). Levcromakalim may induce a voltage-independent K-current in rat portal veins by modifying the gating properties of the delayed rectifier. *Br. J. Pharmacol.*, **110**, 1037–1048.

IBBOTSON, T., EDWARDS, G., NOACK, TH. & WESTON, A.H. (1993). Effects of P1060 and aprikalim on whole-cell currents in rat portal vein; inhibition by glibenclamide and phentolamine. *Br. J. Pharmacol.*, **108**, 991–998.

GARCIA-CALVO, M., LEONARD, J.L., NOVICK, J., STEVENS, S.P., SCHMALHOFER, W., KACZOROWSKI, G.J. & GARCIA, M.L. (1993). Purification, characterization, and biosynthesis of margatoxin, a component of *Centroides margaritatus* venom that selectively inhibits voltage-dependent potassium channels. *J. Biol. Chem.*, **268**, 18866–18874.

HARDING, E.A., JAGGAR, J.H., AYTON, B.J. & DUNNE, M.J. (1993). Properties of diazoxide and cromakalim-induced activation of potassium channels in cultured rat and RINm5F insulin-secreting cells; effects of GTP. *Exp. Physiol.*, **78**, 25–34.

SCHWANSTECHER, M., LÖSER, S., RIETZE, I. & PANTEN, U. (1991). Phosphate and thiophosphate group donating adenine and guanine nucleotides inhibit glibenclamide binding to membranes from pancreatic islets. *Naunyn-Schmied. Arch. Pharmacol.*, **343**, 83–89.

(Received August 13, 1993
Accepted September 3, 1993)

Br. J. Pharmacol. (1993), **110**, 1282–1284

SPECIAL REPORT

© Macmillan Press Ltd., 1993

Endotoxin-induced arterial endothelial barrier dysfunction assessed by an *in vitro* model

Rodney S. Berman, Joyce D. Frew & [1]William Martin

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

Using an *in vitro* model in which albumin transfer across monolayers of bovine aortic endothelial cells (BAEC) was measured, we have shown that lipopolysaccharide (LPS) induces a concentration-dependent increase in endothelial permeability. This increase was biphasic, having an early peak at 2 h and rising again by 24 h. Both peaks were abolished by polymixin B (PMB) but were unaffected by N^{ω}-monomethyl-L-arginine, N^{ω}-nitro-L-arginine methyl ester or dexamethasone. Furthermore, LPS did not stimulate nitric oxide production by BAEC following 24 h exposure. Thus, the LPS-induced increase in permeability may account for the vascular leakage of septic shock, but the L-arginine-nitric oxide system does not appear to be involved.

Keywords: Endothelial barrier function; endotoxin shock; lipopolysaccharide; nitric oxide; polymixin B

Introduction Septic shock is characterized by severe hypotension, hyporeactivity to vasoconstrictors, profound vascular leakage, and ultimately multiple organ failure culminating in death (Pinsky *et al.*, 1993). Lipopolysaccharide (LPS) derived from gram-negative bacteria is a common cause of septic shock with various mediators having been implicated, including catecholamines, prostaglandins, free radicals, cytokines and nitric oxide (Wright *et al.*, 1992). Evidence suggests that nitric oxide is responsible for the hypotension associated with septic shock (Parker & Adams, 1993), however, the mediator(s) of the vascular leakage remains unknown.

Using an *in vitro* model, we wished to determine if LPS causes endothelial barrier dysfunction and so account for the vascular leakage of septic shock. If so, we wished to investigate the involvement of the L-arginine-nitric oxide system.

Methods *Albumin transfer experiments* Albumin transfer experiments were conducted as previously described (Berman & Martin, 1993). Briefly, bovine aortic endothelial cells (BAEC) were isolated, grown in 80 cm^2 culture flasks until confluent and seeded onto Transwell (Costar) polycarbonate membranes (6.5 mm diameter, 3 μm pore size). Following incubation with drugs as indicated in Results, membranes were washed twice in Krebs solution comprising (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 2.4, glucose 11 and HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) 5 at 37°C and pH 7.4, and transferred to 24-well plates. Krebs solution (600 μl) was placed in the lower chamber of each well, with 100 μl of Krebs containing 4% trypan blue-labelled albumin being placed in the upper chamber, above the monolayer. For experiments involving polymixin B (PMB), N^{ω}-monomethyl-L-arginine (L-NMMA) and N^{ω}-nitro-L-arginine methyl ester (L-NAME), these were then re-added to both chambers. The plates were transferred to an orbital shaker and incubated for 90 min at 37°C after which the extent of equilibration of albumin across the monolayers was determined spectrophotometrically at 590 nm.

Drugs Dexamethasone, lipopolysaccharide from *Salmonella typhosa*, N^{ω}-nitro-L-arginine methyl ester and polymixin B sulphate were obtained from Sigma (Poole, Dorset) whilst N^{ω}-monomethyl-L-arginine acetate was a gift from Dr D.D. Rees, Wellcome Laboratories (Beckenham). All drugs were dissolved in distilled water except for dexamethasone which

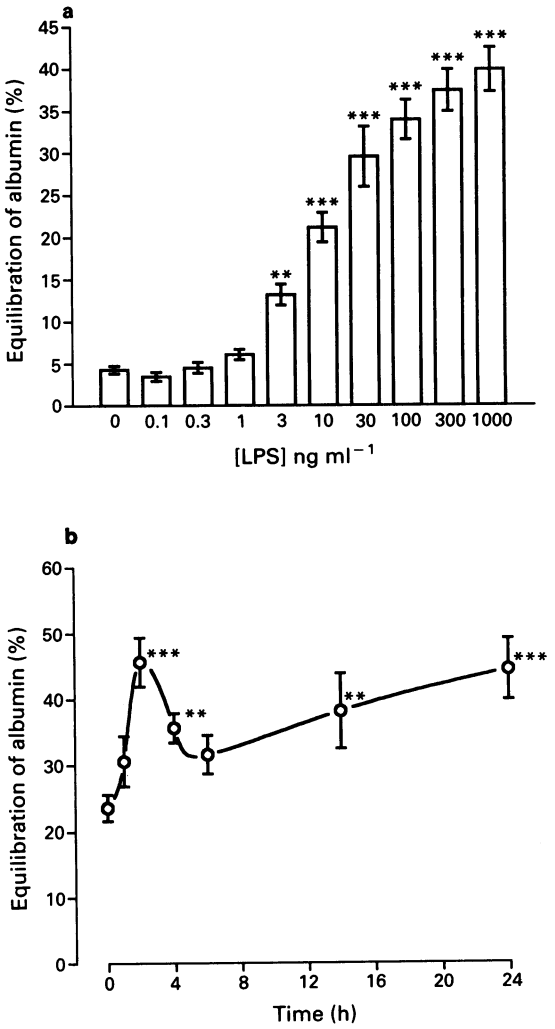


Figure 1 (a) Effects of 24 h exposure to 0–1000 ng ml^{-1} lipopolysaccharide (LPS) on albumin transfer across monolayers of BAEC. Each column is the mean with s.e.mean of 6–23 observations. $^{**}P<0.01$; $^{***}P<0.001$, indicates a significant difference from control (no LPS). (b) Time-course of the development of the increases in albumin transfer across monolayers of BAEC induced by 30 ng ml^{-1} LPS. Each point is the mean with s.e.mean of 12–24 observations. $^{**}P<0.01$; $^{***}P<0.001$, indicates a significant difference from control (0 h).

[1] Author for correspondence.

Table 1 Effects of polymixin B (PMB), N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-monomethyl-L-arginine (L-NMMA) and dexamethasone on the increases in albumin transfer stimulated by 2 h and 24 h exposure to 30 ng ml^{-1} lipopolysaccharide (LPS)

	Equilibration of albumin (%)			
LPS 2 h Treatment	*Control*	*LPS*	*Treatment alone*	*LPS + treatment*
PMB, 10 μg ml^{-1}	9.6 ± 0.5	15.9 ± 2.0**	9.2 ± 0.4	11.1 ± 1.1‡‡
L-NAME, 500 μM	7.8 ± 0.7	26.4 ± 3.6***	8.6 ± 2.0	24.8 ± 1.6***
L-NMMA, 2 mM	4.5 ± 1.1	19.1 ± 1.5***	8.1 ± 1.9	28.3 ± 3.1***
Dexamethasone, 1 μM	9.2 ± 1.4	18.0 ± 1.6***	7.3 ± 1.0	15.6 ± 0.6**
LPS 24 h Treatment	*Control*	*LPS*	*Treatment alone*	*LPS + treatment*
PMB, 10 μg ml^{-1}	11.0 ± 0.7	24.5 ± 0.7***	10.4 ± 0.4	12.4 ± 1.3‡‡‡
L-NAME, 500 μM	8.2 ± 0.9	43.1 ± 2.6***	6.2 ± 0.6	45.8 ± 1.1***
L-NMMA, 2 mM	5.1 ± 0.4	16.1 ± 0.8***	5.3 ± 0.5	19.9 ± 2.3***
Dexamethasone, 1 μM	9.2 ± 0.8	32.1 ± 1.8***	6.6 ± 0.9	26.4 ± 2.6***

Levels of albumin transfer across BAEC monolayers are shown following incubation both in the absence of drugs (control) and in the presence of LPS. Effects of concomitant addition of PMB, L-NAME and L-NMMA, and of 20 h pretreatment with dexamethasone, are also shown. Results are expressed as the mean ± s.e.mean of 6 observations.
**$P<0.01$; ***$P<0.001$, indicates a significant difference from control.
‡‡$P<0.01$; ‡‡‡$P<0.001$, indicates a significant difference from LPS.

was dissolved in ethanol. The final concentration of ethanol in experiments did not exceed 0.2%.

Statistical analysis Results are expressed as means ± s.e.mean and were compared by one-way analysis of variance followed by Fisher's test. $P \leqslant 0.05$ was taken as significant.

Results Following 24 h exposure, LPS (3–1000 ng ml^{-1}) produced a concentration-dependent increase in the equilibration of albumin across BAEC monolayers over 90 min (Figure 1a).

The time-course of the increase in albumin transfer stimulated by 30 ng ml^{-1} LPS is shown in Figure 1b. An early, transient peak was observed after 2 h which then declined towards basal levels before rising again by 24 h.

The increase in albumin transfer stimulated by exposure to LPS for both 2 h and 24 h was abolished by PMB (10 μg ml^{-1}) which binds and inhibits LPS, but was unaffected by the nitric oxide synthase inhibitors, L-NMMA (2 mM) and L-NAME (500 μM), or by 20 h pretreatment with the glucocorticoid, dexamethasone (1 μM) (Table 1).

Using a chemiluminescence assay (Cox, 1980), we found no significant increase over basal levels (29.2 ± 4.0 nmol/10^6 cells) in nitrite accumulation in the medium above BAEC exposed to up to 1000 ng ml^{-1} LPS for 24 h (21.8 ± 4.5 nmol/10^6 cells).

Discussion The principal new finding is that LPS induced a concentration-dependent increase in albumin transfer across BAEC monolayers and this may account for the profound vascular leakage observed in septic shock (Pinsky *et al.*, 1993).

The LPS-induced permeability increase developed with a biphasic time-course, exhibiting a transient peak at 2 h which then declined, before rising again, nearing maximum by 24 h. At both time points PMB, which inhibits LPS activity by binding it with high affinity (Lasfargues *et al.*, 1989), abolished the increases in albumin transfer.

Interestingly, the nature of this biphasic time-course parallels that of the hypotension induced by LPS *in vivo* (Wright *et al.*, 1992). Evidence shows that the early fall in blood pressure results from production of nitric oxide by the vascular endothelium (Salvemini *et al.*, 1990) and that the subsequent delayed fall results from induction of nitric oxide synthase in the vascular smooth muscle (Wright *et al.*, 1992). We found, however, that L-NMMA and L-NAME, inhibitors of nitric oxide synthase, had no effect on LPS-stimulated increases in BAEC permeability, suggesting that nitric oxide is not involved. Two further findings led us to conclude that LPS had not stimulated an increase in permeability through nitric oxide production. Firstly, dexamethasone, a glucocorticoid which prevents induction of the Ca^{2+}-independent, inducible form of nitric oxide synthase (Moncada, 1992), exhibited no inhibitory effect when added 20 h prior to LPS. Secondly, on determining nitrite levels as an indicator of nitric oxide production, we found no stimulated increase by LPS over 24 h.

Our findings differ from those of Palmer *et al.* (1992) who showed that LPS-stimulated cytotoxicity in pig aortic endothelium is inhibited by L-NMMA, dexamethasone and hydrocortisone. However, in their study cell death was taken as a marker of LPS activity, using a standard stimulus 300 times greater than the concentration we used to induce an increase in albumin transfer. To what extent cell viability is impaired by LPS in our study is unclear, but, whilst nitric oxide might be required to cause cell death in response to much higher concentrations of LPS, it does not appear to mediate the observed increases in endothelial permeability.

In conclusion, we have shown that LPS clearly induces endothelial barrier dysfunction and this may account for the profound vascular leakage associated with septic shock. This dysfunction does not appear, however, to be mediated by the L-arginine-nitric oxide system.

This work was supported by the Science and Engineering Research Council and the British Heart Foundation.

References

BERMAN, R.S. & MARTIN, W. (1993). Arterial endothelial barrier dysfunction: actions of homocysteine and the hypoxanthine-xanthine oxidase free radical generating system. *Br. J. Pharmacol.*, **108**, 920–926.

COX, R.D. (1980). Determination of nitrate and nitrite at the parts per billion level by chemiluminescence. *Anal. Chem.*, **52**, 332–335.

LASFARGUES, A., TAHRI-JOUTI, M., GIRARD, R. & CHABY, R. (1989). Effects of lipopolysaccharide on macrophages analyzed with anti-lipid A monoclonal antibodies and polymixin B. *Eur. J. Immunol.*, **19**, 2219–2225.

MONCADA, S. (1992). The L-arginine:nitric oxide pathway. *Acta Physiol. Scand.*, **145**, 201–227.

PALMER, R.M.J., BRIDGE, L., FOXWELL, N.A. & MONCADA, S. (1992). The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids. *Br. J. Pharmacol.*, **105**, 11–12.

PARKER, J.L. & ADAMS, H.R. (1993). Selective inhibition of endothelium-dependent vasodilator capacity by *Escherichia coli* endotoxemia. *Circ. Res.*, **72**, 539–551.

PINSKY, M.R., VINCENT, J., ALEGRE, M. & DUPONT, E. (1993). Serum cytokine levels in human septic shock. *Chest*, **103**, 565–575.

SALVEMINI, D., KORBUT, R., ÅNGGÅRD, E. & VANE, J. (1990). Immediate release of a nitric oxide-like factor from bovine aortic endothelial cells by Escherichia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. USA*, **87**, 2593–2597.

WRIGHT, C.E., REES, D.D. & MONCADA, S. (1992). Protective and pathological roles of nitric oxide in endotoxin shock. *Cardiovasc. Res.*, **26**, 48–57.

(Received August 25, 1993
Accepted September 21, 1993)

Br. J. Pharmacol. (1993), **110**, 1285–1290 © Macmillan Press Ltd, 1993

Effects of ω-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the rabbit urethra and detrusor

P.M. Zygmunt, P.K.E. Zygmunt, [1]E.D. Högestätt & K.-E. Andersson

Department of Clinical Pharmacology, University of Lund, S-22185 Lund, Sweden

1 The effects of ω-conotoxin GVIA (an inhibitor of N-type voltage-operated calcium channels; VOCCs) were compared on adrenergic, cholinergic and non-adrenergic, non-cholinergic (NANC) responses induced by electrical field stimulation (EFS) in the rabbit urethra and detrusor.

2 EFS induced a relaxation in urethral smooth muscle and lamina propria precontracted by arginine vasopressin (AVP). The relaxation was abolished by tetrodotoxin (TTX) or the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine. ω-Conotoxin inhibited the relaxation induced by EFS, but not that elicited by the NO donor 3-morpholino-sydnonimin. The inhibition, however, decreased with increasing frequencies of stimulation. Nimodipine, tetramethrin and nickel did not affect the ω-conotoxin-resistant relaxation in lamina propria, suggesting that neuronal L or T VOCCs were not involved in the response.

3 EFS contracted urethral smooth muscle at resting tension. The contractions were virtually abolished by TTX or prazosin. ω-Conotoxin effectively inhibited the contractile responses to EFS, but not those to exogenous noradrenaline. An ω-conotoxin-resistant contraction was, however, observed at high frequencies of stimulation.

4 The detrusor responded with frequency-dependent contractions upon EFS. A TTX-resistant contraction less than 10% of controls remained at 30 Hz stimulation. At a stimulation frequency of 10 Hz, scopolamine reduced the EFS-induced contraction by 71%. ω-Conotoxin inhibited the responses in both the absence and presence of scopolamine. The inhibition decreased with increasing frequencies of stimulation (examined in the absence of scopolamine). ω-Conotoxin did not affect the contractile responses to carbachol or adenosine 5′-triphosphate.

5 The adrenergic contraction (25 Hz) and NANC relaxation (10 Hz) in the urethra, and cholinergic and NANC contractions (10 Hz) in the detrusor were inhibited concentration-dependently by ω-conotoxin. The adrenergic contraction in the urethra was 10 times and the cholinergic contraction in the detrusor was three times more sensitive to ω-conotoxin than the NANC responses.

6 These results suggest that NANC neurotransmission is less inhibited by ω-conotoxin than transmission mediated by adrenergic and cholinergic nerves in the rabbit lower urinary tract. In the urethra a marked ω-conotoxin-resistant component of the NANC relaxation was observed which increased with increasing stimulation frequencies and was unaffected by inhibitors of L and T type VOCCs. This raises the question whether VOCCs of a type other than L, T, and N is involved in the mediation of this response.

Keywords: ω-Conotoxin; calcium channels; adrenergic neurotransmission; cholinergic neurotransmission; NANC neurotransmission; nitric oxide

Introduction

It is well known that non-adrenergic, non-cholinergic (NANC) nerves use transmitters like adenosine 5′-triphosphate (ATP) and various peptides, e.g., calcitonin gene-related peptide and substance P. NANC transmission closely resembles transmission by classical non-peptide nerves in that the transmitters are stored in vesicles and released by a calcium-activated exocytotic process (Dockray, 1992). Although the exact mechanism for vesicular release is unknown, influx of calcium through voltage-operated calcium channels (VOCCs) seems to be a crucial event. Recently it has been suggested that nitric oxide (NO) may be the transmitter substance responsible for NANC relaxation in several tissues (Persson & Andersson, 1992; Sneddon & Graham, 1992), including rabbit urethral smooth muscle and lamina propria (Andersson *et al.*, 1991; 1992; Zygmunt *et al.*, 1993). NO is formed from L-arginine by the enzyme NO synthase (NOS), which exists in different isoforms (Förstermann *et al.*, 1991). NOS can be competitively inhibited by several N^{ω}-substituted L-arginine analogues, e.g., N^{ω}-nitro-L-arginine (L-NOARG; Mülsch & Busse, 1990). The NOS found in neuronal tissues is a constitutive and Ca^{2+}/calmodulin-dependent enzyme (Bredt *et al.*, 1991; Murad *et al.*, 1992). An important distinction between NO and other neurotransmitters is that exocytosis of preformed NO-containing membrane vesicles is not obligatory for NO release. NO is rather thought to be produced on demand and to diffuse from the site of synthesis to adjacent effector cells (Snyder, 1992). Formation of more stable compounds has been considered, including dinitrosyl iron complexes (Vanin, 1991; Vedernikov *et al.*, 1992) and nitrosothiols (Myers *et al.*, 1990); the latter may subsequently be stored in acidic membrane vesicles (Ignarro, 1989).

Based on electrophysiological findings, VOCCs may be divided into low (LVA) and high (HVA) voltage-activated calcium channels (Sher *et al.*, 1991). The LVA calcium channel is also designated T (transient) due to its rapid inactivation kinetics (Fox *et al.*, 1987; Hagiwara *et al.*, 1988), whereas the HVA calcium channels may be further subdivided into L, N and P subtypes (Snutch & Reiner, 1992). The N-type VOCC antagonist ω-conotoxin GVIA has been shown to be a potent inhibitor of adrenergic and cholinergic neurotransmission in the peripheral nervous system (Keith *et al.*, 1989; Pruneau & Angus, 1990; Zygmunt & Högestätt, 1993). However, there are indications that the susceptibility of ω-conotoxin may differ between NANC nerves on one hand and

[1] Author for correspondence.

adrenergic and cholinergic nerves on the other hand. In the rat bladder, the cholinergic component of the biphasic contractile response to electrical field stimulation (EFS) was more inhibited by ω-conotoxin than was the NANC response (Maggi, 1991). However, in the guinea-pig electrically-stimulated vas deferens, the ATP-mediated component of the biphasic contraction was found to be more susceptible to ω-conotoxin than was the adrenergic component (Hata et al., 1992). In neither of these studies was a full concentration-response curve for ω-conotoxin obtained.

The rabbit urethra exhibits both an adrenergic contraction and a NANC relaxation in response to EFS (Andersson et al., 1983). Such responses can also be elicited in preparations of the rabbit urethral lamina propria (Mattiasson et al., 1985; Zygmunt et al., 1993). In rabbit detrusor muscle, EFS elicits a contraction consisting of both a cholinergic and a NANC component (Ambache & Zar, 1970). The aim of the present study was to investigate whether ω-conotoxin GVIA in the rabbit lower urinary tract affects differently neurotransmission mediated by NANC nerves on one hand, and adrenergic and cholinergic nerves on the other. Therefore, we compared the effects of ω-conotoxin GVIA and some other subtype selective calcium channel blockers on NANC relaxation and adrenergic contractions in the urethra, and cholinergic and NANC contractile responses in the detrusor of the rabbit.

Methods

Tissue preparation

Female rabbits (New Zealand White) with an average weight of 3 kg were stunned by a blow on the head and exsanguinated. The abdomen was opened and the bladder and urethra were removed and placed in ice cold Krebs solution (for composition, see below). The bladder and urethra were opened longitudinally and separated by a transverse cut at the level of the bladder neck. The vesical mucosa was removed and the detrusor muscle tissue was cut into longitudinally-oriented strip preparations (1 × 2 × 5 mm). The lamina propria of the urethra was separated from the smooth muscle layers under the microscope, and the strip preparations (1 × 2 × 5 mm) were prepared as described previously (Zygmunt et al., 1993). Urethral circular muscle strips were prepared from the middle and proximal parts of the urethra (Andersson et al., 1983). The strips were transferred to organ baths (5 ml), containing Krebs solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5 and glucose 11. The Krebs solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 37°C, resulting in a pH of 7.4. The preparations were suspended between two L-formed hooks by means of silk ligatures. One of the hooks was connected to a movable unit allowing adjustment of tension, and the other to a Grass Instruments FTO3C force transducer. The isometric tension was displayed on a Grass Instruments model 7 D polygraph. The resting tension of the preparations was adjusted to 4 mN during an equilibration period of 1 h.

Experimental procedure

EFS was achieved by two platinum wire electrodes placed parallel to the preparations in the organ bath. Square wave pulses with a duration of 0.3 ms were delivered at a frequency of 1 to 30 Hz in 5 s trains at 2 min intervals. Optimum voltage was determined individually for each preparation by increasing the voltage until a maximum response at 10 Hz (relaxations of urethral smooth muscle and lamina propria, and contractions of the detrusor) or 25 Hz (contractions of urethral smooth muscle) was obtained. The polarity was changed after each pulse to minimize oxidation of the electrodes.

In order to study relaxant responses, the preparations were precontracted with 1 nM arginine vasopressin (AVP). This concentration, which produced approximately 50% of the maximal response in lamina propria, was chosen because it produced stable contractions. Phentolamine (1 μM) and propranolol (1 μM) were present in all these experiments. Contractile responses in urethral smooth muscle were examined in the presence of 0.3 mM L-NOARG. Frequency-response relationships were studied by stepwise increasing the frequency of stimulation from 1 to 30 Hz. The same preparation was then treated with ω-conotoxin (0.1 μM) for 20 min, and the stimulation procedure repeated with the same frequencies. The effects of increasing concentrations of ω-conotoxin on electrically-induced responses were studied at a frequency of 10 Hz (relaxations of urethral smooth muscle and lamina propria, and contractions of the detrusor) or 25 Hz (contractions of urethral smooth muscle). The drug was applied cumulatively when reproducible responses were obtained. Experiments run in parallel in the absence of ω-conotoxin served as controls. TTX (1 μM) was added at the end of each experiment to obtain an estimate of the direct muscle stimulation. Responses to cumulatively added noradrenaline (urethral smooth muscle), 3-morpholino-sydnonimin (SIN-1, urethral smooth muscle and lamina propria), carbachol (detrusor), and a single concentration (1 mM) of ATP (detrusor) were examined before and 20 min after exposure to ω-conotoxin.

Drugs

(−)-Noradrenaline hydrochloride (Sigma, St Louis, U.S.A.) was dissolved in 0.9% NaCl, containing 1 mM ascorbic acid. Prazosin hydrochloride (Pfizer, N.Y., U.S.A.) was dissolved in 10% lactic acid. (±)-Propranolol hydrochloride, (−)-scopolamine hydrochloride, carbamylcholine chloride (carbachol), adenosine 5'-triphosphate (ATP), N^{ω}-nitro-L-arginine (L-NOARG), arginine vasopressin acetate (AVP), tetrodotoxin (TTX), ω-conotoxin GVIA, nickel chloride (Sigma, St Louis, U.S.A.), phentolamine methane sulphonate (Ciba-Geigy, Basel, Switzerland) and 3-morpholino-sydnonimin hydrochloride (SIN-1; Casella AG, Germany) were dissolved in distilled water. (±)-Nimodipine (Bayer, Leverkusen, Germany) and (±)-tetramethrin (Nanogen, Watsonville, U.S.A.) were dissolved in absolute ethanol.

Calculations and statistics

Relaxant responses were expressed as a percentage of the response obtained before application of the test drug. When the test drug reduced the baseline tension, R/T values were calculated according to Garcia-Pascual et al. (1991). R/T values express the relaxation (R) as a percentage of the tension (T) recorded immediately before each train of stimulation. The negative logarithm of the drug concentration eliciting half maximum inhibition (pIC_{50}) was determined by linear regression analysis using the values immediately above and below half maximum response. I_{max} refers to the maximum inhibition obtained. The results are expressed as mean values ± s.e.mean, and n denotes the number of experiments (animals) performed. Statistical analysis was performed by using Student's t test (two-tailed). Statistical significance was accepted when $P < 0.01$.

Results

Relaxant responses in urethral smooth muscle and lamina propria

In AVP-contracted (1 nM) urethral smooth muscle and lamina propria, EFS elicited frequency-dependent relaxations (Figure 1). Half maximum relaxation was obtained at a

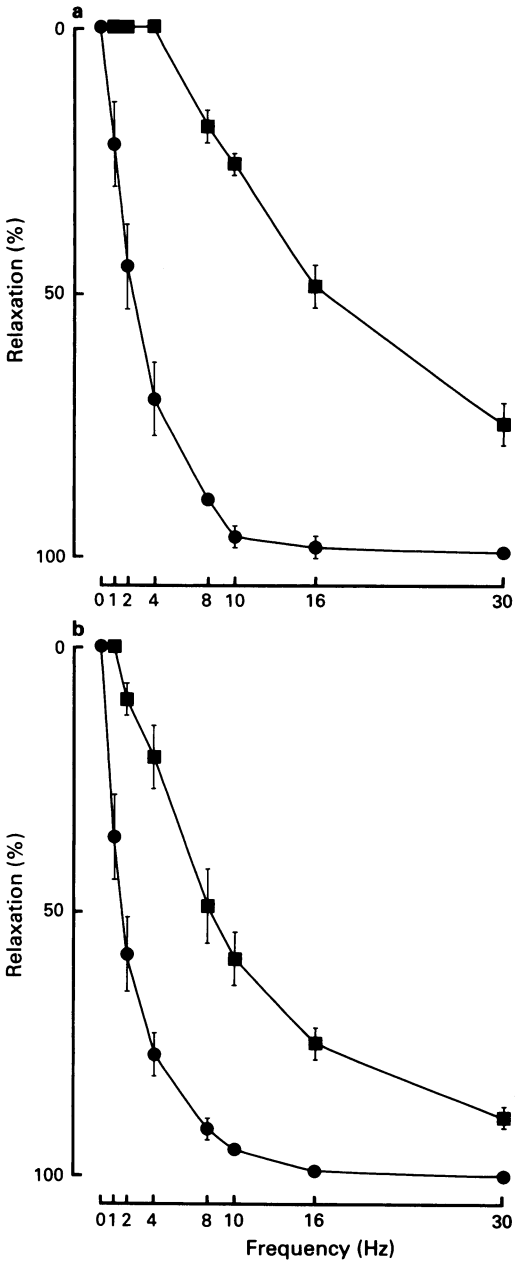


Figure 1 Frequency-dependent non-adrenergic, non-cholinergic relaxation in rabbit urethral smooth muscle (a) and lamina propria (b) precontracted with 1 nM arginine vasopressin before (●) and after (■) application of 0.1 μM ω-conotoxin. Preparations were treated with ω-conotoxin for 20 min before the second stimulation period with the different frequencies. Results are expressed as percentage of the maximum relaxation obtained during the first stimulation period, and given as mean ± s.e.mean ($n = 5$).

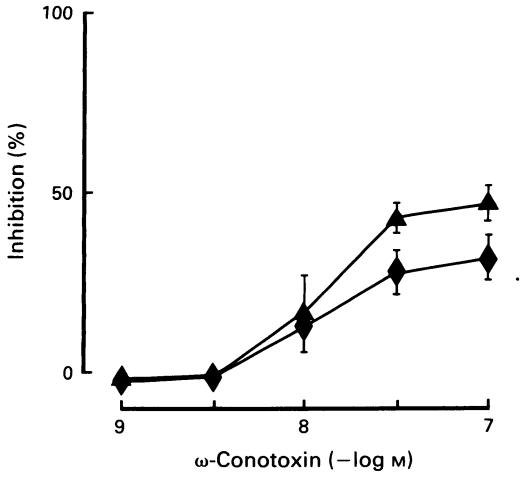


Figure 2 Inhibitory effect of cumulatively added ω-conotoxin on electrically-induced (10 Hz) non-adrenergic, non-cholinergic relaxation in rabbit urethral smooth muscle (▲) and lamina propria (◆). Preparations were precontracted with 1 nM arginine vasopressin. As shown in parallel controls, the electrically-induced relaxations were reproducible throughout the duration of the experiments (not shown). Responses are expressed as percentage of the maximum relaxation before application of ω-conotoxin, and given as mean ± s.e.mean ($n = 6$).

frequency of approximately 2 Hz. The maximum relaxation amounted to 73% and 89% of the AVP-induced contraction in the urethral smooth muscle and lamina propria, respectively. In the presence of 0.1 μM ω-conotoxin, the relaxation was significantly reduced (Figure 1). The inhibition was larger the lower the frequency of stimulation. An ω-conotoxin-resistant relaxation was observed at frequencies higher than 1 Hz (lamina propria) and 4 Hz (urethral smooth muscle), and at 30 Hz stimulation this relaxation was 89% and 75% of the maximum relaxation, respectively. TTX (1 μM) abolished the relaxation (tested at 30 Hz) both in the absence and presence of ω-conotoxin.

The relaxant responses at 10 Hz stimulation amounted to 96% (urethral smooth muscle) and 95% (lamina propria) of the maximum relaxation. These relaxations were concentration-dependently inhibited by ω-conotoxin (Figure 2, Table 1). The pIC_{50} values in the urethral smooth muscle and lamina propria did not differ significantly (Table 1). As can be seen in Figure 2, a significant portion of the relaxation remained in the presence of 0.1 μM ω-conotoxin; 53% (urethral smooth muscle) and 68% (lamina propria). TTX (1 μM, $n = 6$) abolished the ω-conotoxin-insensitive relaxant response in both types of preparation.

In the lamina propria, nimodipine (0.1 μM) and nickel (0.3 mM) reduced the AVP-induced contraction (Figure 3) by 33 ± 5% ($n = 6$) and 55 ± 6% ($n = 4$), respectively. The effects of nimodipine and nickel were therefore evaluated by comparing R/T values (see Methods). These values were not significantly different before and after application of nimodipine (81 ± 6% versus 84 ± 6%, $n = 6$) or nickel (81 ± 6% versus 106 ± 13%, $n = 4$). Tetramethrin (0.3 μM, $n = 3$) did not affect the AVP-induced contraction nor the relaxation elicited by EFS. The NO donor, SIN-1, relaxed both types of preparation ($n = 5-6$) contracted by AVP (1 nM) in a concentration-dependent manner. At the highest SIN-1 concentration tested (0.1 mM), the relaxation amounted to 81 ± 7% and 92 ± 8% of the AVP-induced contraction in the urethral smooth muscle and lamina propria, respectively. ω-Conotoxin (0.1 μM) did not affect the SIN-1-induced relaxation in either type of preparation ($n = 5-6$).

Contractile responses in urethral smooth muscle

As shown previously (Andersson *et al.*, 1983) and confirmed in the present study ($n = 5$, data not shown), EFS induced frequency-dependent contractions in urethral smooth muscle preparations at resting tension. These contractions were virtually abolished by 1 μM prazosin ($n = 3$). Since the frequency-response curve did not reach a plateau, the frequency for half maximum contraction (>14 Hz) could only be estimated. Contractions elicited by frequencies between 4–30 Hz were all significantly inhibited by 0.1 μM ω-conotoxin ($n = 5$). The relative inhibition amounted to 100% (4 Hz), 91% (8 Hz), 86% (12 Hz), 84% (16 Hz), 74% (25 Hz) and 69% (30 Hz). Thus an ω-conotoxin-resistant response was obtained with increasing frequencies of stimulation (≥ 12 Hz) and amounted at 30 Hz to 31%.

Table 1 Inhibitory effects of ω-conotoxin and tetrodotoxin (TTX) on electrically-induced adrenergic, cholinergic and NANC responses

	n	ω-Conotoxin pIC_{50}	ω-Conotoxin I_{max} (%)	TTX (1 μM) (%)
Relaxant responses				
Lamina propria (10 Hz)[a]	6	7.9 ± 0.1	32 ± 6	100 ± 0
Urethral smooth muscle (10 Hz)[a]	6	7.9 ± 0.1[b]	47 ± 5	100 ± 0
Contractile responses				
Urethral smooth muscle (25 Hz)[a]	6	8.9 ± 0.1[b]	77 ± 4	95 ± 1
Detrusor (10 Hz)[a]				
In the absence of scopolamine	7	8.7 ± 0.1[c]	88 ± 2	90 ± 2
In the presence of scopolamine (1 μM)	7	8.2 ± 0.1[c]	85 ± 2	87 ± 4

[a]Frequency of stimulation.
[b,c]Significant ($P < 0.01$).
pIC_{50} = negative logarithm of the ω-conotoxin concentration producing half maximum inhibition. I_{max} = maximum inhibition expressed as percentage reduction of the maximum response.
Results are expressed as mean values ± s.e.mean, and n indicates the number of preparations (animals) examined.

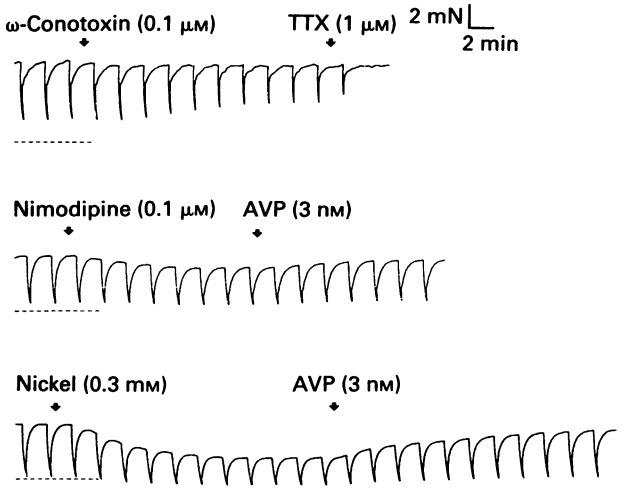


Figure 3 Original tracings showing the effects of 0.1 μM ω-conotoxin (upper panel), 0.1 μM nimodipine (middle panel) and 0.3 mM nickel (lower panel) on electrically-induced (10 Hz) non-adrenergic, non-cholinergic relaxations in rabbit urethral lamina propria. Preparations were precontracted with 1 nM arginine vasopressin (AVP). The second arrow indicates application of tetrodotoxin (TTX) or a higher concentration of AVP. Broken line denotes the baseline tension before application of AVP.

The sensitivity to ω-conotoxin was studied at a frequency of 25 Hz. At this frequency, the contraction was 82% of the response to the highest frequency tested (30 Hz). A small TTX (1 μM)-resistant contraction (5 ± 1%, $n = 5$) remained at 25 Hz; this component increased with increasing stimulation frequencies (data not shown). ω-Conotoxin concentration-dependently inhibited the contractions. The pIC_{50} value for ω-conotoxin was significantly larger for the contractile than for the relaxant responses in the urethra (Table 1). Contractile responses to noradrenaline (0.01 μM– 0.1 mM, $n = 4$) were not affected by 0.1 μM ω-conotoxin (data not shown).

Contractile responses in the detrusor

EFS induced frequency-dependent contractions in the detrusor ($n = 5$, data not shown). Half maximum contraction was obtained at a frequency of approximately 6 Hz. A small TTX (1 μM)-resistant contraction (6 ± 1%, $n = 5$) remained at the highest frequency used (30 Hz). Contractions elicited by frequencies between 4–30 Hz were all significantly reduced by 0.1 μM ω-conotoxin ($n = 5$). The relative inhibition amounted to 88% (4 Hz), 84% (8 Hz), 81% (12 Hz), 78% (16 Hz), 69% (20 Hz) and 53% (30 Hz). Thus an ω-conotoxin-resistant response was obtained with increasing frequencies of stimulation (≥ 20 Hz) and amounted at 30 Hz to 47%.

The contractions obtained at 10 Hz stimulation amounted to 70% of the maximum response. Scopolamine reduced these contractions by 71 ± 3% ($n = 7$). ω-Conotoxin inhibited the response in a concentration-dependent manner in both the absence and presence of 1 μM scopolamine (Table 1). The pIC_{50} value for ω-conotoxin was significantly larger for the 'total' contraction than for the NANC contraction. Contractile responses to carbachol (0.01 μM–0.1 mM, $n = 4$) and 1 mM ATP ($n = 4$) were unaffected by 0.1 μM ω-conotoxin (data not shown).

Discussion

The morphology of the lamina propia and smooth muscle preparations of the rabbit urethra differ in that the former is rich in connective tissue and blood vessels (Mattiasson *et al.*, 1985). NOS localized to neurones has been demonstrated by NADPH diaphorase staining around arteries and in the smooth muscle bundles in the rabbit urethral lamina propria (Zygmunt *et al.*, 1993). NOS has also been demonstrated by immunohistochemistry and/or NADPH diaphorase staining in rat and pig urethral smooth muscle (Burnett *et al.*, 1992; Larsson *et al.*, 1992; Alm *et al.*, 1993). Even though similar studies on the rabbit urethral smooth muscle have not to our knowledge been published, the observation that the electrically-induced NANC relaxation in rabbit urethral smooth muscle and lamina propria was sensitive to L-NOARG and TTX supports the existence of NO releasing nerves in both these preparations (Andersson *et al.*, 1991; 1992; Zygmunt *et al.*, 1993). In the present study, ω-conotoxin inhibited the relaxation with similar potency in both types of preparation. The inhibitory effect was not due to an interaction with the NO effector system, since ω-conotoxin did not affect the relaxation induced by SIN-1. The inhibition, however, decreased with increasing frequencies of stimulation, and at 10 Hz stimulation, a large ω-conotoxin-resistant component remained in the urethral smooth muscle (53%) and lamina propria (68%).

In the lamina propria, nimodipine and nickel reduced the AVP-induced contraction. The effects of these substances on EFS-induced relaxation were therefore evaluated by comparing R/T values before and after their application. A positive correlation has been demonstrated between the amplitude of the electrically-induced relaxation (R) and the agonist-induced tension (T), i.e. the higher the contraction amplitude

the greater the relaxant response (Garcia-Pascual *et al.*, 1991). As shown in the present study, it was possible to regain the initial tension and relaxation recorded before addition of nimodipine or nickel by increasing the AVP concentration. Thus, the effects of nickel and nimodipine on the relaxation were considered to be attributable to the reduction of the AVP-induced tension rather than to inhibition of neurotransmitter release. Nickel is known for its dual action on L and T channels, although it is claimed to have some selectivity towards the T channel (Fox *et al.*, 1987; Hagiwara *et al.*, 1988). However, it is not probable that the effect of nickel on the AVP-induced contraction was caused by T channel blockade in the present study, since the T channel blocker, tetramethrin, had no effect. The tetramethrin concentration used in this study was three times higher than that previously shown to block T channel currents in the rabbit sinoatrial node (Hagiwara *et al.*, 1988). Our results suggest that neither L nor T channels are involved in the NO release process. Furthermore, it appears that influx of calcium through ω-conotoxin-sensitive VOCCs is only partially responsible for NANC relaxation in urethral smooth muscle and lamina propria at stimulation frequencies higher than 1 Hz (lamina propria) and 4 Hz (urethral smooth muscle).

The ω-conotoxin-resistant relaxation may have been caused by calcium influx through yet another type of VOCC (i.e. a non-L, N and T channel). A third HVA calcium channel, insensitive to ω-conotoxin and dihydropyridines, has been demonstrated in cerebellar Purkinje cells and therefore denoted P (Llinas *et al.*, 1989). Lundy *et al.* (1992) recently suggested that P channels were responsible for calcium influx in mammalian brain synaptosomes, since the influx was only slightly reduced by ω-conotoxin and dihydropyridines, but blocked to a greater extent by the venom from the spider *Hololena curta*. Another venom from the spider *Grammostola spatula* blocked calcium influx in rat synaptosomes, [^{3}H]-D-aspartate release from rat hippocampal brain slices and the whole cell calcium current of guinea-pig hippocampal CA1 neurones to a greater extent than did ω-conotoxin or dihydropyridine calcium antagonists (Keith *et al.*, 1992). It has also been suggested that P channels mediate calcium influx into mouse motor nerve terminals, since neuromuscular transmission was blocked by the funnel-web spider venom FTX, but not by ω-conotoxin and dihydropyridine calcium antagonists (Uchitel *et al.*, 1992). There is also evidence of a VOCC-independent release of VIP from rat enteric nerve and noradrenaline and GABA from rat brain slices (Minchin, 1980; Sandoval, 1980; Schoffelmeer & Mulder, 1983; Belai *et al.*, 1987). It was proposed that an intracellular Na^+ accumulation in the nerve endings may increase the cytoplasmic calcium level through a Na^+/Ca^{2+} exchange mechanism with intracellular calcium stores, e.g., in mitochondria (Minchin, 1980; Sandoval, 1980; Schoffelmeer & Mulder, 1983).

In accordance with a previous study by Andersson *et al.* (1983), electrically-induced contractile responses in the urethral smooth muscle were virtually abolished by TTX or α-adrenoceptor blockade, suggesting that the contractions were caused by neuronal release of mainly noradrenaline. ω-Conotoxin inhibited the contractions in a concentration-dependent manner without affecting the exogenous response to noradrenaline. The adrenergic contraction was approximately 10 times more sensitive to ω-conotoxin than was the NANC relaxation in the same preparation. The responses also differed with regard to the frequency-response relationship and the magnitude of the ω-conotoxin-resistant component, which appeared to be less prominent for the adrenergic than for the NANC responses. This may indicate that calcium influx through N-type VOCCs is more important for transmitter release in adrenergic than in NANC nerves in the rabbit urethra. Only approximately 20% of the EFS-induced contraction remained in the presence of ω-conotoxin at 25 Hz stimulation. Since TTX and prazosin virtually abolished this response, the ω-conotoxin-resistant contraction could not be attributed to a direct electrical stimulation of the smooth muscle. As shown by Larsson *et al.* (1984), the electrically-induced [^{3}H]noradrenaline release in the rabbit urethra was insensitive to the L channel blocker, nifedipine. This does not favour the view that influx of calcium through neuronal L channels was involved in the ω-conotoxin-resistant adrenergic response.

The electrically-induced contractile response in the rabbit detrusor consists of an atropine-sensitive and an atropine-resistant component, which have been attributed to neuronal release of acetylcholine and ATP, respectively (Ambache & Zar, 1970; Igawa *et al.*, 1993). A relatively small scopolamine-resistant component was found in the present study at 10 Hz stimulation. ω-Conotoxin concentration-dependently inhibited and eventually abolished the cholinergic and NANC contractile responses at this frequency. The effect of ω-conotoxin was considered to be prejunctional, since the contractile responses to carbachol and ATP were unaffected. A small but significant difference in the sensitivity to ω-conotoxin was found between the cholinergic and NANC contractile responses; the 'total' contraction was approximately three times more sensitive to ω-conotoxin than the scopolamine-resistant component. In the rat bladder, the cholinergic component was also inhibited more by ω-conotoxin than was the atropine-resistant response (Maggi, 1991).

The results of the present study demonstrate differences between adrenergic, cholinergic and NANC nerves with regard to the frequency-response relationship and the susceptibility to inhibition by ω-conotoxin in the rabbit lower urinary tract. The stimulation frequency for half maximum response was significantly smaller for the NANC relaxation (2 Hz) than for the contractile responses in the urethra (>14 Hz) and detrusor (6 Hz). ω-Conotoxin concentration-dependently inhibited the adrenergic contraction and NANC relaxation in the urethra, and the cholinergic and NANC contractions in the detrusor. The NANC responses in the urethra and detrusor appeared to be less sensitive to ω-conotoxin than the adrenergic and cholinergic contractions, respectively. An ω-conotoxin-resistant response was observed in all preparations. This response increased with increasing frequencies of stimulation, and was more prominent for the NANC relaxation than for the adrenergic and cholinergic contractions. Calcium influx through neuronal L or T channels did not seem to be involved in the ω-conotoxin-resistant relaxation in the lamina propria. This component may have been caused by calcium influx through another type of neuronal VOCC, e.g., a P channel, or by calcium mobilized via routes independent of VOCCs.

This work was supported by the Swedish Medical Research Council (no 06837) and the Medical Faculty at the University of Lund.

References

ALM, P., LARSSON, B., EKBLAD, E., SUNDLER, F. & ANDERSSON, K.-E. (1993). Immunohistochemical localization of peripheral nitric oxide synthase-containing nerves using antibodies raised against synthesized C- and N-terminal fragments of a cloned enzyme from rat brain. *Acta Physiol. Scand.*, **148**, 421–429.

AMBACHE, N. & ZAR, M.A. (1970). Non-cholinergic transmission by post-ganglionic motor neurones in the mammalian bladder. *J. Physiol.*, **210**, 761–783.

ANDERSSON, K.-E., MATTIASSON, A. & SJÖRGREN, C. (1983). Electrically induced relaxation of the noradrenaline contracted isolated urethra from rabbit and man. *J. Urol.*, **129**, 63–67.

ANDERSSON, K.-E., GARCIA PASCUAL, A., FORMAN, A. & TÖTTRUP, A. (1991). Non-adrenergic, non-cholinergic nerve-mediated relaxation of rabbit urethra is caused by nitric oxide. *Acta Physiol. Scand.*, **141**, 133–134.

ANDERSSON, K.-E., GARCIA PASCUAL, A., PERSSON, K., FORMAN, A. & TÖTTRUP, A. (1992). Electrically-induced, nerve-mediated relaxation of rabbit urethra involves nitric oxide. *J. Urol.*, **147**, 253–259.

BELAI, A., RALEVIC, A. & BURNSTOCK, G. (1987) VIP release from enteric nerves is independent of extracellular calcium. *Regul. Pept.*, **19**, 79–89.

BREDT, D.S., HWANG, P.M., GLATT, C.E., LOWENSTEIN, C., REED, R.R. & SNYDER, S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, **351**, 714–718.

BURNETT, A.L., LOWENSTEIN, C.J., BREDT, D.S., CHANG, T.S.K. & SNYDER, S.H. (1992). Nitric oxide: a physiologic mediator of penile erection. *Science*, **257**, 401–403.

DOCKRAY, G.J. (1992). Transmission: Peptides. In *Autonomic Neuroeffector Mechanisms*. ed. Burnstock, G. & Hoyle, C.H.V., pp. 409–464. Chur, Switzerland: Harwood Academic Publishers.

FÖRSTERMANN, U., SCHMIDT, H.H.H.W., POLLOCK, J.S., SHENG, H., MITCHELL, J.A., WARNER, T.D., NAKANE, M. & MURAD, F. (1991). Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem. Pharmacol.*, **42**, 1849–1857.

FOX, A.P., NOWYCKY, M.C. & TSIEN, R.W. (1987). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *J. Physiol.*, **394**, 149–172.

GARCIA-PASCUAL, A., COSTA, G., GARCIA-SACRISTAN, A. & ANDERSSON, K.-E. (1991). Relaxation of sheep urethral muscle induced by electrical stimulation of nerves: involvement of nitric oxide. *Acta Physiol. Scand.*, **141**, 531–539.

HAGIWARA, N., IRISAWA, H. & KAMEYAMA, M. (1988). Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. *J. Physiol.*, **395**, 233–253.

HATA, F., FUJITA, A., SAEKI, K., KISHI, I., TAKEUCHI, T. & YAGASAKI, O. (1992). Selective inhibitory effects of calcium channel antagonists on the two components of the neurogenic response of guinea-pig vas deferens. *J. Pharmacol. Exp. Ther.*, **263**, 214–220.

IGAWA, Y., MATTIASSON, A. & ANDERSSON, K.-E. (1993). Functional importance of cholinergic and purinergic neurotransmission for micturition contraction in the normal, unanaesthetized rat. *Br. J. Pharmacol.*, **198**, 473–479.

IGNARRO, L.J. (1989). Endothelium-derived nitric-oxide: actions and properties. *FASEB J.*, **3**, 31–36.

KEITH, R.A., MANGANO, T.J., PACHECO, M.A. & SALAMA, A.I. (1989). Characterization of the effects of ω-conotoxin GVIA on the responses of voltage-sensitive calcium channels. *J. Auton. Pharmacol.*, **9**, 243–252.

KEITH, R.A., DEFEO, P.A., LAMPE, R.A., SPENCE, K.T., LO, M.M.S. & FFRENCH-MULLEN, J.M.H. (1992). Inhibition of neuronal calcium channels by a fraction from *grammostola spatulata* venom. *Pharmacol. Commun.*, **1**, 19–25.

LARSSON, B., ALM, P., PERSSON, K. & ANDERSSON, K.-E. (1992). Studies on the localization of some neurotransmitters, nerve markers, and NADPH diaphorase activity in tissues from the pig lower urinary tract. *Neurourol. Urodyn.*, **11**, 444–445.

LARSSON, B., HÖGESTÄTT, E.D., MATTIASSON, A. & ANDERSSON, K.-E. (1984). Differential effects of nifedipine, verapamil, and diltiazem on noradrenaline-induced contractions, adrenergic transmitter release, and alpha-adrenoceptor binding in the female rabbit urethra. *Naunyn-Schmied. Arch. Pharmacol.*, **326**, 14–21.

LLINAS, R., SUGIMORI, M., LIN, J.-W. & CHERKSEY, B. (1989). Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 1689–1693.

LUNDY, P.M., HONG, A. & FREW, R. (1992). Inhibition of a dihydropyridine, ω-conotoxin insensitive Ca^{2+} channel in rat synaptosomes by venom of the spider *Hololena curta*. *Eur. J. Pharmacol.*, **225**, 51–56.

MAGGI, C.A. (1991). Omega conotoxin and prejunctional modulation of the biphasic response of the rat isolated urinary bladder to single pulse electrical field stimulation. *J. Auton. Pharmacol.*, **11**, 295–304.

MATTIASSON, A., ANDERSSON, K.-E. & SJÖGREN, C. (1985). Contractant and relaxant properties of the female rabbit urethral submucosa. *J. Urol.*, **133**, 304–310.

MINCHIN, M.C.W. (1980). The role of Ca^{2+} in the protoveratrine-induced release of γ-aminobutyrate from rat brain slices. *Biochem. J.*, **190**, 333–339.

MÜLSCH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^5-[imino-(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmied. Arch. Pharmacol.*, **341**, 143–147.

MURAD, F., FÖRSTERMANN, U., NAKANE, M., SCHMIDT, H., POLLOCK, J., SHENG, H., MATSUMOTO, T., WARNER, T., MITCHELL, J., TRACEY, R. & BUECHLER, W. (1992). The nitric oxide-cyclic GMP signal transduction pathway in vascular smooth muscle preparations and other tissues. *Jpn. J. Pharmacol.*, **58**, 150P–157P.

MYERS, P.R., MINOR Jr, R.L., GUERRA Jr, R., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345**, 161–163.

PERSSON, K. & ANDERSSON, K.-E. (1992). Nitric oxide and relaxation of pig lower urinary tract. *Br. J. Pharmacol.*, **106**, 416–422.

PRUNEAU, D. & ANGUS, J.A. (1990). ω-Conotoxin GVIA is a potent inhibitor of sympathetic neurogenic responses in rat small mesenteric arteries. *Br. J. Pharmacol.*, **100**, 180–184.

SANDOVAL, M.E. (1980). Sodium-dependent efflux of [^{3}H]GABA from synaptosomes probably related to mitochondrial calcium mobilization. *J. Neurochem.*, **35**, 915–921.

SCHOFFELMEER, A.N.M. & MULDER, A.H. (1983). [^{3}H]noradrenaline release from brain slices induced by an increase in the intracellular sodium concentration: role of intracellular calcium stores. *J. Neurochem.*, **40**, 615–621.

SHER, E., BIANCARDI, E., PASSAFARO, M. & CLEMENTI, F. (1991). Physiopathology of neuronal voltage-operated calcium channels. *FASEB J.*, **5**, 2677–2683.

SNEDDON, P. & GRAHAM, A. (1992). Role of nitric oxide in the autonomic innervation of smooth muscle. *J. Auton. Pharmacol.*, **12**, 445–456.

SNUTCH, T.P. & REINER, P.B. (1992). Ca^{2+} channels: diversity of form and function. *Curr. Opinion Neurobiol.*, **2**, 247–253.

SNYDER, S.H. (1992). Nitric oxide: first in a new class of neurotransmitters? *Science*, **257**, 494–496.

UCHITEL, O.D., PROTTI, D.A., SANCHEZ, V., CHERKSEY, B.D., SUGIMORI, M. & LLINAS, R. (1992). P-type voltage-dependent calcium channel mediates presynaptic calcium influx and transmitter release in mammalian synapses. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3330–3333.

VANIN, A.F. (1991). Endothelium-derived relaxing factor is a nitrosyl iron complex with thiol ligands. *FEBS*, **289**, 1–3.

VEDERNIKOV, Y.P., MORDVINTCEV, P.I., MALENKOVA, I.V. & VANIN, A.F. (1992). Similarity between the vasorelaxing activity of dinitrosyl iron cysteine complexes and endothelium-derived relaxing factor. *Eur. J. Pharmacol.*, **211**, 313–317.

ZYGMUNT, P.K.E., PERSSON, K., ALM, P., LARSSON, B. & ANDERSSON, K.-E. (1993). The L-arginine/nitric oxide pathway in the rabbit urethral lamina propria. *Acta Physiol. Scand.*, **148**, 431–439.

ZYGMUNT, P.M. & HÖGESTÄTT, E.D. (1993). Calcium channels at the adrenergic neuroeffector junction in the rabbit ear artery. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, 617–623.

(Received April 13, 1993
Revised July 16, 1993
Accepted July 23, 1993)

Br. J. Pharmacol. (1993), **110**, 1291–1296 © Macmillan Press Ltd, 1993

Solubilization and characterization of $GABA_B$ receptor binding sites from porcine brain synaptic membranes

M. Facklam & [1]N.G. Bowery

Department of Pharmacology, School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX

1 The characteristics of membrane bound $GABA_B$ receptors in pig brain are similar to those in rat brain as judged by *in vitro* binding experiments and sensitivity to GTP. The rank order of affinity of $GABA_B$ receptor ligands was CGP 54626 > GABA ~(−)-baclofen >> CGP 35348 = CGP 36742 > (+)-baclofen in membranes from both species.

2 For solubilization of $GABA_B$ receptors from pig brain, washed membranes were preincubated with 5 mM $MgSO_4$ and subsequently incubated with various detergents. 3-[(3-Cholamidopropyl)dimethylammoniol]-1-propane sulphonate (CHAPS) (0.5%) proved to be the most successful, solubilizing 22.7 ± 4.7% (mean ± s.e.mean, $n = 6$) of $GABA_B$ receptors.

3 Binding of [^{3}H]-GABA to $GABA_B$ receptors solubilized with 0.5% CHAPS exhibited similar characteristics to the binding at membrane bound receptors since, firstly, the K_d and B_{max} values (around 30 nM and 450 fmol mg^{-1} protein, respectively) were comparable; secondly, stereospecific binding for baclofen was obtained in both forms; thirdly, the affinity for the agonists GABA and (−)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626 were the same; fourthly, comparable sensitivity to Ca^{2+} (2.5 mM) was observed and finally, a similar sensitivity to GTP was apparent.

4 Saturation experiments performed with the $GABA_B$ antagonist, [^{3}H]-CGP 54626, indicated a higher K_d value and a lower B_{max} value for solubilized (7.7 ± 2.6 nM and 1033 ± 41 fmol mg^{-1} protein, mean ± s.e.mean, $n = 3$) than for membrane bound receptors (1.35 ± 0.08 nM, 1171 ± 20 fmol mg^{-1} protein, $n = 3$).

Keywords: γ-Aminobutyric acid; $GABA_B$ receptor; solubilization; baclofen; CGP 35348; CGP 36742; CGP 54626

Introduction

$GABA_B$ receptors in the mammalian brain are coupled to adenylyl cyclase, Ca^{2+} channels and K^+ channels via G-proteins. But at present little information is available about the possible structure of the receptor. The affinity of agonists for $GABA_B$ receptors is decreased by guanyl nucleotides, in particular guanosine 5′ triphosphate (GTP, Hill *et al.*, 1984), and pertussis toxin, which ADP ribosylates the associated G-protein (Asano *et al.*, 1985; Knott *et al.*, 1993).

Kuriyama and colleagues have recently described the solubilization and purification of $GABA_B$ receptors from bovine brain. To solubilize the receptors, they used either 0.2% deoxycholate sodium (DOC Na) plus 1 M KCl (Ohmori *et al.*, 1990) or 1.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) plus 0.2% asolectin (Ohmori & Kuriyama, 1990). Treatment with DOC Na completely eliminated the stimulating effects of calcium ions as well as the inhibitory effects of GTP on $GABA_B$ receptor binding. Since they did not characterize their solubilized binding sites, we are currently performing experiments to characterize the sites solubilized from porcine brain.

The aim of the present study was therefore to solubilize $GABA_B$ receptors in a GTP-sensitive form, which bind agonists and antagonists with similar affinities to membrane bound receptors. Part of our results have already been presented in abstract form to the British Pharmacological Society (Facklam & Bowery, 1993).

Methods

Tissue preparation

Whole pig brains were obtained from a local slaughterhouse, transported in ice-cold 0.32 M sucrose, dissected free from white matter and stored at − 80°C. Crude synaptic membranes were prepared from pig cerebrum according to the method of Zukin *et al.* (1974). All steps were carried out on ice, solutions were kept at 4°C. The tissue was thawed and homogenized in 0.32 M sucrose + 0.1 mM phenylmethylsulphonylfluoride (PMSF) + 20 μg ml^{-1} bacitracin (1 g tissue per 6 ml) in a Potter-Elvehjem homogenizer fitted with a teflon pestle. The homogenate was centrifuged for 10 min at 1600 *g*, the supernatant collected and recentrifuged for 20 min at 31,000 *g*. The resulting pellet was lysed by resuspension in distilled water at 0.1 mM PMSF + 20 μg ml^{-1} bacitracin and the mixture centrifuged for 20 min at 12,000 *g*. The supernatant was used to rinse off the upper, soft layer of the pellet. The combined suspension was centrifuged for 20 min at 48,000 *g*. The final crude synaptic membranes were resuspended and centrifuged once again with distilled water + PMSF + bacitracin and stored at − 20°C for at least 18 h.

Washing of crude synaptic membranes

Membranes were suspended in ice-cold distilled water (0.5 g original tissue per 40 ml), centrifuged for 10 min at 13,000 *g* and washed twice in distilled water. The pellet was then suspended in 50 mM Tris HCl buffer, pH 7.4, incubated at room temperature for 15 min, centrifuged and washed once again by resuspension, incubation and centrifugation. The washed membranes were either used directly or stored at − 20°C with no loss of binding activity.

Solubilization

Washed membranes were preincubated with 50 mM Tris HCl buffer pH 7.4 + 5 mM $MgSO_4$ for 30 min at room temperature, subsequently incubated at a protein concentration of 2.5–3.5 mg ml^{-1} with various detergents in the presence of 5 mM $MgSO_4$ + 20 μg ml^{-1} bacitracin at 4°C for 90 min and centrifuged for 60 min at 100,000 *g*. The supernatant con-

[1] Author for correspondence.

tained the solubilized fraction. Digitonin was dissolved by heating the solution in a waterbath at 90°C for 20 min.

Dialysis

If inhibition or saturation experiments were performed with the preparations solubilized with 0.5% CHAPS, they were dialysed for 3 h against 100 volumes Tris HCl buffer pH 7.4 + 5 nM $MgSO_4$ + 20 $\mu g\ ml^{-1}$ bacitracin at 4°C (molecular weight cut-off: 12,000–14,000).

[^{3}H]-GABA$_B$ binding

Crude, preincubated (see solubilization) or solubilized preparations (about 0.3 and 0.06 mg protein per tube, respectively) were incubated in the presence of 2.5 mM $CaCl_2$, 40 μM isoguvacine HCl, varying concentrations of unlabelled test compound and [^{3}H]-GABA (5 nM final concentration) in 50 mM Tris HCl buffer, pH 7.4, for 10 min at room temperature. The final volume was 0.8 ml. Nonspecific binding was detected by addition of 100 μM (−)-baclofen. The maximum concentrations of the detergents tolerated were 0.05% for CHAPS and 0.1% for the remainder. In the case of soluble receptors γ-globulin and polyethyleneglycol 8000 were added (final concentration 0.15 w/v% and 16 w/v%, respectively, final volume 1.2 ml). The mixture was left to precipitate for 10 min at room temperature. The assay was terminated by centrifugation at 7 500 *g* for 3 min. The supernatant was aspirated off and the pellet quickly washed twice with 1.5 ml ice cold, distilled water.

The tissue was digested overnight in Soluene-350 solubilizer. The test tube and its contents were transferred to a scintillation vial containing 0.4 ml of 0.2 M HCl and 10 ml Optiphase. The tritium content of each sample was determined by liquid scintillation spectrometry.

[^{3}H]-GABA$_A$ binding

This was performed as [^{3}H]-GABA$_B$ binding with the following exceptions: no $CaCl_2$ was present; 100 μM (−)-baclofen was used to block GABA$_B$ sites and isoguvacine was omitted generally but used to define nonspecific binding (40 μM).

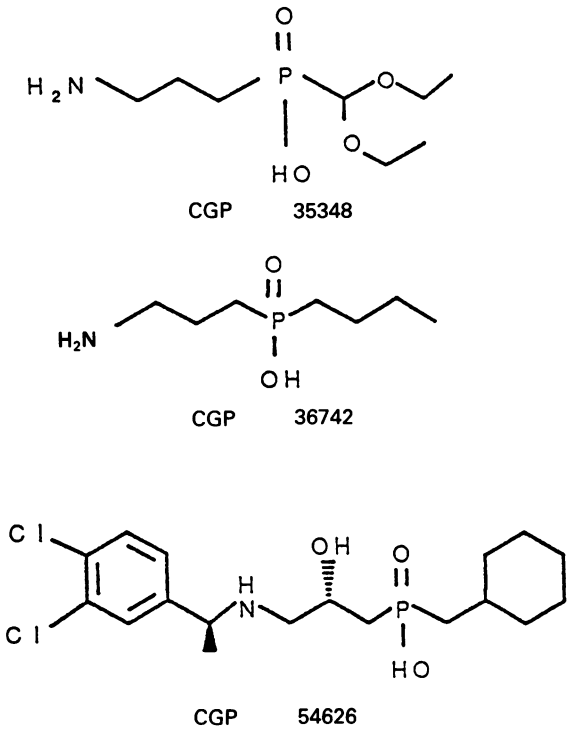


Figure 1 Formulae of GABA$_B$ antagonists from CIBA, Basel.

[^{3}H]-CGP 54626 binding (Bittiger *et al.*, 1992)

This was performed as [^{3}H]-GABA$_B$ binding with the following exceptions: [^{3}H]-CGP 54626 (final concentration: 1 nM for crude and 2 nM for solubilized preparations) was used; no isoguvacine was present; nonspecific binding was assayed in presence of 10 μM CGP 54626; incubation time was 15 min and the mixture was left to precipitate for 1 min after addition of γ-globulin and polyethyleneglycol 8000.

Protein determination

Estimates of the protein content were made by the bicinchoninic acid assay (Dunn, 1989). This assay was found not to interact with any of the detergents used.

Analysis of binding data

Nonspecific binding was subtracted from the total amount of radioactivity bound to yield the amount of ligand specifically bound. In the case of inhibition experiments, specific binding (% of control) was plotted versus the inhibitor concentration and fitted with nonlinear least squares regression analysis to the following equation:

$$y = 100/(1 + (x/IC_{50})^n)$$

where y = specific binding, x = inhibitor concentration, IC_{50} = concentration of inhibitor reducing specific binding by 50% and n = slope factor.

In the case of saturation experiments specific binding (fmol mg^{-1} protein) was plotted versus the free concentration (nM) of the radioligand and fitted with nonlinear least squares regression analysis to the following equation:

$$y = B_{max} * x/(K_d + x)$$

where y = specific binding, x = free concentration of radioligand, B_{max} = maximum amount bound and K_d = affinity constant.

Materials

[^{3}H]-GABA (60 Ci $mmol^{-1}$) was purchased from Amersham, [^{3}H]-CGP 54626 (60 Ci $mmol^{-1}$), CGP 54626, CGP 36742, CGP 35348 (structural formulae, see Figure 1), (−)-baclofen and (+)-baclofen were a generous gift from CIBA, Basel, Switzerland. All other materials were obtained from commercial sources.

Results

Solubilization of GABA$_B$ receptors

For solubilization of the receptors, washed membranes were preincubated with 5 mM $MgSO_4$, subsequently incubated with various detergents and centrifuged for 60 min at 100,000 *g*. Specific GABA$_A$ and GABA$_B$ binding was determined in the pellet and the supernatant, containing the solubilized fraction (Table 1).

Digitonin (1%), sodium cholate (1%) and CHAPS (0.5%) solubilized around 20% of GABA$_B$ receptors. DOC Na produced little bindng in the supernatant whilst reducing the residual binding in the pellet to a low level. By comparison, these detergents, solubilized between 24% and 65% of GABA$_A$ sites. From these three conditions 0.5% CHAPS was chosen for further investigation (Table 2), since it showed the most favourable ratio of GABA$_B$ to GABA$_A$ receptors and the highest concentration of GABA$_B$ sites in the pellet, which might indicate that this detergent maintains the receptor G-protein complex. Adding 1 M KCl to DOC Na, sodium cholate or CHAPS did not increase the recovery of GABA$_B$ sites in the supernatant (data not shown).

Table 1 Solubilization of $GABA_A$ and $GABA_B$ receptors with various detergents

	n	Protein [%]	$GABA_A$ binding pellet [%]	$GABA_A$ binding supernatant [%]	$GABA_B$ binding pellet [%]	$GABA_B$ binding supernatant [%]
Digitonin 0.5%	3	10.9 ± 0.6	108.3 ± 12.5	12.2 ± 6.3	31.6 ± 2.1	0
Digitonin 1.0%	4	32.3 ± 4.4	151.1 ± 22.5	52.5 ± 19	14.2 ± 2.0	19.3 ± 9.6
DOC Na 1.0%	4	54.6 ± 4.4	114.8 ± 2.2	65.5 ± 6.2	4.3 ± 1.6	8.4 ± 3.1
Sodium cholate 1.0%	4	27.5 ± 4.4	34.5 ± 0.2	42.6 ± 7.4	0.3 ± 0.3	20.7 ± 6.8
CHAPS 0.5%	6	20.5 ± 0.8	37.4 ± 5.0	24.3 ± 8.5	40.9 ± 6.2	22.7 ± 4.7

Washed membranes were preincubated with 5 mM $MgSO_4$, subsequently incubated with various detergents and centrifuged for 60 min at 100,000 *g*. Specific $GABA_A$ and $GABA_B$ binding (5 nM [^{3}H]-GABA + 100 μM (−)-baclofen or 40 μM isoguvacine, respectively) was determined in the pellet and the supernatant, containing the solubilized fraction. Values are expressed as a percentage of starting material (mean ± s.e.mean). Experiments were performed in triplicate, *n* equals number of individual experiments.

Table 2 Solubilization of $GABA_A$ and $GABA_B$ receptors with CHAPS under various conditions

	n	Protein [%]	$GABA_A$ binding pellet [%]	$GABA_A$ binding supernatant [%]	$GABA_B$ binding pellet [%]	$GABA_B$ binding supernatant [%]
CHAPS (0.5%), Mg^{2+} absent	4	21.8 ± 0.5	28.4 ± 0.7	33.0 ± 5.9	26.2 ± 4.0	13.7 ± 7.1
CHAPS 0.5%	6	20.5 ± 0.8	37.4 ± 5.0	24.3 ± 8.5	40.9 ± 6.2	22.7 ± 4.7
CHAPS 0.75%	4	24.9 ± 0.7	53.7 ± 6.7	42.4 ± 4.8	37.3 ± 4.7	25.6 ± 3.8
CHAPS 1.0%	4	24.7 ± 1.1	54.8 ± 4.6	45.2 ± 8.4	33.0 ± 3.3	4.8 ± 3.2

Washed membranes were preincubated with 5 mM $MgSO_4$, subsequently incubated with various detergents and centrifuged for 60 min at 100,000 *g*. Specific $GABA_A$ and $GABA_B$ binding (5 nM [^{3}H]-GABA + 100 μM (−)-baclofen or 40 μM isoguvacine, respectively) was determined in the pellet and the supernatant, containing the solubilized fraction. Values are expressed as a percentage of starting material (mean ± s.e.mean). Experiments were performed in triplicates, *n* equals number of individual experiments.

Table 3 Saturation experiments to membrane bound and 0.5% CHAPS-solubilized $GABA_B$ receptors performed with the agonist [^{3}H]-GABA and the antagonist [^{3}H]-CGP 54626

		n	Membrane bound receptors mean ± s.e.mean	n	Solubilized receptors mean ± s.e.mean
[^{3}H]-GABA	K_d [nM]	3	25.9 ± 1.9	6	38.6 ± 5.9
	B_{max} [fmol mg^{-1} protein]	3	454 ± 99	6	409 ± 70
[^{3}H]-CGP 54626	K_d [nM]	3	1.35 ± 0.08	3	7.7 ± 2.6
	B_{max} [fmol mg^{-1} protein]	3	1171 ± 20	3	1033 ± 41

Experiments were performed in triplicate, *n* equals number of individual experiments.

Preincubation of crude membranes in the absence of $MgSO_4$ decreased the recovery of $GABA_B$ sites solubilized with 0.5% CHAPS by about 10% (Table 2). Increasing the CHAPS concentration to 0.75% did not increase the yield of $GABA_B$ sites in the supernatant and 1% CHAPS solubilized only 5% of the $GABA_B$ sites. Addition of 0.2% asolectin to 0.5% CHAPS did not result in a higher recovery of solubilized $GABA_B$ sites (data not shown). Since 0.5% CHAPS showed a ratio of solubilized $GABA_B$ to $GABA_A$ sites of about 1, compared to 0.6 with 0.75% CHAPS, 0.5% (= 8.1 mM CHAPS) was chosen to solubilize $GABA_B$ receptors.

To support the view, that the $GABA_B$ sites were truly solubilized, the solubilized material was subjected to additional centrifugation for 2 h at 100,000 *g* and then subsequently to filtration through filters of a pore size of 0.22 μm (Millipore, type GVWP). Neither treatment decreased specific $GABA_B$ binding.

Characterization of $GABA_B$ sites solubilized with 0.5% CHAPS

The characteristics of solubilized, dialysed $GABA_B$ sites were compared to membrane-bound receptors. Saturation experiments (Table 3, Figure 2) were performed with the agonist [^{3}H]-GABA and the antagonist [^{3}H]-CGP 54626. With [^{3}H]-GABA the K_d values for membrane bound and solubilized receptors were approximately 30 nM and the B_{max} values about 450 fmol mg^{-1} protein. With [^{3}H]-CGP 54626 the K_d value was 5 times higher for solubilized receptors and the B_{max} value was significantly lower ($P<0.05$, Student's unpaired, two-tailed *t* test).

Inhibition experiments against [^{3}H]-GABA (Table 4, Figure 3) were carried out with the agonist (−)-baclofen, (+)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626 (Figure 1) revealing comparable affinities for membrane bound and solubilized $GABA_B$ sites. Total binding was around 7000 d.p.m. for crude and 2 000 d.p.m. for solubilized preparations. The high nonspecific binding of 70% of total binding observed for solubilized sites, compared to 50% for membrane-bound receptors, probably stems from the fact that some free unbound radioligand is precipitated by the addition of γ-globulin and polyethyleneglycol 8000.

Detection of specific $GABA_B$ binding was assayed in the presence of Ca^{2+} or Mg^{2+} (Bowery *et al.*, 1983). Normally, $GABA_B$ binding was assayed in the presence of 2.5 mM Ca^{2+}. If no Ca^{2+} was added, specific [^{3}H]-$GABA_B$ binding was decreased to 53.5 ± 4.0% (mean ± s.e.mean, $n = 5$) of the value observed in presence of 2.5 mM Ca^{2+} for membrane

bound receptors and to 43.8 ± 9.7% ($n = 6$) for solubilized receptors. If crude membranes were either preincubated in the absence of $MgSO_4$, or if 5 mM EDTA was added to the binding assay, no specific binding was detected.

Agonist binding to $GABA_B$ receptors is GTP-sensitive, i.e., affinity decreases in presence of guanyl nucleotides (Hill *et al.*, 1984). Specific [^{3}H]-$GABA_B$ binding was measured in the

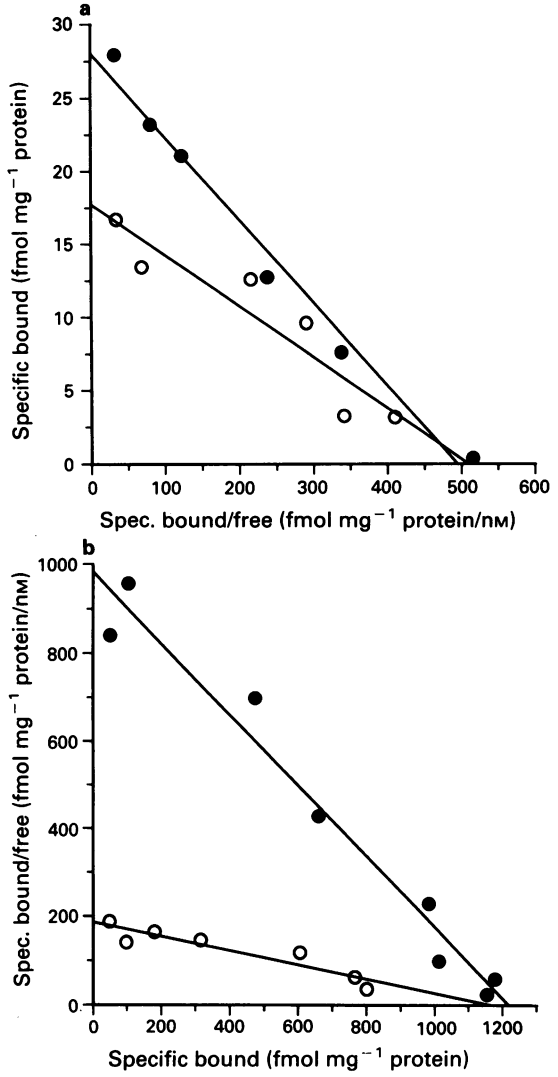


Figure 2 Saturation experiments shown as Scatchard plots of a representative of 3–6 experiments performed in triplicate of the agonist [^{3}H]-GABA (a) and the antagonist [^{3}H]-CGP 54626 (b) to membrane bound (●) and solubilized (○) $GABA_B$ receptors.

Table 4 Displacement of 5 nM [^{3}H]-GABA binding to membrane bound and 0.5% CHAPS-solubilized $GABA_B$ receptors

		Membrane-bound receptors	*Solubilized receptors*
(−)-Baclofen	IC_{50} [μM]	0.048 ± 0.002	0.024 ± 0.004
	slope factor	0.94 ± 0.03	0.86 ± 0.13
(+)-Baclofen	IC_{50} [μM]	>30	>30
CGP 35348	IC_{50} [μM]	15.4 ± 1.6	25.6 ± 5.0
	slope factor	0.86 ± 0.07	0.85 ± 0.13
CGP 36742	IC_{50} [μM]	14.2 ± 1.8	8.2 ± 2.3
	slope factor	0.73 ± 0.06	0.69 ± 0.13
CGP 54626	IC_{50} [μM]	0.0024 ± 0.0003	0.0018 ± 0.0005
	slope factor	1.08 ± 0.14	0.72 ± 0.13

Values ± s.d. of curve fitted to mean data of 3–5 experiments, performed in triplicate.

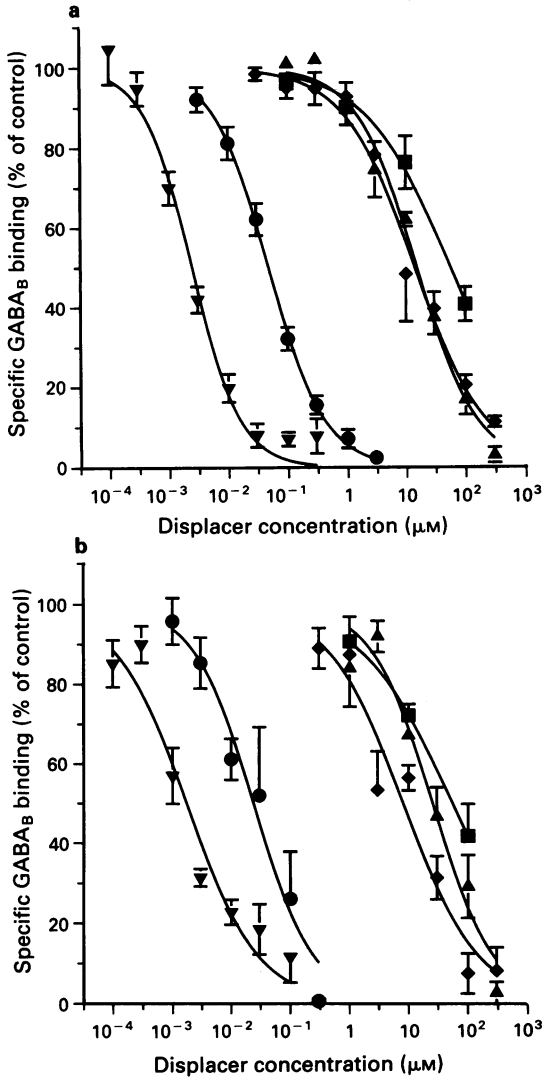


Figure 3 Displacement of 5 nM [^{3}H]-GABA binding to membrane bound (a) and solubilized $GABA_B$ receptors (b). Symbols represent means ± s.e. mean of 3–6 experiments performed in tripicate. Lines are best theoretical fits to data as determined by nonlinear least squares regression analysis: (●) (−)-baclofen; (■) (+)-baclofen; (▲) CGP 35348; (◆) CGP 36742; (▼) CGP 54626.

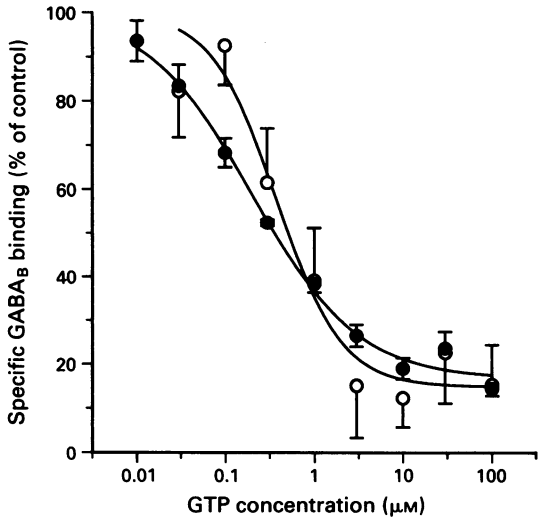


Figure 4 Displacement of 5 nM [^{3}H]-GABA binding to membrane bound (●) and solubilized $GABA_B$ receptors (○) by GTP. Symbols represent means ± s.e.mean of 4 experiments performed in triplicate. Lines are best theoretical fits to data as determined by nonlinear least squares regression analysis.

presence of increasing concentrations of GTP (Figure 4). For membrane-bound receptors the IC_{50} value of the curve fitted to the main data of 4 experiments was 0.21 ± 0.03 μM (mean ± s.e.mean) and the slope factor 0.74 ± 0.06. The corresponding values for solubilized $GABA_B$ sites were 0.38 ± 0.10 μM and 1.20 ± 0.32, respectively.

Specific [^{3}H]-CGP 54626 binding to membrane bound receptors showed no GTP-sensitivity up to 100 μM. Specific binding to solubilized receptors, however, was increased from 100% to 160.2 ± 8.9% with 100 μM GTP (mean ± s.e.mean, $n = 5$; $P < 0.005$, one sample, Student's two tailed t test) and to 137.2 ± 17.6% with 10 μM.

Discussion

Solubilization of $GABA_B$ receptors

The detergents chosen, digitonin, sodium cholate, DOC Na and CHAPS, were mainly those, which have been used successfully in solubilizing other G-protein coupled receptors (Poyner, 1990). For 1% digitonin and 1% DOC Na the sum of the $GABA_A$ sites found in the pellet and the supernatant, expressed as a percentage of starting material, was higher than 150%. This might be explained by an increase in the B_{max} value or the affinity for GABA. Indeed Enna & Snyder (1977) found that treatment of rat crude brain membranes with 0.05% Triton X-100 increased specific [^{3}H]-$GABA_A$ binding about fivefold; 0.03% DOC Na, however, reduced [^{3}H]-$GABA_A$ binding.

A difficulty, experienced with the solubilization of G-protein coupled receptors, e.g. α_2-adrenoceptors (Smith & Limbird, 1981) or D_2 dopamine receptors (Kilpatrick & Caron, 1983), is that the soluble receptors are insensitive to GTP and bind only antagonists, but not agonists with an affinity similar to membrane bound receptors. This could be explained by dissociation of the G-protein from its receptor during the solubilization procedure. We found for all the detergents and conditions tested that the sum of the $GABA_B$ sites in the pellet and the supernatant, assayed with the agonist [^{3}H]-GABA, was clearly lower than 100%. This could be due to dissociation of the G-protein. The only situations, where this sum was observed to be greater than 60%, were with 0.5% and 0.75% CHAPS. This compared with 35% or lower in the other cases. The zwitterionic detergent CHAPS has proved to be successful in solubilizing α_2-adrenoceptors (Sladceczek *et al.*, 1984; Matsui *et al.*, 1985), D_2 dopamine (Kuno *et al.*, 1983) and opioid receptors (Chow & Zukin, 1983) in a form, which is GTP-sensitive and binds antagonists and agonists with an affinity similar to membrane-bound receptors. If α_2-adrenoceptors (Smith & Limbird, 1981) and D_2 dopamine receptors (Kilpatrick & Caron, 1983) were solubilized with digitonin, the receptors were insensitive to GTP and agonists exhibited a lower affinity than for membrane bound receptors. In our study the $GABA_B$ receptors solubilized with 0.5% CHAPS were GTP sensitive and bound antagonists and agonists with affinities comparable to these obtained with membrane bound sites.

CHAPS (1.0%) solubilized only 5% $GABA_B$ sites in contrast to 25% with 0.5% and 0.75% CHAPS. This marked concentration-dependence is in line with previous reports by Guicheney *et al.* (1983), Kuno *et al.* (1983) and Simonds *et al.* (1980), where the highest CHAPS concentration tolerated was 10 mM (= 0.61%).

Compared to the high yield observed for $GABA_A$ sites, a recovery of 25% for $GABA_B$ sites is rather low. This figure, however, matches with the reports of Kuno *et al.* (1983) who solubilized 26% of D_2 dopamine receptors with 10 mM CHAPS + 0.72 M NaCl and Simonds *et al.* (1980) who obtained 20% of opioid binding sites after using 10 mM CHAPS.

Kuriyama and colleagues (Ohmori & Kuriyama, 1990) reported, in contrast to our findings, a yield of 30–40% of $GABA_B$ sites with 1.2% CHAPS and 0.2% asolectin. However, the authors did not indicate the protein concentration they used and if it was higher than in the present study, this might provide an explanation for the different results.

Characterization of $GABA_B$ sites solubilized with 0.5% CHAPS

Membrane-bound $GABA_B$ receptors in pig brain were compared to those in rat brain. For [^{3}H]-GABA, K_d values between 20 and 80 nM and B_{max} values between 600 and 1 200 fmol mg^{-1} protein have been described in rat brain membranes (Bowery *et al.*, 1983; Cross & Horton, 1987; Szekely *et al.*, 1987). Thus, our values in pig brain (26 nM and 454 fmol mg^{-1} protein respectively) are at the lower range of the ones reported in rat brain. Bittiger *et al.* (1992) found for [^{3}H]-CGP 54626 a K_d of 1.5 nM and a B_{max} of 3 700 fmol mg^{-1} protein, the K_d being the same as in pig brain with a three fold higher B_{max} value. The ratio of the B_{max} value obtained with the antagonist to the value obtained with an agonist was in both cases 2.6.

The IC_{50} values for (−)-baclofen, (+)-baclofen, CGP 35348, CGP 36742 and CGP 54626 in pig brain were, within a factor of three, comparable to those in rat brain (Bittiger *et al.*, 1990; Bowery *et al.*, 1983; Froestl *et al.*, 1992; Bowery, 1993).

An important determinant of binding characteristics and affinity is the removal of endogenous GABA. Crude membranes were thoroughly washed until no more $GABA_B$ binding inhibitory activity could be detected in the supernatant after incubation and centrifugation with buffer solution. It was also important to establish whether the characteristics of the solubilized, dialysed $GABA_B$ sites were comparable to those of the membrane-bound receptors. Inhibition experiments against [^{3}H]-GABA showed in both cases stereospecific binding for baclofen with similar affinities for the agonist (−)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626. Saturation experiments with the agonist [^{3}H]-GABA revealed no statistical differences (Student's two-tailed, unpaired t test) in the K_d and B_{max} values. However, if the antagonist [^{3}H]-CGP 54626 was used, the K_d value for solubilized receptors was 5 times higher and the B_{max} value 10% lower than for membrane bound receptors. As expected the maximal amount of receptors labelled with the antagonist was 2–3 times higher than with the agonist for membrane bound and solubilized sites. For membrane bound receptors the K_i value of CGP 54626, calculated according to Cheng & Prusoff (1973) from the IC_{50} value and the K_d value were comparable (2.0 nM and 1.4 nM respectively). For solubilized sites, however, the K_i value was 1.6 nM and the K_d 7.7 nM.

To measure the amount of radioligand bound, soluble receptors have to be precipitated and this was performed by the addition of γ-globulin and polyethyleneglycol 8000 which increased the total incubation volume from 0.8 to 1.2 ml. Bound radioligand was then separated from unbound by centrifugation. Hence dissociation of the radioligand cannot be excluded. Indeed, if the solubilized receptors were left to precipitate for 1 min specific binding of [^{3}H]-CGP 54626 was somewhat higher than after 10 min, for [^{3}H]-GABA no difference was observed. It might be that dissociation accounts for the slightly, however statistically significant, lower B_{max} value labelled with [^{3}H]-CGP 54626 in solubilized than in membrane-bound receptors.

Binding of the agonist [^{3}H]-GABA to both membrane-bound and solubilized receptors were sensitive to GTP, the IC_{50} values being around 0.3 μM. Hill *et al.* (1984) reported an IC_{50} value for GTP of 0.7 μM if the assay was performed in Krebs-Henseleit buffer and 16 μM in Tris HCl + 2.5 mM Ca^{2+}. GTP failed to affect the binding of the antagonist [^{3}H]-CGP 54626 to membrane bound receptors, however it increased specific binding to solubilized receptors by 60% at 100 μM. Schütz & Freissmuth (1992) proposed that guanine nucleotides initiate a conversion of a low-affinity antagonist/

high-affinity agonist state into a high-affinity antagonist/low-affinity agonist state. Thus guanine nucleotides not only decrease the affinity of agonists but in addition increase the affinity of antagonists with negative intrinsic efficacy (= inverse agonists). The latter was shown to be the case for muscarinic receptors (Hulme *et al.*, 1981) and A_1 adenosine receptors (Ramkumar & Stiles, 1988), the EC_{50} value for GTP being in both cases around 2 μM. Since in the present study a comparatively high GTP concentration was needed to increase specific [^{3}H]-CGP 54626 binding, the observed effect is probably nonspecific.

In summary, $GABA_B$ receptors from pig brain can be solubilized by 0.5% CHAPS. Solubilized $GABA_B$ receptors appear to be comparable to membrane-bound-receptors, since they show stereospecific binding for baclofen, they exhibit similar affinities for the agonists GABA and (−)-baclofen and the antagonists CGP 35348, CGP 36742 and CGP 54626 and they exhibit comparable sensitivities to Ca^{2+} and GTP.

We wish to thank CIBA, Basel, Switzerland for the very generous gift of [^{3}H]-CGP 54626, CGP 54626, CGP 36742, CGP 35348, (−)-baclofen and (+)-baclofen and Mr Derek King for skilful computer assistance. M.F. is grateful for financial support by Roche Research Foundation and The Royal Society.

References

ASANO, T., UI, M. & OGASAWARA, N. (1985). Prevention of the agonist binding to γ-aminobutyric $acid_B$ receptors by guanine nucleotides and islet-activating protein, pertussis toxin, in bovine cerebral cortex. *J. Biol. Chem.*, **260**, 12653–12658.

BITTIGER, H., FROESTL, W., HALL, R., KARLSSON, G., KLEBS, K., OLPE, H.R., POZZA, M.F., STEINMANN, M.W. & VAN RIEZEN, H.(1990). Biochemistry, electrophysiology and pharmacology of a new $GABA_B$ antagonist: CGP 35348. In *$GABA_B$ Receptors in Mammalian Function*. ed. Bowery, N.G., Bittiger, H. & Olpe, H.-R. pp. 47–60. Chichester: John Wiley and Sons Ltd.

BITTIGER, H., REYMANN, N., FROESTL, W. & MICKEL, S.J. (1992). ^{3}H-CGP 54626: a potent antagonist radioligand for $GABA_B$ receptors. *Pharmacol. Commun.*, **2**, 23.

BOWERY, N.G. (1993). $GABA_B$ receptor pharmacology. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 109–147.

BOWERY, N.G., HILL, D.R. & HUDSON, A.L. (1983). Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes. *Br. J. Pharmacol.*, **78**, 191–206.

CHENG, Y.-C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3102.

CHOW, T. & ZUKIN, S. (1983). Solubilization and preliminary characterization of *mu* and *kappa* opiate receptor subtypes from rat brain. *Mol. Pharmacol.*, **24**, 203–212.

CROSS, J.A. & HORTON, R.W. (1987). Are increases in $GABA_B$ receptors consistent findings following chronic antidepressant administration. *Eur. J. Pharmacol.*, **141**, 159–162.

DUNN, M.J. (1989). Determination of total protein concentration. In *Protein Purification Methods: A Practical Approach*. ed. Harris, E.L.V. & Angal, S. pp. 10–17. Oxford: IRL Press.

ENNA, S.J. & SNYDER, S.H. (1977). Influence of ions, enzymes and detergents on γ-aminobutyric acid-receptor binding in synaptic membranes of rat brain. *Molec. Pharmacol.*, **13**, 442–453.

FACKLAM, M. & BOWERY, N.G. (1993). Solubilization and characterization of $GABA_B$ receptors from pig brain. *Br. J. Pharmacol.*, **108**, 46P.

FROESTL, W., MICKEL, S.J., VON SPRECHER, G., BITTIGER, H. & OLPE, H.R. (1992). Chemistry of new $GABA_B$ antagonists. *Pharmacol. Commun.*, **2**, 52–56.

GUICHENEY, P., RAPPAPORT, A. & MARCEL, D. (1983). Solubilization of active brain α_1-adrenoceptors by a zwitterionic detergent. *J. Neurochem.*, **41**, 56–61.

HILL, D.R., BOWERY, N.G. & HUDSON, A.L. (1984). Inhibition of $GABA_B$ receptor binding by guanyl nucleotides. *J. Neurochem.*, **42**, 652–657.

HULME, E.C., BERRIE, C.P., BIRDSALL, N.J.M. & BURGEN, A.S.V. (1981). Two populations of binding sites for muscarinic antagonists in the rat heart. *Eur. J. Pharmacol.*, **73**, 137–142.

KILPATRICK, B.F. & CARON, M.G. (1983). Agonist binding promotes a guanine nucleotide reversible increase in the apparent size of the bovine anterior pituitary dopamine receptors. *J. Biol. Chem.*, **258**, 13528–13534.

KNOTT, C., MAGUIRE, J.J., MORATALLA, R. & BOWERY, N.G. (1993). Regional effects of pertussis toxin *in vivo* and *in vitro* on $GABA_B$ receptor binding in rat brain. *Neurosci.*, **52**, 73–82.

KUNO, T., SAIJOH, K. & TANAKA, C. (1983). Solubilization of D_2 dopamine receptor coupled to guanine nucleotide regulatory protein from bovine striatum. *J. Neurochem.*, **41**, 841–847.

MATSUI, H., ASAKURA, M., TSUKAMOTO, T., IMAFUKU, J., INO, M., SAITOH, N., MIYAMURA, S. & HASEGAWA, K. (1985). Solubilization and characterization of rat brain α_2-adrenergic receptor. *J. Neurochem.*, **44**, 1625–1632.

OHMORI, Y., HIROUCHI, M., TAGUCHI, J. & KURIYAMA, K. (1990). Functional coupling of the γ-aminobutyric $acid_B$ receptor with calcium ion channel and GTP-binding protein and its alteration following solubilization of the γ-aminobutyric $acid_B$ receptor. *J. Neurochem.*, **54**, 80–85.

OHMORI, Y. & KURIYAMA, K. (1990). Solubilization and partial purification of $GABA_B$ receptor from bovine brain. *Biochem. Biophys. Res. Commun.*, **172**, 22–27.

POYNER, D. (1990). Receptor-G-protein complexes in solution. In *Receptor-Effect Coupling, A Practical Approach*. ed. Hulme, E.C. pp. 31–58. Oxford: University Press.

RAMKUMAR, V. & STILES, G.L. (1988). Reciprocal modulation of agonist and antagonist binding to A_1 adenosine receptors by guanine nucleotides is mediated via a pertussis toxin-sensitive G-protein. *J. Pharmacol. Exp. Ther.*, **246**, 1194–1200.

SCHÜTZ, W. & FREISSMUTH, M. (1992). Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol. Sci.*, **13**, 376–380.

SIMONDS, W.F., KOSKI, G., STREATY, R.A., HJELMELAND, L.M. & KLEE, W.A. (1980). Solubilization of active opiate receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 4623–4627.

SLADECZEK, F., BOCKAERT, J. & ROUOT, B. (1984). Solubilization of brain α_2-adrenoceptor with a zwitterionic detergent: preservation of agonist binding and its sensitivity to GTP. *Biochem. Biophys. Res. Commun.*, **119**, 1116–1121.

SMITH, S.K. & LIMBIRD, L.E. (1981). Solubilization of human platelet α-adrenergic receptors: evidence that agonist occupancy of the receptor stabilizes receptor-effector interactions. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 4026–4030.

SZEKELY, A.M., BARBACCIA, M.L. & COSTA, E. (1987). Effect of a protracted antidepressant treatment on signal transduction and ^{3}H(−)baclofen binding at $GABA_B$ receptors. *J. Pharmacol. Exp. Ther.*, **243**, 155–159.

ZUKIN, S.R., YOUNG, A.B. & SNYDER, S.H. (1974). γ-Aminobutyric acid binding to receptor sites in the rat central nervous system. *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4802–4807.

(Received May 18, 1993
Revised July 22, 1993
Accepted July 26, 1993)

Br. J. Pharmacol. (1993), **110**, 1297–1304 © Macmillan Press Ltd, 1993

Action of purine and pyrimidine nucleotides on the rat superior cervical ganglion

[1]G.P. Connolly, P.J. Harrison & *T.W. Stone

Department of Physiology, University College of London, Gower Street, London WC1E 6BT and *Department of Pharmacology, Glasgow University, Glasgow G12 8QQ

1 Using a grease-gap technique, we have investigated the effects of purine and pyrimidine nucleotides on the d.c. potential of the rat isolated superior cervical ganglion (SCG).

2 Of the purines tested, adenosine, adenosine 5'-triphosphate (ATP), β,γ-methylene-adenosine 5'-triphosphate (β,γ-MeATP) at up to 300 μM produced concentration-dependent hyperpolarizations, whereas 2-methyl-thio-ATP (2-Me.S.ATP) and α,β-methylene-ATP (α,β-MeATP) depolarized ganglia. Of the pyrimidines tested, uridine 5'-triphosphate (UTP) produced concentration-dependent depolarizations and cytosine 5'-triphosphate (CTP) at 1000 μM produced considerably smaller but significant depolarizations. In contrast uridine 5'-monophosphate (UMP) at 1000 μM hyperpolarized ganglia. The relative order of potency of purines and pyrimidines to depolarize ganglia was:- UTP > α,β-MeATP >> CTP > 2-Me.S.ATP and to hyperpolarize ganglia was:- adenosine = β,γ-MeATP > ATP > UMP.

3 The ability of purines and pyrimidines to alter the depolarizing response caused by muscarine and of purines to alter depolarization induced by γ-aminobutyric acid (GABA) was determined. The relative order of potency of nucleotides in depressing submaximal depolarization caused by muscarine (100 nM) was: adenosine = ATP > β,γ-MeATP whereas 2-Me.S.ATP, α,β-MeATP and UTP did not significantly alter depolarization caused by muscarine. At 100 μM β,γ-MeATP and adenosine but not ATP potentiated GABA-induced depolarizations.

4 Hyperpolarizations caused by adenosine, ATP, β,γ-MeATP and UMP and depolarizations caused by α,β-MeATP were enhanced in medium containing reduced concentrations of calcium (0.1 mM) and potassium (2 mM). In this medium 8-phenyltheophylline abolished hyperpolarizations caused by adenosine and reversed hyperpolarizations caused by ATP into depolarizations. Suramin (300 μM), a P_2-purinoceptor antagonist, significantly reduced the depolarizing response caused by α,β-MeATP and significantly increased hyperpolarizations caused by ATP and β,γ-MeATP. Suramin (300 μM) did not significantly alter depolarizations caused by 1,1-dimethyl-4-phenylpiperazinium (10 μM), potassium (3 mM) or muscarine (100 nM) and significantly potentiated depolarizations caused by UTP (100 μM).

5 It is concluded that the rat SCG contains P_1-purinoceptors that hyperpolarize the ganglion and diminish sensitivity to muscarine, and P_{2X}-purinoceptors that depolarize the SCG. There is also some evidence to suggest the presence of receptors for UTP, i.e., pyrimidinoceptors, which depolarize SCG neurones.

Keywords: Superior cervical ganglion (SCG); adenosine; ATP; P_1-purinoceptors; P_{2X}-purinoceptors, pyrimidines; 8-phenyltheophylline; suramin

Introduction

It has been suggested that adenosine and ATP might act as neurotransmitters in peripheral ganglia or act as modulators of transmitter sensitivity (Potter *et al.*, 1983; Akasu *et al.*, 1984; Wolinsky & Patterson, 1985; Furshpan *et al.*, 1986). There are, however, conflicting reports as to the effects of adenosine on the resting membrane potential of sympathetic ganglia, adenosine being reported to have either no effect (Siggins *et al.*, 1977; Akasu *et al.*, 1981; Henon & McAfee, 1983a), to depolarize (Gallagher & Shinnick-Gallagher, 1978; Gruol *et al.*, 1981) or hyperpolarize sympathetic ganglia (Brown *et al.*, 1979; Connolly & Stone, 1988; 1993a,b). In contrast to receptors for adenosine which are widely distributed in neuronal tissues, ATP receptor distribution is restricted to dorsal root ganglion cells and selected areas of the CNS (Salt & Hill, 1983; Krishtal *et al.*, 1983; Salter & Henry, 1985; Edwards *et al.*, 1993), they are absent in the hippocampus (Stone & Cusack, 1989). In peripheral ganglia, ATP has a variety of effects, hyperpolarizing rat superior cervical ganglia (SCG) (Brown *et al.*, 1979) and depolarizing bullfrog cultured sympathetic neurones (Siggins *et al.*, 1977) and bullfrog paravertebral ganglia (Akasu *et al.*, 1981).

Using an extracellular grease-gap recording technique we have now compared the effects of adenosine and ATP analogues on the rat SCG to see whether they are mediated via any of the recognized subtypes of purinoceptors defined by Burnstock (1978), i.e. P_1-purinoceptors that show greater selectivity for adenosine than ATP, and P_2-purinoceptors that show the opposite selectivity. Furthermore, receptors for ATP on vascular and visceral smooth muscle have been subclassified into P_{2X} and P_{2Y} subtypes by the action of selective agonists (Burnstock & Kennedy, 1985). P_{2X}-purinoceptor-mediated responses are characterized by the following order of potency: α,β-methylene ATP (α,β-MeATP) > β,γ-methylene ATP (β,γ-MeATP) >> ATP = 2-methylthio-ATP (2-Me.S.ATP), whereas P_{2Y}-purinoceptors exhibit a rank order of potency of 2-Me.S.ATP > ATP > α,β-MeATP = β,γ-MeATP.

There is evidence suggesting that purines modify the sensitivity of neurones to neurotransmitters, particularly to acetylcholine (ACh) (Siggins *et al.*, 1977; de Groat & Booth, 1980; Akasu *et al.*, 1981; 1982; 1983; Henon & McAfee, 1983b; Silinsky & Ginsborg, 1983). Akasu and his colleagues (Akasu *et al.*, 1981; 1983; Akasu & Koketsu, 1985) demonstrated that ATP (but not adenosine) depolarized and potentiated responses to acetylcholine (ACh) mediated via nicotinic receptors on bullfrog ganglia and the frog skeletal muscle endplate (Akasu *et al.*, 1983). The former action was not due

[1] Author for correspondence.

to a direct interaction with nicotinic receptors, being mediated via a change in either the number of ion channels or an increase in the single channel conductance. Other studies have shown that adenosine enhances the response of the rabbit gastrointestinal tract to ACh (Gustafsson, 1981), and reduces responses of hippocampal neurones to carbachol (Brooks & Stone, 1988). Cox & Walker (1987) have observed either an increase or decrease in the muscarinic sensitivity of snail ganglion neurones with adenosine. We have preliminary evidence which shows that adenosine selectively reduced the response of the rat SCG to muscarine and not the response caused by γ-aminobutyric acid (GABA) (Connolly & Stone, 1988). On frog dorsal root ganglia, ATP has been found to depolarize the ganglia and also to potentiate GABA-mediated depolarizations (Morita *et al.*, 1984). It was therefore the second aim of this study to extend our preliminary observations and to examine the effect of ATP and some of its analogues on the depolarizing responses caused by muscarine and GABA.

A third objective was to compare the actions of purines and some pyrimidines on the SCG as it is known that pyrimidines such as uridine 5'-triphosphate (UTP) depolarize bullfrog cultured SCG (Siggins *et al.*, 1977), bullfrog paravertebral sympathetic ganglia (Gruol *et al.*, 1981) and intact rat SCG (Adams *et al.*, 1982). The ability of pyrimidines to depolarize sympathetic neurones may be due to the activation of 'nucleotide receptors' which are defined as having equal sensitivity to ATP and UTP, and at which α,β-MeATP has little or no activity (O'Connor *et al.*, 1991). Alternatively UTP may activate 'pyrimidinoceptors' that are distinct from purinoceptors (von Kugelgen *et al.*, 1987; Haussinger *et al.*, 1987) as reviewed by Seifert & Schultz (1989). We have investigated the effect of suramin, a P_2-purinoceptor antagonist (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989; Schlicker *et al.*, 1989; Nakazawa *et al.*, 1990), on the response of the rat SCG to purines and UTP to determine if the effects of these compounds are mediated via the same receptor, i.e. a P_2-purinoceptor.

Methods

Male Wistar rats (120 to 390 g) were killed by a lethal dose of urethane (3 g kg^{-1}). Ganglia were removed and desheathed in physiological salt solution (PSS) at room temperature (19 to 23°C) and prepared for recording of d.c. potentials via the internal carotid nerve and the body of the ganglion as described by Connolly & Stone (1993a,b). In brief, desheathed ganglia were mounted in a tissue bath divided into three compartments by insulating grease-sealed partitions. The ganglion body was situated in the central chamber (bath volume ca. 0.5 ml) with the pre- and postganglionic trunks protruding through lightly greased slots into the outer chambers. All chambers were filled with PSS and the central chamber was superfused with PSS at 25 ± 1°C, pH 7.4, equilibrated with 5% CO_2/95% O_2, containing (mM): NaCl 125, $NaHCO_3$ 25, KCl 5; KH_2PO_4 1, $MgSO_4$ 1, glucose 10 and $CaCl_2$ 2.5. For low K^+/Ca^{2+} the standard PSS was altered so that $CaCl_2$ and KCl were reduced to 0.1 mM and 1 mM respectively.

Muscarine, GABA, purines and pyrimidines were dissolved in PSS as 10 or 100 mM stock solutions and frozen (− 20°C) as aliquots. New aliquots of stock solutions were used for each experiment. Just before use, suramin was dissolved in about 20 ml of PSS and then diluted to a final concentration of 300 μM in perfusate. To avoid precipitation of insoluble 8-phenyltheophylline (8-PT), 5–10 mg of 8-PT was dissolved in up to 100 μl of alkali (1 M NaOH) and made up in perfusate as a 10 mM stock solution which was further diluted into pre-oxygenated perfusate at a final concentration of 10 μM; the pH of the perfusate was adjusted to 7.4 with dilute hydrochloric acid.

Experimental protocols were derived from initial experiments and agonists were applied for times that were found to be sufficient for potential changes to reach a plateau and allow repeated applications without desensitization (see results). Ganglia were equilibrated for 2 to 3 h before use to allow increases in sensitivity to agonists to occur. For hyperpolarizing responses, purines were applied for 2 min periods at 20 min intervals and concentration-response curves were constructed by applying single concentrations of an analogue near or at subthreshold concentrations, and then increasing by three fold or ten fold, until the response size was maximal. Peak responses to drugs were measured from a baseline drawn between the potential at the start of the application of a drug to the finish of the response and are expressed as a mean ± s.e.mean (n), where n is the number of ganglia studied. Responses to a maximum of three different agonists or two concentration-response curves to a single agonist were examined on each ganglion.

For antagonist studies, ganglia were incubated with antagonists for a minimum of 30 min before testing responses to purines or pyrimidines. Two concentration-response curves to a single purine were made on each ganglion, the first being the control responses followed by the response to the same adenosine analogue in the presence of 8-PT. The first and second concentration-response curves to adenosine were reproducible (Connolly, 1991). Responses caused by a given concentration of a purine in the absence and presence of antagonist were compared by Student's paired t test and P values were considered statistically significant if less than 0.05.

Muscarine (100 nM) and GABA (10 μM) were applied for 1 min at 20 min intervals. To determine the effect of purines and UTP on the peak response caused by muscarine or GABA, two or three reproducible responses to muscarine or GABA were obtained, and the last response of this series was used as the control response. For the test response, a ganglion was perfused with a purine or UTP for 5 min and muscarine or GABA applied for 1 min, a minute from the start of perfusion with a purine or UTP. The effects of the purines or UTP on the peak response to muscarine or GABA is expressed as a percentage of the control response. The concentrations and the adenosine analogues tested on each ganglia were randomized so that at least 3 ganglia from different animals were used for each analogue. The depolarizing response caused by muscarine after washing out test compounds returned to the control level within 20–40 min of application. Each ganglion was used as its own control and statistical differences between the peak responses caused by muscarine or GABA before (control) and in the presence of a purine or UTP were determined by a Student's paired t test, and are expressed as the percentage inhibition of the control response caused by muscarine or GABA respectively. P values were considered statistically significant if less than 0.05.

(±)-Muscarine and 8-PT were purchased from Research Biochemicals Inc. Suramin hexasodium salt was a generous gift from Bayer U.K. Adenosine hemisulphate, atropine sulphate, ATP, α,β-MeATP, β-γ,-MeATP, cytosine 5'-triphosphate (CTP), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), GABA, 2-Me.S.ATP, uridine 5'-monophosphate (UMP) and UTP were obtained from Sigma Chemical Co., U.K. and salts for the perfusate PSS were of Analar grade.

Results

Effect of muscarine and adenosine on the d.c. potential

Ganglia showed a marked increase in sensitivity to submaximal concentrations of potassium (12 mM) and muscarine (100 nM) during the first 2 to 3 h of perfusion. To ensure reproducibility of responses throughout an experiment (up to 12 h) ganglia were equilibrated in the recording chamber and

Table 1 Effects of adenosine, adenine nucleotides and UMP on the d.c. potential of the rat SCG in standard and low K^+/Ca^{2+} physiological salt solution (PSS) and the effect of suramin on the response caused by purines, UTP and depolarizing agents

Compound	Conc (μM)	n	Response (μV ± s.e.mean) PSS	Low K^+/Ca^{2+} PSS Control[a]	+ Suramin[b]
Hyperpolarization					
Adenosine	100	8	64 ± 11	310 ± 61***	
	100	7		140 ± 35	161 ± 40
	1000	6		224 ± 58	281 ± 64**
ATP	100	4	26 ± 10	121 ± 25*	
	100	7		83 ± 22	145 ± 38**
	1000	4		98 ± 35	175 ± 35*
β,γ-MeATP	30	7		88 ± 23	116 ± 21*
	100	4	93 ± 25	124 ± 24	
	100	5		145 ± 46	189 ± 55*
UMP	100	7	0 ± 0	23 ± 8*	
	1000	7	31 ± 12	53 ± 15	
DMPP	10	4		131 ± 46	158 ± 83
Depolarization					
α,β-MeATP	100	4	66 ± 9	240 ± 25***	
	100	9		76 ± 11	− 5 ± 10***
UTP	100	6		216 ± 38	284 ± 44**
Muscarine	0.1	6		231 ± 32	268 ± 29
Potassium	3000	4		136 ± 33	114 ± 28
DMPP	10	4		121 ± 27	139 ± 35

Differences between responses in (a) and low K^+/Ca^{2+} PSS and (b) responses in low K^+/Ca^{2+} PSS in the absence or presence of 300 μM suramin. Statistical differences (paired t test) are denoted by $*P<0.05$; $**P<0.01$ and $***P<0.001$.

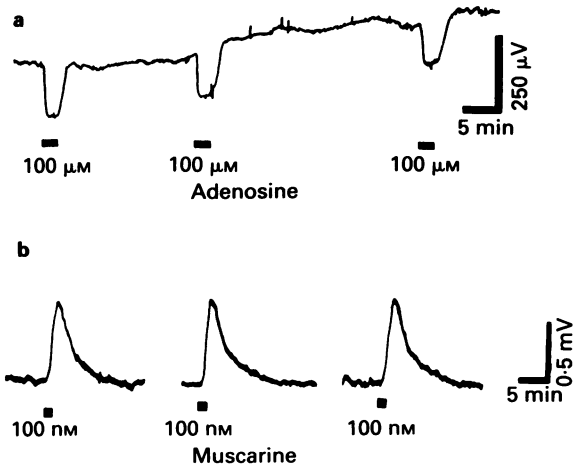


Figure 1 Sample responses of rat SCG in standard PSS to three consecutive applications of (a) adenosine (100 μM) and (b) muscarine (100 nM) to illustrate the nature of the responses and their reproducibility.

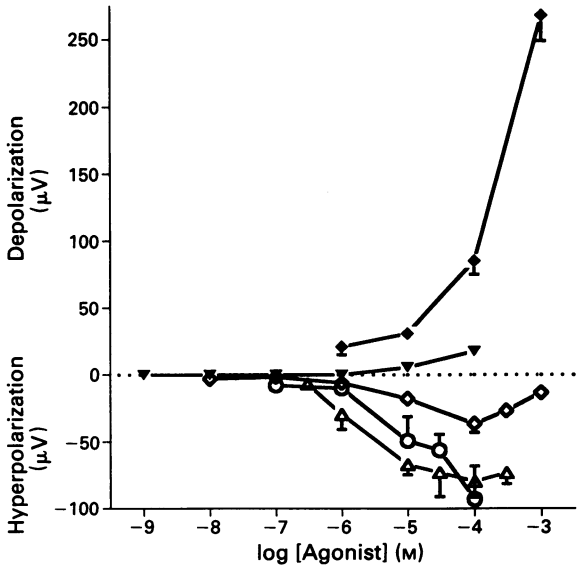


Figure 2 Effect of adenosine, ATP, β,γ-MeATP, ATP, 2-Me.S.ATP and UTP on the d.c. potential of the rat isolated SCG. Response to adenosine Δ, β,γ-MeATP (○), ATP (◇), 2-Me.S.ATP (▼) and UTP (◆) were recorded in standard PSS and each point represents the average results obtained from at least four ganglia. In this and subsequent figures, the vertical bars represent the s.e.mean response (symbol) and are shown when they exceed the symbols. For abbreviations, see text.

perfused for at least 2 h before experimental observations. Although the size of the response to agonists recorded from different ganglia was variable (see Table 1), responses recorded from a single ganglion in either low K^+/Ca^{2+} or standard PSS were consistent upon repeated application, e.g., in standard PSS the first, second and third responses (μV ± s.e.mean) to 100 μM adenosine ($n = 10$) and also to 100 nM muscarine ($n = 19$) were 70 ± 9, 69 ± 6, 73 ± 8 and 294 ± 36, 289 ± 36, 304 ± 38 respectively. There were no significant differences between the first and second responses. A sample trace of the responses to consecutive applications of adenosine and muscarine is shown in Figure 1.

When examining the effect of purines or UTP on muscarine-induced depolarizations, two or more reproducible responses to muscarine were obtained before testing the effects of purines and UTP. To avoid desensitization of responses, drugs were applied for 1 or 2 min (producing their maximal response during this period) with a minimum of 15 min between applications (Figure 1).

The effect of purines on the d.c. potential

The effects of purines on the d.c. potentials of ganglia in PSS are shown in Figure 2. Adenosine (0.3 to 1,000 μM) elicited concentration-dependent hyperpolarizations and was about equipotent with β,γ-MeATP. ATP hyperpolarized ganglia, but was over ten fold less potent and was less efficacious than adenosine or β,γ-MeATP. Between 10 nM and 10 μM, 2-Me.S.ATP was inactive ($n = 4$ to 8) and at 100 μM produced small but significant depolarizations ($P<0.01$, $n = 8$). In contrast α,β-MeATP produced concentration-dependent depolarizations (Figure 3).

When PSS was replaced by low K^+/Ca^{2+}, hyperpolarizations caused by adenosine and ATP (Table 1) as well as depolarizations to α,β-MeATP (Figure 3) were significantly enhanced. In this medium, ATP produced concentration-dependent hyperpolarizations (Figures 4 and 5) which were followed by an after-depolarization at a concentration of 300 μM ATP. At 1000 μM, ATP produced a compound response that included an initial hyperpolarization, a depolarization and then a late hyperpolarization and a slow, long-lasting after-depolarization (Figure 4a).

The effect of pyrimidines on the d.c. potential

In PSS, UTP (1–1000 μM) produced concentration-dependent depolarizations (Figure 2) and on the same ganglia was more potent than α,β-MeATP. CTP (1000 μM) produced smaller depolarizations (34 ± 2 μV, $n = 4$; $P < 0.001$) than UTP at an equivalent concentration. In contrast, UMP was inactive at 100 μM but produced a small and prolonged hyperpolarization at 1000 μM. When PSS was replaced by low K^+/Ca^{2+} PSS, responses caused by UMP were enhanced and UMP at 100 and 1000 μM produced concentration-dependent hyperpolarizations (Table 1).

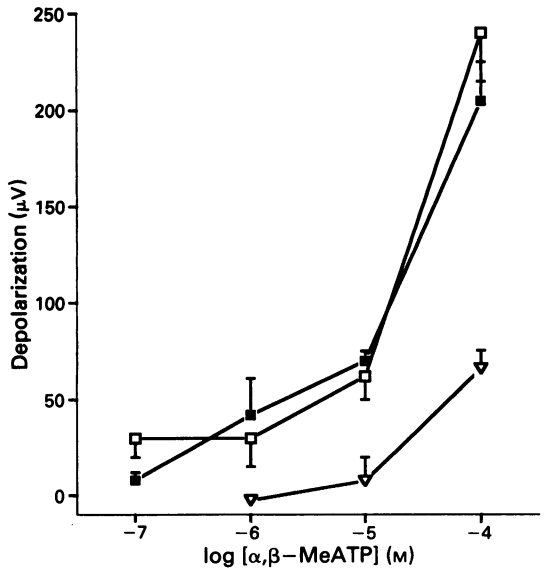


Figure 3 Effect of α,β-MeATP on the d.c. potential of the rat isolated SCG in standard and low K^+/Ca^{2+} PSS in the absence and presence of 10 μM 8-phenyltheophylline (8-PT). Responses caused by α,β-MeATP in PSS (▽), and response caused by α,β-MeATP in low K^+/Ca^{2+} PSS in the absence (□) and presence of 10 μM 8-PT (■) were obtained from four ganglia. For abbreviations, see text.

Antagonist studies

Since low K^+/Ca^{2+} PSS enhanced responses to most of the agonists tested (Table 1), making changes of response size easier to quantify, this medium was used for the determination of the effect of antagonists.

Effect of 8-PT on the response caused by purines

In low K^+/Ca^{2+} PSS containing 10 μM 8-PT, the hyperpolarizing component of responses to ATP were reversed to depolarizations (Figures 4 and 5). There was no desensitization of the response caused by ATP at up to 1000 μM and responses at this concentration were reproducible when applied at 30 min intervals (Figure 4a). Both the initial and second hyperpolarizations caused by 1000 μM ATP were dramatically reduced by 8-PT to leave a small hyperpolarization superimposed on a prolonged depolarization (Figure 4b). The depolarizing component of ATP responses seen at concentrations of 300 μM or above were unaffected by 8-PT (Figure 5).

In low K^+/Ca^{2+} PSS, depolarizations caused by α,β-MeATP (0.1 to 100 μM) were not significantly altered by the presence of 10 μM 8-PT (Figure 3), whereas on the same ganglia, hyperpolarizations caused by 100 μM adenosine (− 263 ± 41 μV) were significantly reduced (to − 50 ± 6 μV; $P < 0.05$, $n = 3$). Hyperpolarization caused by adenosine and β,γ-MeATP were affected similarly by 10 μM 8-PT with concentration-response curves to adenosine and β,γ-MeATP being displaced rightwards (Figure 6).

Effect of suramin on the response of ganglia to purines, UTP and depolarizing agents

In low K^+/Ca^{2+} PSS, suramin at 300 μM did not alter hyperpolarizations-induced by adenosine (100 μM) or depolarizations induced by muscarine (100 nM), DMPP (10 μM) or potassium chloride (3 mM) (Table 1). In contrast, suramin significantly potentiated hyperpolarizations caused by adenosine (1000 μM), ATP (100 μM and 1000 μM) and β,γ-MeATP

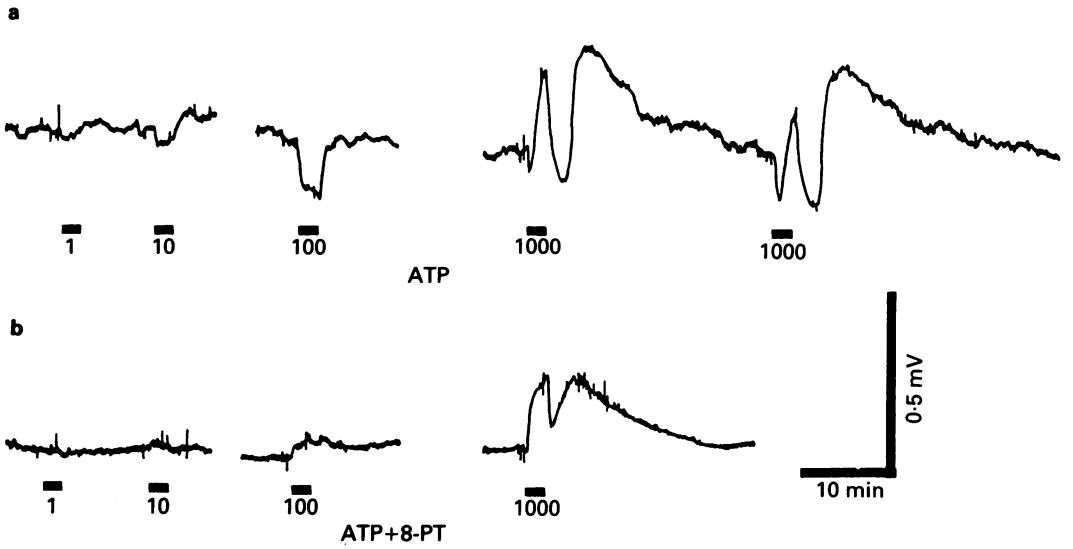


Figure 4 Antagonism by 8-phenyltheophylline (8-PT) of the response of the rat SCG to adenosine 5'-triphosphate (ATP). Responses of a single ganglion to increasing concentrations of ATP in low K^+/Ca^{2+} PSS (a) in the absence and (b) in the presence of 10 μM 8-PT. Numbers under responses refer to concentration of ATP in μM.

(30 μM and 100 μM); it also abolished depolarizations induced by α,β-MeATP (100 μM) and enhanced UTP (100 μM)-induced depolarizations (Table 1).

On three ganglia the response caused by 100 μM α,β-MeATP was reduced by suramin (300 μM) from 160 ± 35 μV to 31 ± 15 μV ($P<0.05$). On the same ganglia in the presence of 300 μM suramin, α,β-MeATP at 1,000 μM depolarized ganglia by 88 ± 45 μV. In the presence of suramin (300 μM), atropine (2 μM) abolished depolarizations caused by 100 nM muscarine (suramin = 276 ± 33 μV cf. suramin + atropine = 6 ± 7 μV, $n=4$, $P<0.01$) but not depolarizations caused by 100 μM UTP (suramin = 263 ± 67 μV cf. suramin + atropine = 200 ± 41 μV, $n=4$, NS).

Effect of purines and UTP on the responses to muscarine or GABA

Adenosine, β,γ-MeATP and ATP significantly reduced the depolarizing response caused by 100 nM muscarine. ATP was approximately equipotent with adenosine, whereas β,γ-MeATP produced a smaller reduction of the response caused by muscarine (Figure 7). The depolarizing response caused by muscarine was not significantly altered by either 2-Me.S.ATP or α,β-MeATP (Table 2). Adenosine or β,γ-MeATP but not ATP significantly increased the depolarization produced by application of 10 μM GABA (Table 2).

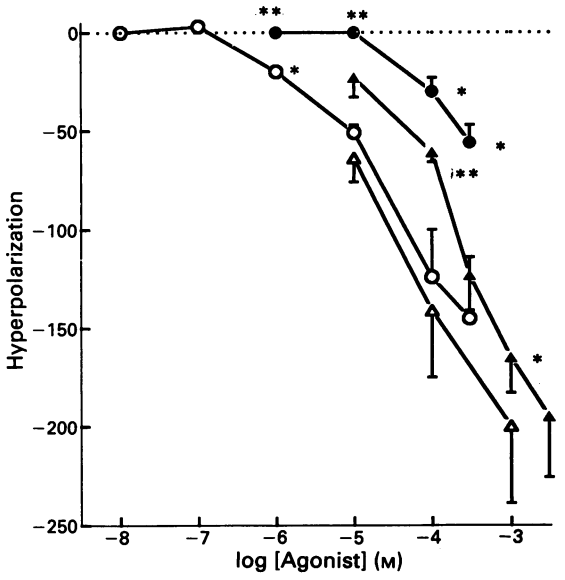


Figure 6 Effect of 8-phenyltheophylline (8-PT) on the response of the rat SCG in low K^+/Ca^{2+} PSS to adenosine and β,γ-MeATP. Responses to adenosine (Δ) and β,γ-MeATP (○) are plotted in the absence and in the presence (▲, ●) of 10 μM 8-PT. Statistical significance (paired t test) between responses in the presence and absence of 8-PT is indicated by * and ** for $P<0.05$ and $P<0.01$ respectively. For abbreviations, see text.

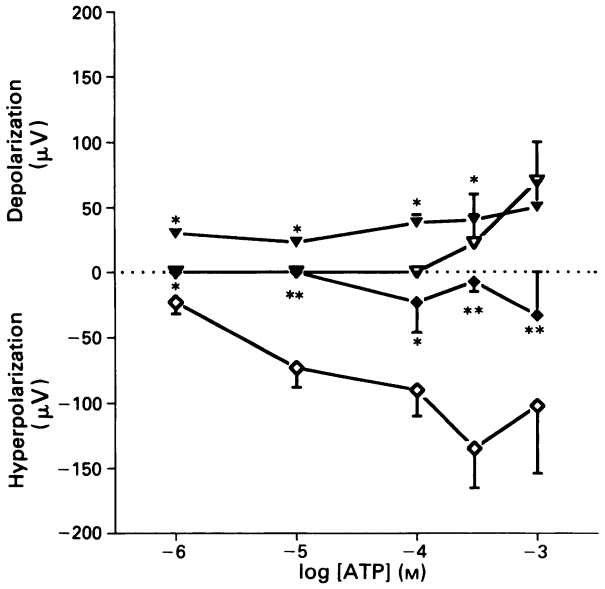


Figure 5 Effect of 8-phenyltheophylline (8-PT) on the response caused by ATP of the rat SCG in low K^+/Ca^{2+} PSS. The hyperpolarizing component (◇) and the depolarizing component (▽) of responses caused by ATP are plotted in the absence and presence (◆, ▼) of 8-PT, 10 μM. Statistical significance (paired t test) between responses in the presence and absence of 8-PT is indicated by *, ** and *** for $P<0.05$, $P<0.01$ and $P<0.001$ respectively.

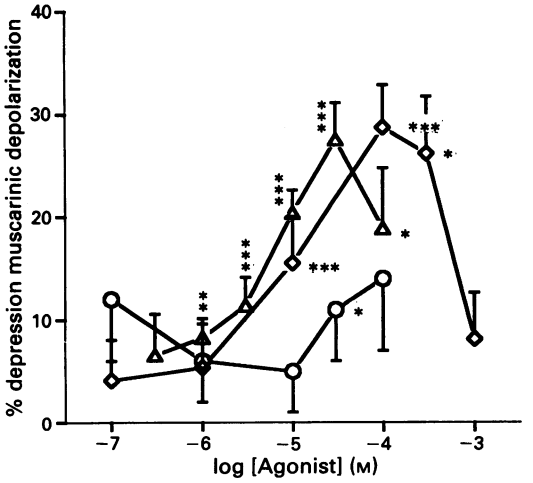


Figure 7 Effect of adenosine, ATP and β,γ-MeATP on the depolarizing response of rat SCG to muscarine. The ordinate scale represents the percentage decrease in response to 100 nM muscarine in standard PSS, caused by adenosine (Δ), ATP (◇) and β,γ-MeATP (○). Statistical significance (paired t test) between responses in the presence and absence of purines is indicated by *, ** and *** for $P<0.05$, $P<0.01$ and $P<0.001$ respectively. For abbreviations, see text.

Table 2 Effect of adenosine, ATP and its analogues and UTP on the depolarizing response of the rat SCG to 100 nM muscarine and 10 μM GABA

Compound (100 μM)	*Response caused by muscarine* (μV ± s.e.mean)	*% change in response to muscarine by compound*	*Response caused by GABA* (μV ± s.e.mean)	*% change in response to GABA by compound*
Adenosine	515 ± 55	− 36 ± 3 (10)***	594 ± 137	9 ± 4 (7)*
ATP	225 ± 21	− 29 ± 4 (12)***	217 ± 37	7 ± 6 (3)
β,γ-MeATP	497 ± 133	− 14 ± 7 (3)*	274 ± 42	15 ± 5 (8)**
2-Me.S.ATP	219 ± 16	− 9 ± 6 (8)		
α,β-MeATP	188 ± 25	− 1 ± 6 (4)		
UTP	251 ± 32	10 ± 6 (7)		

Number of ganglia in parentheses. *$P<0.05$, **$P<0.01$, ***$P<0.001$

Discussion

As responses to purines and pyrimidines were substantially enhanced when recordings were made using PSS containing low K^+/Ca^{2+}, this solution was used for the majority of our studies. The reasons for this facilitation of responses are unclear, but are consistent with previous studies that reported reductions in K^+ or Ca^{2+} enhanced muscarinic agonist-induced depolarization and hyperpolarization (Brown *et al.*, 1980; Newberry & Priestley, 1987; Newberry & Connolly, 1989) and adenosine-induced hyperpolarization of the rat SCG (Connolly & Stone, 1993b). Hyperpolarizations of the rat SCG caused by adenosine were reported to be dependent upon $[Ca^{2+}]_e$ but are not due to an interaction with calcium channels as calcium channel antagonists were ineffective or enhanced the response to adenosine (Connolly & Stone, 1993b). As adenosine is thought to act by increasing potassium efflux (Connolly & Stone, 1993b) and reduced $[Ca^{2+}]_e$ can alter the gating of potassium channels (Armstrong & Lopez-Barneo, 1987) the possibility that a similar phenomenon may explain the ability of low $[Ca^{2+}]_e$ PSS to potentiate agonist-induced responses of SCG neurones cannot be excluded. Given the ability of low $[Ca^{2+}]_e$ to potentiate the response of the rat SCG to a variety of agonists (present study, see also Brown *et al.*, 1980; Connolly & Stone, 1993b) this potentiation may result from a general increase in neuronal sensitivity.

Another action of reducing $[Ca^{2+}]_e$ could have been to convert weakly-effective agonist responses into more strongly-effective ones, altering agonist efficacy and the shape of the transducer relationship (Dougall & Leff, 1987). This latter possibility appears unlikely, as low K^+/Ca^{2+} PSS enhanced the size of purine-evoked hyperpolarizations but did not affect the EC_{50} value for N^6-cyclopentyladenosine-induced hyperpolarizations (Connolly & Stone, 1993c). Furthermore, although Ca^{2+} is required for a number of agonist-induced responses, e.g., ACh at muscarinic receptors (El-Fakahany & Richelson, 1980), ACh and histamine in the taenia of the guinea-pig and α-adrenoceptor agonists in vas deferens of the rat, Ca^{2+} ions are not believed to be involved in the interactions of antagonists with these receptors (Takagi *et al.*, 1972). In the present study, depolarizing responses caused by α,β-MeATP but not UTP were antagonized in either normal or low K^+/Ca^{2+} PSS (unpublished results). Similarly a reduction of $[Ca^{2+}]_e$ from 2.5 to 0.1 mM did not alter the ability of DPCPX (8-cyclopentyl-1,3-dipropylxanthine, an A_1-adenosine receptor antagonist) to shift the concentration-response curve for adenosine (Connolly, 1991). Overall, our observations suggest that low K^+/Ca^{2+} PSS is unlikely to alter qualitatively the antagonistic properties of 8-PT or suramin and provides a useful means of enhancing the sensitivity of the SCG to purines and pyrimidines.

The role of purinoceptor subtypes

The action of 8-PT in blocking the hyperpolarizing responses to adenosine and converting the initial hyperpolarizing response caused by ATP into a depolarization suggested that ATP hyperpolarized and depolarized the rat SCG via P_1- and P_2-purinoceptors respectively. Our results differ from those of Siggins *et al.* (1977) who found that both ATP and adenosine caused depolarization of cultured bullfrog sympathetic ganglion cells and of Akasu *et al.* (1983) who found ATP caused depolarization of intact bullfrog sympathetic ganglion cells. In the rat SCG, ATP may have been rapidly metabolized to adenosine (Bruns, 1980; Zimmerman *et al.*, 1985) which then mediated hyperpolarization via P_1-purinoceptors. The similarity of the concentration-dependent hyperpolarizations caused by adenosine and the P_{2X}-purinoceptor agonist β,γ-MeATP and the fact that both responses were antagonized by 8-PT show that the hyperpolarization caused by adenosine and β,γ-MeATP were due to activation of P_1-purinoceptors. The hyperpolarization by β,γ-MeATP most probably occurred after metabolism of the nucleotide to adenosine (Cusack *et al.*, 1988). The depolarization caused by the stable P_{2X}-purinoceptor agonist α,β-MeATP, and its insensitivity to antagonism by 8-PT, is consistent with the fact that this nucleotide is much less prone to degradation to adenosine than ATP (Cusack *et al.*, 1988) and with its depolarizing action having been mediated via P_{2X}-purinoceptors. The small depolarizing effect shown here of high concentrations of the P_{2Y}-purinoceptor agonist 2-Me.S.ATP (Burnstock & Kennedy, 1985) suggested that there was little, if any, P_{2Y}-purinoceptor mediated effects on the rat SCG. Our results therefore suggest that purine nucleotides *per se* depolarized the SCG via P_{2X}-purinoceptors whereas hyperpolarization via P_1-purinoceptors was caused by adenosine and by the purine nucleotides after metabolism to adenosine.

The depression by ATP of the depolarizing response to muscarine was reduced by 8-PT suggesting that this action of ATP was also mediated via P_1-purinoceptors. The depression of muscarinic depolarization by ATP is selective since depolarization by other agents such as GABA are not depressed by analogues of ATP (Table 2; Connolly, 1991). Indeed adenosine and β,γ-MeATP slightly increased GABA-induced depolarization. ATP was of similar potency to adenosine in depressing muscarinic depolarization while β,γ-MeATP was less active. Neither α,β-MeATP nor 2-Me.S.ATP increased or decreased the depolarizing response caused by muscarine, indicating that neither P_{2X}- nor P_{2Y}-purinoceptors were involved.

Pyrimidines

On tissues reported to contain pyrimidinoceptors it has been observed that UTP is generally the most active pyrimidine (Seifert & Schultz, 1989) and here we report that UTP was more potent than α,β-MeATP in depolarizing the rat SCG. Similarly UTP was found to depolarize bullfrog ganglion cells whereas CTP was weak or inactive (Gruol *et al.*, 1981). The less potent effect of CTP and the hyperpolarizing effect of UMP may also have been mediated though a pyrimidinoceptor, although the effect of suramin was not examined on these responses. The ability of UTP to depolarize the rat SCG as reported here is consistent with the reported inhibition of the M-current of rat SCG neurones by Adams *et al.* (1982), who found that 100 μM UTP strongly inhibited this potassium current. Here we noted that atropine abolished equivalent sized depolarizations to muscarine but not UTP, suggesting that UTP did not depolarize the ganglia by releasing ACh or by activating muscarinic receptors directly. The possibility that ATP depolarizes the rat SCG by inhibiting the M-current as reported for bullfrog ganglion cells (Akasu *et al.*, 1983) may be indicative that ATP and UTP share common ionic and biochemical mechanisms.

Effects of suramin

Suramin has been used as a specific antagonist of both P_{2X}- and P_{2Y}-purinoceptors in smooth muscles (Dunn & Blakeley, 1988; Mallard *et al.*, 1989; Den Hertog *et al.*, 1989; Schlicker *et al.*, 1989), as a competitive selective antagonist of P_{2X}-purinoceptors of the rabbit ear artery (Wood *et al.*, 1990) and as a reversible and selective antagonist of an ATP-receptor-operated membrane current in PC12 cells (Nakazawa *et al.*, 1990). In the present study suramin increased ATP-induced hyperpolarizations suggesting that these were in part functionally opposed by depolarizations mediated by P_2-purinoceptors. The ability of suramin to antagonize the depolarizations caused by α,β-MeATP and to potentiate the hyperpolarization produced by ATP and β,γ-MeATP supports the view that the depolarization was mediated via P_{2X}-purinoceptors. The lack of antagonism of the depolarizations induced by UTP suggest that this nucleotide does not depolarize the rat SCG by activating P_2-purinoceptors and

may therefore activate distinct pyrimidinoceptors. The reason for the potentiating effect of suramin on the UTP-induced depolarization is unknown, but it may be speculated that UTP may also activate hyperpolarizing receptors that are antagonized by suramin.

Effect of purines on the GABA-induced depolarization

The potentiation of GABA-induced depolarization by adenosine and β,γ-MeATP and the non-significant effect of ATP clearly contrasts with the effect of these compounds in depressing muscarinic depolarization. It has been reported that ATP strongly depressed the M current of bullfrog sympathetic neurones (Akasu *et al.*, 1983) and sensory ganglia (Morita *et al.*, 1984) and augmented the response caused by GABA (Morita *et al.*, 1984). It is possible that the M-current is involved in the depolarizing component of responses to ATP and α,β-MeATP but it seems unlikely that it is involved in the effect of adenosine and β,γ-MeATP in augmenting GABA-induced depolarizations. The effect of GABA on the rat SCG involves an increase in efflux of K^+ and chloride ions (Ballanyi & Grafe, 1985). Therefore the ability of adenosine and β,γ-MeATP to increase depolarization caused by GABA in the SCG may involve augmentation of the increased chloride efflux as reported by Ferrero & Frischknecht (1983) on guinea-pig taenia coli, or may be due to a change in the GABA-receptor/ion channel complex as described for bullfrog sensory ganglia by Morita *et al.* (1984). However, adenosine-induced hyperpolarization of the SCG is independent of the extracellular chloride concentration but is potentiated by low K^+ solutions (Connolly, 1991; Connolly & Stone, 1993b). In this study the hyperpolarizing responses caused by purines and UMP were also potentiated in low K^+/Ca^{2+} PSS, suggesting that the hyperpolarization induced by all these compounds is mediated via increased K^+ efflux. Likewise the ability of adenosine and β,γ-MeATP to potentiate GABA-mediated depolarizations may arise from increased K^+ efflux.

In conclusion, the rat SCG has P_1-purinoceptors that mediate hyperpolarization and depression of depolarizations induced by muscarine, and P_{2X}-purinoceptors that mediate depolarization but do not modulate the muscarinic depolarization. There are also receptors for UTP that mediate depolarization but do not modulate the muscarinic depolarization. These UTP receptors are insensitive to suramin, raising the possibility that UTP and other pyrimidines may act at distinct pyrimidinoceptors. The presence of receptors in the rat SCG for purines and the pyrimidine, UTP, supports the hypothesis that purines and pyrimidines may have a neurotransmitter or neuromodulatory role in autonomic ganglia.

G.P.C. was the recipient of an S.E.R.C. studentship. Part of this work was performed at SmithKline Beecham (SB), Pharmaceuticals Research and Development, Harlow and G.P.C. wishes to thank SB for the provision of laboratory and other facilities, and SB employees for their help. We also wish to thank Dr Frank Brown (SB) for his helpful comments and suggestions on an earlier version of this manuscript.

References

ADAMS, P.R., BROWN, D.A. & CONSTANTI, A. (1982). Pharmacological inhibition of the M-current. *J. Physiol.*, **332**, 223–262.

AKASU, T., HIRAI, K. & KOKETSU, K. (1981). Increase of acetylcholine receptor sensitivity by adenosine triphosphate: a novel action of ATP on ACh-sensitivity. *Br. J. Pharmacol.*, **74**, 505–507.

AKASU, T., HIRAI, K. & KOKETSU, K. (1982). Modulatory effect of adenosine triphosphate on the release of acetylcholine from presynaptic nerve terminals in bullfrog sympathetic ganglia. *Kuhume Med. J.*, **29**, 75–83.

AKASU, T., HIRAI, K. & KOKETSU, K. (1983). Modulatory actions of adenosine triphosphate on membrane potentials of bullfrog sympathetic ganglion cells. *Brain Res.*, **258**, 313–317.

AKASU, T. & KOKETSU, K. (1985). Effect of adenosine triphosphate on the sensitivity of the nicotinic acetylcholine-receptor in the bullfrog sympathetic ganglion cell. *Br. J. Pharmacol.*, **84**, 525–531.

AKASU, T., SHINNICK-GALLAGHER, P. & GALLAGHER, J.P. (1984). Adenosine mediates a slow hyperpolarisation synaptic potential in autonomic neurones. *Nature*, **311**, 62–64.

ARMSTRONG, C.M. & LOPEZ-BARNEO, J. (1987). External calcium ions are required for potassium channel gating in squid neurones. *Science*, **236**, 712–714.

BALLANYI, K. & GRAFE, P. (1985). An intracellular analysis of gamma-aminobutyric acid associated ion movements in rat sympathetic neurones. *J. Physiol.*, **365**, 41–58.

BROOKS, P.A. & STONE, T.W. (1988). Purine modulation of cholinomimetic responses in the rat hippocampal slice. *Brain Res.*, **458**, 106–114.

BROWN, D.A., CAULFIELD, M.P. & KIRBY, P.J. (1979). Relation between catecholamine-induced cyclic AMP changes and hyperpolarisation in isolated rat sympathetic ganglia. *J. Physiol.*, **290**, 441–451.

BROWN, D.A., FATHERAZI, S., GARTHWAITE, J.G. & WHITE, R.D. (1980). Muscarinic receptors in rat sympathetic ganglia. *Br. J. Pharmacol.*, **70**, 577–592.

BRUNS, R.F. (1980). Adenosine receptor activity in human fibroblasts: nucleotide agonists and antagonists. *Can. J. Physiol. Pharmacol.*, **58**, 673–691.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.* ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing between two types of P2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CONNOLLY, G.P. (1991). Pharmacology of adenosine receptors of the rat isolated superior cervical ganglion. *Ph.D. Thesis. University of Glasgow.*

CONNOLLY, G.P. & STONE, T.W. (1988). Purine receptors in rat isolated superior cervical ganglia. *Br. J. Pharmacol.*, **95**, 841P.

CONNOLLY, G.P. & STONE, T.W. (1993a). On the site of action and inactivation of adenosine by the rat superior cervical ganglion. *J. Auton. Pharmacol.*, **13**, 213–224.

CONNOLLY, G.P. & STONE, T.W. (1993b). The ionic mechanism of action of adenosine on the rat superior cervical ganglion. *J. Auton. Pharmacol.*, **13**, 253–266.

CONNOLLY, G.P., STONE, T.W. & BROWN, F. (1993c). Characterisation of the adenosine receptors of the rat superior cervical ganglion. *Br. J. Pharmacol.*, (in press).

COX, R.T.L. & WALKER, R.J. (1987). An analysis of the adenosine receptors responsible for modulation of an excitatory acetylcholine response of an identified helix neuron. *Comp. Biochem. Physiol.*, **88C**, 121–130.

CUSACK, N.J., HOURANI, S.M.O. & WELFORD, L.A. (1988). The role of ectonucleotidases in pharmacological responses to nucleotide analogues. In *Adenosine and Adenine nucleotides: Physiology and Pharmacology*, ed. D.M. Paton. pp. 93–102. London: Taylor & Francis.

DE GROAT, W.C. & BOOTH, A.M. (1980). Inhibition and facilitation in parasympathetic ganglia of the urinary bladder. *Fed. Proc.*, **39**, 2990–2996.

DEN HERTOG, A., NELEMANS, A. & VAN DEN AKKER, J. (1989). The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **166**, 531–534.

DOUGALL, I.G. & LEFF, P. (1987). Pharmacological analysis of the calcium-dependence of μ-receptor agonism. *Br. J. Pharmacol.*, **92**, 723–731.

DUNN, P.M. & BLAKELEY, I. (1988). Suramin: a reversible P2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

EDWARDS, F.A., GIBB, A.J. & COLQUHOUN, D. (1993). ATP receptor-mediated synaptic currents in the central nervous system. *Nature*, **359**, 144–147.

EL-FAKAHANY, E. & RICHELSON, E. (1980). Effects of lanthanides on muscarinic acetylcholine receptor function. *Mol. Pharmacol.*, **19**, 282–290.

FERRERO, J.D. & FRISCHKNECHT, R. (1983). Different effector mechanisms for ATP and adenosine-hyperpolarisation in the guinea-pig taenia coli. *Eur. J. Pharmacol.*, **87**, 151–154.

FURSHPAN, E.J., POTTER, D.D. & MATSUMOTO, S.G. (1986). Synaptic functions in rat sympathetic neurones in microcultures. III. A purinergic effect on cardiac myocytes. *J. Neurosci.*, **6**, 1099–1107.

GALLAGHER, J.P. & SHINNICK-GALLAGHER, P. (1978). Electrophysiological effects of nucleotides injected intracelullarly into rat sympathetic ganglion cells: In *Ionotophoresis and Transmitter Mechanisms in the Mammalian Central Nervous System.* ed. Ryall, R.W. & Kelly, J.S. pp. 152–154. Amsterdam: North-Holland Biomedical Press, Elsevier.

GRUOL, D.L., SIGGINS, G.R., PADJEN, A. & FORMAN, D. (1981). Explant cultures of adult amphibian sympathetic ganglia: electrophysiological and pharmacological investigation of neurotransmitter and nucleotide action. *Brain Res.*, **223**, 81–106.

GUSTAFSSON, L.E. (1981). Influence of adenosine on responses to vagal nerve stimulation in the anaesthetized rabbit. *Acta Physiol. Scand.*, **11**, 263–268.

HAUSSINGER, D., STEHLE, T. & GEROK, W. (1987). Actions of extracellular UTP and ATP in perfused rat liver. *Eur. J. Pharmacol.*, **167**, 65–71.

HENON, B.K. & MCAFEE, D.A. (1983a). The ionic basis of adenosine receptor actions on postganglionic neurones in the rat superior cervical ganglion. *J. Physiol.*, **336**, 607–620.

HENON, B.K. & MCAFEE, D.A. (1983b). Modulation of calcium currents by adenosine receptors on mammalian sympathetic neurones. In *Regulatory Function of Adenosine.* ed. Berne, R.M., Rall, T.W. & Rubio, R. pp. 455–466. The Hague: Martinis Nijhoff Publ.

KRISHTAL, O.A., MARCHENKO, S.M. & PIDOPLICHKO, V.I. (1983). Receptors for ATP in the membrane of mammalian sensory neurones. *Neurosci. Lett.*, **35**, 41–45.

MALLARD, N.J., MARSHALL, R.W. & SPRIGGS, T.L.B. (1989). Suramin a selective inhibitor of NANC transmission in rat vas deferens: comparison with nifedipine. *Br. J. Pharmacol.*, **98**, 618P.

MORITA, K., KATAYAMA, Y., KOKETSU, K. & AKASU, T. (1984). Actions of ATP on the soma of bullfrog afferent neurones and the modulating action on the GABA-induced response. *Brain Res.*, **293**, 360–363.

NAKAZAWA, K., FUJIMORI, K., TAKANAKA, A. & INOUE, K. (1990). Reversible and selective antagonism by suramin of ATP-activated inward current in PC12 phaeochromocytoma cell. *Br. J. Pharmacol.*, **101**, 224–226.

NEWBERRY, N.R. & CONNOLLY, G.P. (1989). Selective antagonism of muscarinic potentials on the superior cervical ganglion of the rat. *Neuropharmacology*, **28**, 487–493.

NEWBERRY, N.R. & PRIESTLEY, T. (1987). Pharmacological differences between two muscarinic responses of the rat superior cervical ganglion *in vitro*. *Br. J. Pharmacol.*, **92**, 817–826.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol Sci.*, **12**, 137–141.

POTTER, D.D., FURSHPAN, E.J. & LANDIS, S.C. (1983). Transmitter status in cultured rat sympathetic neurones: plasticity and multiple function. *Fed. Proc.*, **42**, 1626–1632.

SALT, T.E. & HILL, R.G. (1983). Excitation of single sensory neurones in the rat caudal trigeminal nucleus by ionophoretically applied ATP. *Neurosci. Lett.*, **35**, 53–58.

SALTER, M.W. & HENRY, J.L. (1985). Effects of cyclic AMP and ATP on functionally identified units in the cat spinal dorsal horn. Evidence for a differential affect of ATP on nociceptive versus non-nociceptive units. *Neuroscience*, **15**, 815–825.

SCHLICKER, E., URBANEK, E. & GOTHERT, M. (1989). ATP, α,β-methylene ATP and suramin as tools for characterisation of vascular P_{2X} receptors in the pithed rat. *J. Auton. Pharmacol.*, **9**, 357–366.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides. *Trends Pharmacol. Sci.*, **10**, 365–369.

SIGGINS, G.R., GRUOL, D.L., PADJEN, A.L. & FORMAN, D.S. (1977). Purine and pyrimidine mononucleotides depolarize neurones of explanted amphibian sympathetic ganglia. *Nature*, **270**, 263–265.

SILINSKY, E.M. & GINSBORG, B.L. (1983). Inhibition of acetylcholine release from preganglionic frog nerves by ATP but not adenosine. *Nature*, **305**, 327–8.

STONE, T.W. & CUSACK, N.J. (1989). Absence of P_2-purinoceptors in hippocampal pathways. *Br. J. Pharmacol.*, **97**, 631–635.

TAKAGI, K., TAKAYANAGI, I. & LIAO, C.S. (1972). The effects of calcium and magnesium ions on drug-receptor interactions. *Eur. J. Pharmacol.*, **19**, 330–342.

VON KUGELGEN, I., HAUSSINGER, D. & STARKE, K. (1987). Evidence for a vasoconstriction-mediating receptors for UTP, distinct from the P_{2X}-purinoceptor, in rabbit ear artery. *Naunyn Schmied. Arch. Pharmacol.*, **336**, 556–560.

WOLINSKY, E.J. & PATTERSON, P.H. (1985). Potassium-stimulated purine release by cultured sympathetic neurones. *J. Neurosci.*, **5**, 1680–1687.

WOOD, B.E., O'CONNOR, S.E.O., FEAR, N.T. & LEFF, P. (1990). Suramin is a slowly equilibrating but competitive antagonist at P_{2X}-purinoceptors in the rabbit ear artery. *Br. J. Pharmacol.*, **100**, 342P.

ZIMMERMAN, H., GRONDAL, E.J.M. & KELEER, F. (1985). Hydrolysis of ATP and formation of adenosine at the surface of cholinergic nerve endings. In *Purines: Pharmacology and Physiological Roles.* ed. Stone, T.W. pp. 35–48. London: Macmillan.

(Received April 30, 1993
Revised July 12, 1993
Accepted July 26, 1993)

Br. J. Pharmacol. (1993), **110**, 1305–1310 © Macmillan Press Ltd, 1993

Increases in intracellular calcium via activation of an endogenous P_2-purinoceptor in cultured CHO-K1 cells

[1]Philip A. Iredale & Stephen J. Hill

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 Increases in intracellular calcium ($[Ca^{2+}]_i$) were measured in chinese hamster cultured ovary cells (clone, CHO-K1), by use of the fluorescent, calcium-sensitive dye, fura-2.

2 Addition of both ATP and UTP elicited rapid increases in $[Ca^{2+}]_i$ due to mobilization from intracellular stores and calcium entry across the plasma membrane.

3 Omission of calcium from the extracellular medium and pre-incubation with the inorganic calcium channel blocker, nickel (Ni^{2+}) prevented the calcium entry components of the responses.

4 Investigation of the concentration-response relationships of various analogues of ATP suggests the presence of a purinoceptor which cannot be characterized as P_{2X} or P_{2Y}. In addition, there appears to be a sub-population of P_{2Y}-purinoceptors which do not cross-react with the 'nucleotide' receptor population.

5 Cross-desensitization and additivity experiments suggest that both ATP and UTP activate the same receptor.

6 Pre-incubation with the tumour-promoting agent, β-phorbol-12,13 dibutyrate (PDBu), caused a reduction in the increases in $[Ca^{2+}]_i$, suggesting a role for protein kinase C in feedback inhibition of purinoceptor responses in this cell line.

7 In summary, we present evidence for the existence of an endogenous P_{2U}-purinoceptor (or 'nucleotide receptor') which is linked to increases in $[Ca^{2+}]_i$ in CHO-K1 cells.

Keywords: ATP; nucleotides; P_2-purinoceptors; P_{2U} purinoceptors; intracellular calcium; phorbol esters; fura-2

Introduction

The classification of purinoceptors has advanced considerably since Burnstock originally divided these receptors into P_1- (now sub-divided into A_1 and A_2-adenosine receptors) and P_2-purinoceptors on 1978 (Burnstock, 1978). The same author subsequently proposed a subdivision of the latter into P_{2X} and P_{2Y} (Burnstock & Kennedy, 1985) based on the rank order of potency of ATP analogues in a number of tissues. In general, the rank order of potency at P_{2X} is α,β-methylene ATP (α,β-MeATP) > β,γ-methylene ATP > ATP = ADP > 2-methylthio ATP (2-MeSATP) and at P_{2Y}, 2-MeSATP > ATP = ADP > α,β-MeATP > β,γ-methylene ATP. The extracellular effects of ATP and its related agonists have been thoroughly investigated in cultured cell lines (Hallam & Pearson, 1986; Phaneuf *et al.*, 1987; Rice & Singleton, 1987; Ehrlich *et al.*, 1988; Cowen *et al.*, 1990; Kastritsis *et al.*, 1992; Koike *et al.*, 1992; Sato *et al.*, 1992) and current classification suggests the existence of at least three additional subtypes of P_2-purinoceptors (see TIPS Receptor Nomenclature supplement, 1993). These include a receptor found primarily on platelets (P_{2T}; Gordon, 1986) and one responsible for permeabilisation of mast cells (P_{2Z}; Cockroft & Gomperts, 1980). However, the proposed receptor which has received the most attention recently is the 'nucleotide' or P_{2U} receptor (for review see O'Connor, 1992).

This 'atypical' purinoceptor has now been reported to exist in a variety of cell lines and has been linked to phospholipase C activation (Brown *et al.*, 1991; Murrin & Boarder, 1991) phospholipase D activation (Murrin & Boarder, 1991) and the production of inositol phosphates and subsequent mobilisation of intracellular calcium (Fine *et al.*, 1989; Iredale *et al.*, 1992a; Lustig *et al.*, 1992; Pavenstadt *et al.*, 1992). Futhermore, it has been suggested that the pyrimidine, UTP might also be an agonist at the same receptor (Pfeilschifter, 1990; Brown *et al.*, 1991; Iredale *et al.*, 1992a). However, the existence of a *separate* pyrimidine receptor has also been proposed (Stutchfield & Cockcroft, 1990; von Kugelgen *et al.*, 1990).

Chinese hamster ovary cells contain very few endogenous receptors which makes them ideal transfection recipients. However, a constitutive ATP receptor linked to arachidonic acid release has been reported (Felder *et al.*, 1991; Traiffort *et al.*, 1992). We now present evidence for the existence of a purinoceptor linked to the mobilisation of $[Ca^{2+}]_i$ in the Chinese hamster ovary cell line clone, CHO-K1. Furthermore, its pharmacological properties suggest that it is of the P_{2U}-purinoceptor (or 'nucleotide' receptor) subtype.

Methods

Cell culture

CHO-K1 cells, passages 20–35, were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagles Medium/Nutrient Mix F12 (1:1, with glutamine) containing 10% foetal calf serum without antibiotics.

Calcium measurements

$[Ca^{2+}]_i$ was measured as previousy described (Iredale *et al.*, 1992b). Briefly, the monolayers from two near confluent flasks were detached with Trypsin/EDTA (trypsin, 0.5 g; EDTA 0.2 g and NaCl, 0.85 g per litre) and resuspended in a simple saline HEPES buffer (composition, mM: $CaCl_2$ 2, NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1 and HEPES 10; pH 7.45). This was followed by incubation with fura-2 acetoxy methyl ester (5 μM) at 37°C (in the presence of 10% foetal calf serum) for 20 min and for a further 5 min following a three fold dilution (to ensure maximum hydrolysis of ester to the acid form). At the end of this loading period, excess dye was removed by centrifugation, the cells resuspended in fresh buffer (no serum), and left at room temperature until use. Each calcium time course was preceded by

[1] Author for correspondence.

a rapid spin in a microcentrifuge followed by resuspension in fresh buffer (2 ml).

All experiments were carried out using a Perkin Elmer LS 50 Spectrometer, with excitation ratioing between 340 and 380 nm, recording at 500 nm. The time course for each calcium measurement was 200 s with drugs added in 10 μl aliquots.

Calibration

At the end of each time course, ionomycin (20 μM) was added followed by EGTA (6.25 mM, pH greater than 8.5) in order to calculate R_{max} and R_{min}. Autofluorescence was determined in a separate cuvette following the addition of manganese (5 mM) after the ionophore ionomycin (20 μM). Using these values and those obtained with fura-2 free acid, $[Ca^{2+}]_i$ was calculated according to the method of Grynciewicz *et al.* (1985). There was, however, some difficulty in obtaining reproducible calibrated traces for the higher concentrations of the agonists used, as the ratios obtained were close to the calculated value for R_{max}. Therefore the measured 340/380 nm fluorescence ratios were used for statistical comparisons between responses.

Statistics and data analysis

EC_{50} (concentrations of drug producing 50% of maximal stimulation or inhibition) values were obtained by computer-assisted curve fitting by using the computer programme Graph-Pad (ISI). Calcium data were captured using the ICBC programme supplied by Perkin-Elmer and imported to the graphics programme Sigma Plot (Jandel).

Significance testing was carried out with Student's unpaired *t* test ($P<0.05$ was considered statistically significant).

Materials

CHO-K1 cells were a generous gift from Dr Andrea Townsend-Nicholson, Garvan Institute, Sydney, Australia. Cell culture flasks were obtained from Costar with Dulbecco's Modified Eagles Medium and foetal calf serum from NBL Ltd (Cramlington, U.K.). PDBu and cholecystokinin were supplied by Sigma (Poole, U.K.), with fura-2 AM and ionomycin from Calbiochem/Novobiochem (Nottingham, U.K.). All nucleotides were supplied by Boehringer Mannheim (Lewes, U.K.).

Results

The calcium responses to ATP and UTP

Addition of ATP (10 μM) to populations of fura-2-loaded CHO-K1 cells resulted in a rapid increase in 340/380 nm fluorescence ratio of 4.7 ± 0.5 ($n = 3$) which returned to a new maintained plateau after approximately 60 s. When the responses were calibrated using ionomycin and EGTA this change corresponded to an initial increase in $[Ca^{2+}]_i$ of 972 ± 111 nM ($n = 3$) with a plateau level of 145 ± 20 nM above basal (Figure 1a). The resting $[Ca^{2+}]_i$ level measured over a number of experiments was found to be 138 ± 6 nM ($n = 16$). Addition of UTP (10 μM) produced a calcium response with similar characteristics (an initial rapid increase in $[Ca^{2+}]_i$ of 710 nM ± 90 ($n = 3$) returning to a secondary plateau level of 116 ± 6 nM above basal). The results suggested that the response to UTP was smaller than that to ATP; however, some day to day variation was observed in the absolute sizes of the responses. When this was accounted for by comparing responses on the same day, the two agonists produced similarly sized increases in $[Ca^{2+}]_i$ (Table 1). Furthermore, the calcium responses to both ATP and UTP were found to be concentration-dependent with EC_{50} values of 2.3 ± 0.5 μM (Figure 1b) and 2.8 ± 0.6 μM ($n = 3$) respectively.

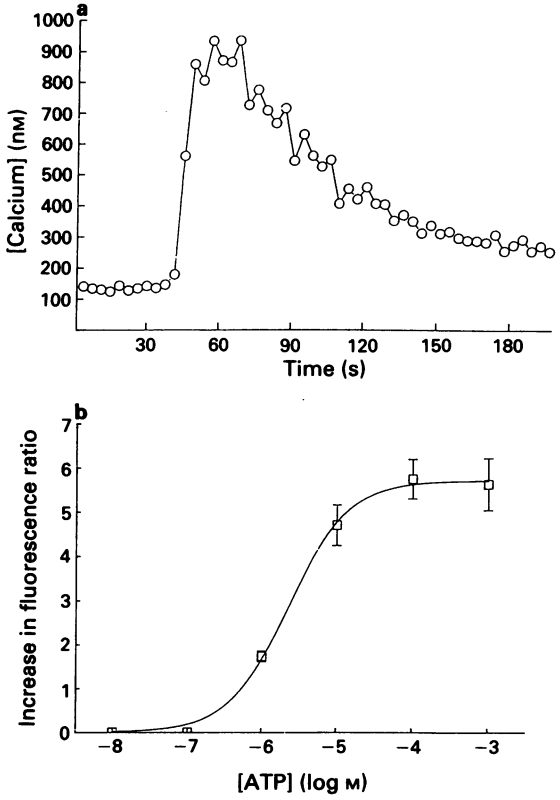


Figure 1 Panel (a) shows the effect of ATP (10 μM) on $[Ca^{2+}]_i$ in populations of fura-2-loaded CHO-K1 cells. Fluorescence values were measured following excitation at 340 and 380 nm and the ratio used to determine $[Ca^{2+}]_i$. The graph shown is typical of two others. Panel (b) shows the concentration-effect relationship for ATP. Data are the means ± s.e.mean of three separate determinations.

The calcium entry component

In order to investigate the role of extracellular calcium in the ATP- and UTP-induced changes in $[Ca^{2+}]_i$, experiments were carried out in the absence of extracellular calcium and in the presence of the non-specific calcium channel antagonist, Ni^{2+} (1 mM). As can be seen, from Figure 2 and Table 2, under both conditions, the secondary plateau was no longer seen, suggesting a significant role for calcium entry in the overall response.

Evidence that ATP and UTP act at the same receptor

In order to determine whether or not ATP and UTP increased $[Ca^{2+}]_i$ via stimulating a common 'nucleotide' receptor, any cross-desensitization or additivity of the calcium responses were investigated. A comparison between cells stimulated with a sub-maximally effective concentration of ATP (6 μM) and those stimulated with a mixture of ATP and UTP (both 3 μM) failed to reveal any significant difference in the relative sizes of the calcium responses (Figure 3a). Furthermore, when the cells were pre-incubated for 20 min with adenosine 5'-(3-thio) triphosphate (ATPγS, 100 μM), the poorly hydrolysable analogue of ATP, and then challenged with UTP (100 μM), there was a significant decrease in the calcium response compared with untreated cells (Figure 3b). The calcium response to the non-purinoceptor agonist, cholecystokinin (2.2 μM) was unaffected by pre-incubation with ATPγS (101 ± 12% of the control response; $n = 4$).

Table 1 The relative potencies and efficacies of ATP, its analogues and other nucleotides in increasing intracellular calcium in CHO-K1 cells

Agonist	*EC_{50}*	*% ATP response* (300 μM)
ATP	2.3 ± 0.5 μM	100
ADP	69 ± 14 μM	84 ± 6
AMP	–	not detectable
2-Chloroadenosine	–	not detectable
ATPγS	12 ± 0.5 μM	96 ± 6
2MeSATP	11.5 ± 4 μM	37 ± 2
α,β-MeATP	–	not detectable
UTP	2.8 ± 0.6 μM	104 ± 2
GTP	–	53 ± 3
CTP	–	7 ± 3

The results are the mean of three separate determinations carried out on at least two different days. Agonists without calculated EC_{50} values were added at a concentration of 300 μM.

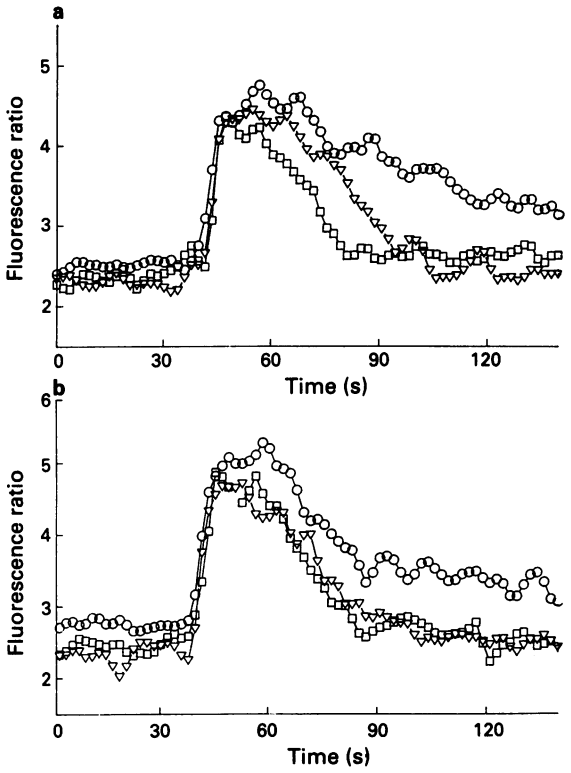


Figure 2 The effects of extracellular calcium and the inorganic calcium channel blocker, Ni^{2+}, on the calcium responses (340/380 nm fluorescence ratios shown) elicited by ATP (a, 100 μM) and UTP (b, 100 μM). Under control conditions (extracellular calcium 2 mM; O) the initial rapid increase in $[Ca^{2+}]_i$ was followed by a slowly degrading secondary plateau phase. However, omission of calcium from the extracellular medium (□) or pre-incubation for 5 min with Ni^{2+} (1 mM; Δ) resulted in a characteristically different response which clearly lacked the secondary plateau. Each of the traces shown is typical of three others.

The responses to ATP analogues and other related compounds

In order to identify the particular receptor(s) involved in the observed calcium response, a number of other possible agonists were investigated (Table 1). 2-Chloroadenosine (300 μM) and AMP (300 μM) were without effect suggesting that P_1-purinoceptors, which are preferentially activated by these compounds, were not involved in the calcium response. There was, however, a significant rise in $[Ca^{2+}]_i$ elicited by ADP with a maximal response of 84 ± 6% of the ATP signal; however ADP was 30 times less potent than ATP (69 ± 14 μM, $n = 3$). GTP (300 μM) was also able to elicit a response (53% of the ATP signal), but CTP (300 μM) was without effect.

Table 2 The effect of the omission of extracellular calcium and Ni^{2+} on the responses to ATP and UTP in CHO-K1 cells

Agonist		*Basal*	*Peak*	*120 s*
ATP	+ Ca^{2+}	2.6 ± 0.1	5.6 ± 0.2	3.4 ± 0.1**
	− Ca^{2+}	2.3 ± 0.1	4.7 ± 0.1	2.5 ± 0.1
	+ Ni^{2+}	2.5 ± 0.2	4.4 ± 0.2	2.6 ± 0.04
UTP	+ Ca^{2+}	2.6 ± 0.03	4.9 ± 0.1	3.3 ± 0.03***
	− Ca^{2+}	2.2 ± 0.03	4.3 ± 0.03	2.3 ± 0.04
	+ Ni^{2+}	2.4 ± 0.03	4.2 ± 0.2	2.6 ± 0.03

The data are the 340/380 nm fluorescence ratios ($n = 4$) of cells unchallenged with ATP (100 μM) and UTP (100 μM) in the presence and absence of extracellular calcium and following pre-incubation for 5 min with the inorganic calcium channel blocker, Ni^{2+} (1 mM). Those responses still significantly above basal at 120 s are shown with asterisks (**$P < 0.01$, ***$P < 0.001$, Student's unpaired t test).

A number of ATP analogues were also investigated: ATPγS exhibited a similar maximal response to ATP but was 5 times less potent (12.4 ± 0.5 μM, $n = 3$). The P_{2X}-selective agonist, α,β-MeATP (300 μM) was without effect, however, the P_{2Y}-selective agonist, 2MeSATP (300 μM) appeared to behave as a partial agonist with an EC_{50} of 11.5 ± 4 μM ($n = 3$) and a very much reduced maximal response (37% of the ATP response). This hypothesis was investigated further by carrying out interaction studies between 2MeSATP and ATP (and UTP). However, 2MeSATP at a concentration of 300 μM (30 times its calculated EC_{50}) failed to have any effect on the calcium responses to 30 μM (a maximally effective concentration) of either agonist (102 ± 1% and 98 ± 2% ($n = 3$) of the control responses, respectively). Furthermore, when the cells were pre-incubated with 2MeSATP (300 μM) for 20 min, there was no significant effect on the calcium response to ATP (30 μM; 98 ± 5% ($n = 3$) of the control response).

Phorbol pretreatment

Pre-incubation (for 20 min during the fura-2 loading period) with the phorbol ester, β-phorbol-12,13 dibutyrate (PDBu; 1.5 μM) failed to have any significant effect on the calcium response to 100 μM ATP (94 ± 2% of the control responses, $n = 3$). However, the responses to sub-maximally effective concentrations of both ATP and UTP (1 μM) were highly attenuated following phorbol pre-treatment (Figure 4).

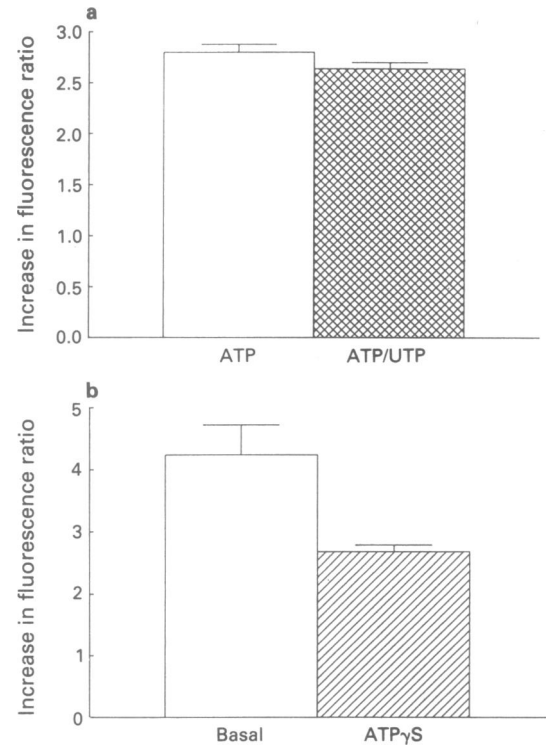


Figure 3 Panel (a) shows a comparison of the calcium response to ATP (6 μM) with that to a mixture of ATP (3 μM) and UTP (3 μM). There was no significant difference between the two responses. Data are the means with s.e.mean of five separate determinations. Panel (b) shows the cross-desensitization of the calcium response to UTP (100 μM). The cells were pre-incubated for 20 min with the poorly-hydrolysable analogue of ATP, adenosine 5'-(3-thio)-triphosphate ATPγS (100 μM). The open column shows the control response to UTP. The hatched column shows the effect of the same concentrations of UTP after the cells were pretreated with ATPγS. Significant ($P<0.01$) reductions in the calcium responses were seen. Data are the means with s.e.mean of five separate determinations.

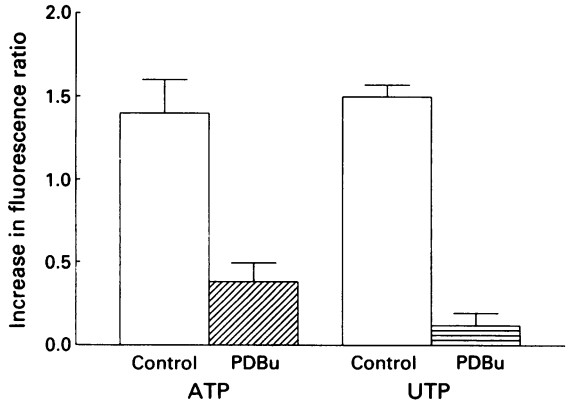


Figure 4 The effect of β-phorbol-12,13 dibutyrate (PDBu, 1.5 μM) on the calcium responses to ATP (1 μM) and UTP (1 μM). Cells were preincubated with the phorbol ester for 25 min, which was present for the remainder of the experiment, resulting in significant reductions ($P<0.001$ and $P<0.0001$ respectively) in the responses to both agents. Data are the means with s.e.mean of five separate determinations.

Discussion

In populations of CHO-K1 cells, addition of both ATP and UTP resulted in rapid, concentration-dependent increases in $[Ca^{2+}]_i$ which involved both mobilisation of calcium from intracellular stores and entry from the extracellular environment. The results of further studies with ATP analogues accompanied by cross-desensitization and additivity experiments indicate that a P_{2U} or 'nucleotide' receptor is present on this cell type.

Results from a number of different cell lines have revealed that some agonist responses are biphasic, involving an initial transient release of calcium from intracellular stores followed by a secondary sustained influx across the plasma membrane, resulting in a maintained plateau (Baird *et al.*, 1989; Jacob, 1990). This plateau is maintained as long as the agonist occupies the receptor. The characteristics of the responses to both ATP and UTP in CHO-K1 cells are consistent with this theory. Following an initial rapid spike, stimulation with either agonist resulted in a secondary, maintained response. Omission of calcium, or inclusion of the inorganic channel blocker, Ni^{2+} in the extracellular medium, prevented the formation of this plateau phase, providing further evidence that influx of calcium forms part of the overall response.

The lack of activity of 2-chloroadenosine and AMP suggests the absence of a P_1-purinoceptor agonist component of the observed calcium response. The original classification of P_2-purinoceptors was based upon the rank order of potencies of ATP analogues (Burnstock & Kennedy, 1985). In the CHO-K1 cell line the highly potent P_{2X}-purinoceptor, α,β-MeATP, was without effect and 2MeSATP, the highly potent P_{2Y}-purinoceptor agonist, elicited only a small increase in $[Ca^{2+}]_i$ and was 5 times less potent than ATP. Indeed only ATPγS was able to increase $[Ca^{2+}]_i$ with an efficacy similar to that of ATP. Similar observations have been made in other cell lines (Allsup & Boarder, 1990; Brown *et al.*, 1991; Lustig *et al.*, 1992; Pavenstadt *et al.*, 1992) and the pharmacological characteristics of the receptor in CHO-K1 cells are very similar to those of the atypical P_2 receptor we have previously reported in the neuroblastoma cell line, N1E-115 (Iredale *et al.*, 1992a).

ATP can be readily hydrolysed and it is therefore necessary to consider the possibility that ATP itself is not the agonist at this receptor. However, this would appear unlikely as ADP was considerably less potent, and AMP and the adenosine analogue, 2-chloroadenosine were without effect. Furthermore, the presence of a triphosphate group within the agonist structure is not sufficient for activity, since although UTP exhibited similar potency and efficacy, GTP was less potent and CTP was without effect. Nevertheless, the triphosphate group might contribute to the activity of the agonist since ATP was more potent than ADP.

Perhaps a more important consideration is the similarity in potency and efficacy between the two structurally distinct molecules, ATP (a purine) and UTP (a pyrimidine). The existence of separate receptors for each agonist has been suggested (Stutchfield & Cockcroft, 1990; von Kugelgen *et al.*, 1990), although it would appear that the idea of a 'common receptor' for both agonists is also a possibility (Pfeilschifter, 1990; Brown *et al.*, 1991; Iredale *et al.*, 1992a; Murrin & Boarder, 1992). In CHO-K1 cells the evidence is consistent with the latter theory. Pre-incubation of the cells with the poorly-hydrolysable analogue of ATP, ATPγS for 20 min resulted in a significant reduction in the response to a maximally effective concentration of UTP, suggesting a possible cross-desensitization of the response. Further evidence for a 'common receptor' was the lack of additivity of the calcium responses to ATP and UTP. Unfortunately these types of experiments are not easily performed when $[Ca^{2+}]_i$ is the parameter of interest since it still remains possible that separate receptors mobilise calcium from a common store, the state of which might be the limiting factor. However, given that the concentrations of the agonists used were submaximal, depletion of calcium stores should not have been a problem. Overall the evidence points towards the two agonists increasing $[Ca^{2+}]_i$ via the same receptor but the lack of any specific antagonists makes further characterization difficult.

The initial results obtained with 2MeSATP suggest th

either (i) it is a low efficacy agonist at the nucleotide receptor or (ii) that 2MeSATP is selectively activating a sub-population of P_{2Y}-purinoceptors. However, 2MeSATP failed to have any effect on the calcium responses elicited by both ATP and UTP. Furthermore, there was no observed desensitization of the calcium response to ATP following pre-incubation with 2MeSATP for 20 min. Thus, it would appear that 2MeSATP might be acting on a separate population of receptors as has been reported in the rat aorta (Dainty *et al.*, 1990). If this is the case, the data obtained here provide no evidence that part of the response to ATP is mediated by these 2MeSATP-sensitive receptors. However, without a selective antagonist, the response to 2MeSATP needs to be interpreted with caution.

Phorbol ester inhibition of the nucleotide response is a feature common to a number of phospholipase C signal transduction pathways (Nishizuka, 1986). We have previously shown that ATP- and UTP-induced mobilisation of $[Ca^{2+}]_i$ can be inhibited by pre-treatment with PDBu in N1E-115 cells (Iredale *et al.*, 1993). Indeed this feature was common to a number of calcium mobilising receptors found in this cell line (Iredale *et al.*, 1993). It would appear likely that the inhibition found in CHO-K1 cells is the result of protein kinase C-mediated phosphorylation. A number of possible sites for this phosphorylation exist including the receptor, the G protein, phosphoinositidase C or even the inositol-1,4,5-triphosphate receptor. The lack of effect of incubation on a maximally-effective concentration of ATP is perhaps quite surprising. However, a similar lack of sensitivity was seen in N1E-115 cells when stimulated with a maximally-effective concentration of bradykinin (Iredale *et al.*, 1993).

In summary these data suggest the presence of a P_2-purinoceptor in CHO-K1 cells which is linked to both calcium mobilisation and entry. The receptor is activated by both ATP and UTP and its characteristics suggest that it can be tentatively classified as a P_{2U} or 'nucleotide' receptor. Unfortunately neither of these definitions describes accurately the type of receptor involved and until a specific antagonist is developed further classification is difficult.

CHO-K1 cells are fibroblastomas in origin. The presence of P_2-purinoceptors on cultured cells derived from fibroblasts has also been reported elsewhere (Fine *et al.*, 1989) and raises the important question as to their possible function *in vivo*. Fibroblasts are essentially present in all tissues and their main functions include deposition, maintenance, degradation and rearrangement of the extracellular matrix. Certainly ATP is released from nerve endings at the site of an injury, thus it might be possible that these receptors are involved in wound healing and tissue formation.

We would like to thank the MRC for their financial support.

References

ALLSUP, D.J. & BOARDER, M.R. (1990). Comparison of P_2-purinergic receptors of aortic endothelial cells with those of the adrenal medulla: evidence for heterogeneity of receptor subtype and inositol phosphate response. *Mol. Pharmacol.*, **38**, 84–91.

BAIRD, J.G., LAMBERT, D.G., MCBAIN, J. & NAHORSKI, S.R. (1989). Muscarinic receptors coupled to phosphoinositide hydrolysis and elevated cytosolic calcium in a human neuroblastoma cell line SK-N-SH. *Br. J. Pharmacol.*, **98**, 1328–1334.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5'-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40**, 648–655.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptors. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*. ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptors? *Gen. Pharmacol.*, **16**, 433–440.

COCKCROFT, S. & GOMPERTS, B.D. (1980). The ATP^4-receptor of rat mast cells. *Biochem. J.*, **188**, 789–798.

COWEN, D.S., SANDERS, M. & DUBYAK, G. (1990). P_2-purinergic receptors activate a guanine nucleotide-dependent phospholipase C in membranes from HL-60 cells. *Biochem. Biophys. Acta.*, **1053**, 195–203.

DAINTY, I.A., STEPTOE, J.E., O'CONNOR, S.E. & LEFF, P. (1990). Is 2-methylthio-ATP an appropriate tool for the identification of P_{2Y}-purinoceptors? *Br. J. Pharmacol.*, **101**, 507P.

EHRLICH, Y.H., SNIDER, R.M., KORNECKI, E., GARFIELD, M.G. & LENOX, R.H. (1988). Modulation of neuronal signal transduction systems by extracellular ATP. *J. Neurochem.*, **50**, 295–301.

FELDER, C., WILLIAMS, H. & AXELROD, J. (1990). A transduction pathway associated with receptors coupled to the inhibitory guanine nucleotide binding protein Gi that amplifies ATP-mediated arachidonic acid release. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6477–6480.

FINE, J., COLE, P. & DAVIDSON, J.S. (1989). Extracellular nucleotides stimulate receptor-mediated calcium mobilisation and inositol phosphate production in human fibroblasts. *Biochem. J.*, **263**, 371–376.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biochem. Sci.*, **260**, 3440–3450.

HALLAM, T.J. & PEARSON, J.D. (1986). Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells. *FEBS Lett.*, **207**, 95–99.

IREDALE, P.A., MARTIN, K.F., HILL, S.J. & KENDALL, D.A. (1992a). Inositol 1,4,5-trisphosphate generation and calcium mobilisation via activation of an atypical P_2 receptor in the neuronal cell line, N1E-115. *Br. J. Pharmacol.*, **107**, 1083–1087.

IREDALE, P.A., MARTIN, K.F., HILL, S.J. & KENDALL, D.A. (1992b). Agonist-induced changes in $[Ca^{2+}]_i$ in N1E-115 cells: differential effects of bradykinin and carbachol. *Eur. J. Pharmacol.*, **226**, 163–168.

IREDALE, P.A., MARTIN, K.F., HILL, S.J. & KENDALL, D.A. (1993). The effects of β-phorbol-12,13 dibutyrate on agonist-induced increases in $[Ca^{2+}]_i$ in N1E-115 cells. *Biochem. Pharmacol.*, **45**, 611–617.

JACOB, R. (1990). Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *J. Physiol.*, **421**, 55–77.

KASTRITSIS, C.H.C., SALM, A.K. & MCCARTHY, K. (1992). Stimulation of the P_{2Y} purinergic receptor on type 1 astroglia results in inositol phosphate formation and calcium mobilisation. *J. Neurochem.*, **58**, 1277–1284.

KOIKE, M., KASHIWAGURA, T. & TAKEGUCHI, T. (1992). Gluconeogenesis stimulated by extracellular ATP is triggered by the initial increase in the intracellular Ca^{2+} concentration of the periphery of hepatocytes. *Biochem. J.*, **283**, 265–272.

LUSTIG, K.D., SPORTIELLO, M.G., ERB, L. & WEISMAN, G.A. (1992). A nucleotide receptor in vascular endothelial cells is specifically activated by the fully ionized forms of ATP and UTP. *Biochem. J.*, **284**, 733–739.

MURRIN, R.J.A. & BOARDER, M.R. (1992). Neuronal 'nucleotide' receptor linked to phospholipase C and phospholipase D? Stimulation of PC12 cells by ATP analogues and UTP. *Mol. Pharmacol.*, **41**, 561–568.

NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. *Science*, **233**, 305–312.

O'CONNOR, S.E. (1992). Recent developments in the classification and functional significance of receptors for ATP and UTP, evidence for nucleotide receptors. *Life Sci.*, **50**, 1657–1664.

PAVENSTADT, H., SPATH, M., SCHLUNCK, G., NAUCK, M., FISCHER, R., WANNER, C. & SCHOLLMEYER, P. (1992). Effect of nucleotides on the cytosolic free calcium activity and inositol phosphate formation in human glomerular epithelial cells. *Br. J. Pharmacol.*, **107**, 189–195.

PFEILSCHIFTER, J. (1990). Comparison of extracellular ATP and UTP signalling in rat renal mesangial cells. *Biochem. J.*, **272**, 469–472.

PHANEUF, S., BERTA, P., CASANOVA, J. & CAVADORE, J. (1987). ATP stimulates inositol phosphates accumulation and calcium mobilisation in a primary culture of rat aortic myoctyes. *Biochem. Biophys. Res. Commun.*, **143**, 454–460.

RICE, W.R. & SINGLETON, F.M. (1987). P_{2Y}-purinoceptor regulation of surfactant secretion from isolated alveolar type II cells is associated with mobilisation of intracellular calcium. *Br. J. Pharmacol.*, **91**, 833–838.

SATO, K., OKAJIMA, F. & KONDO, Y. (1992). Extracellular ATP stimulates three different receptor-signal transduction systems in FRTL-5 thyroid cells. *Biochem. J.*, **283**, 281–287.

STUTCHFIELD, J. & COCKROFT, S. (1990). Undifferentiated HL60 cells respond to extracellular ATP and UTP by stimulating phospholipase C activation and exocytosis. *FEBS Lett.*, **262**, 256–258.

TRAIFFORT, E., RUAT, M., ARRANG, J., LEURS, R., PIOMELLI, D. & SCHWARTZ, J. (1992). Expression of a cloned rat histamine H_2 receptor mediating inhibition of arachidonate release and activation of cAMP accumulation. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 2649–2653.

VON KUGELGEN, I., BULTMANN, R. & STARKE, K. (1990). Interaction of adenine nucleotides, UTP and suramin in mouse vas deferens: suramin-sensitive and suramin-insensitive components in the contractile effect of ATP. *Naunyn-Schmied Arch. Pharmacol.*, **342**, 198–205.

(Received July 5, 1993
Revised July 26, 1993
Accepted July 27, 1993)

Br. J. Pharmacol. (1993), **110**, 1311–1316 © Macmillan Press Ltd, 1993

Effects of two β_3-adrenoceptor agonists, SR 58611A and BRL 37344, and of salbutamol on cholinergic and NANC neural contraction in guinea-pig main bronchi *in vitro*

[1]Corinne A.E. Martin, Emmanuel Naline, *Luciano Manara & Charles Advenier

Département de Pharmacologie, Faculté de Médecine Paris-Ouest, 15 rue de l'Ecole de Médecine, F-75270 Paris Cedex 06, France and *SANOFI-MIDY S.p.A. Research Center, via Piranesi 38, 20137 Milan, Italy

1 The aim of the present study was to investigate the type of adrenoceptor which modulates constriction of the guinea-pig isolated main bronchus in response to electrical field stimulation (EFS). Drugs used were salbutamol and two agonists reportedly selective for the putative β_3-adrenoceptor: BRL 37344 and SR 58611A.

2 At basal tone, all three drugs induced relaxation, however, SR 58611A and BRL 37344 (10^{-9} to 10^{-6} M) relaxed guinea-pig isolated main bronchus more weakly than salbutamol (10^{-9} to 10^{-6} M). The effects observed at 10^{-6} M were 43% ± 9%, 63% ± 4% and 98% ± 1% of the maximal effect induced by theophylline (3×10^{-3} M) for SR 58611A, BRL 37344 and salbutamol, respectively.

3 SR 58611A and BRL 37344 (10^{-8} to 10^{-6} M) did not significantly modify the cholinergic component of the response to EFS, but caused a concentration-dependent reduction of the nonadrenergic non-cholinergic (NANC) excitatory component (41.8% ± 10.1% and 56.8% ± 7.4% respectively at 10^{-6} M, $n = 6-7$). Salbutamol (10^{-9} to 10^{-7} M) strongly inhibited both components, with 91.1% ± 4.2% of inhibition for the NANC contraction and 62.0% ± 5.2% of inhibition for the cholinergic contraction (10^{-7} M, $n = 7$).

4 Whereas the inhibitory effects of salbutamol were strongly inhibited by both propranolol (10^{-6} M) and ICI 118,551 (10^{-6} M), those of BRL 37344 were only slightly, albeit significantly reduced by both propranolol and ICI 118,551, and those of SR 58611A were unaffected by treatment with either β-adrenoceptor antagonist. An α_2-adrenoceptor antagonist, yohimbine, did not influence the inhibitory effects of any of the β-adrenoceptor agonists tested.

5 Concentration-response curves to acetylcholine (10^{-8} to 10^{-3} M), [Nle^{10}]NKA(4–10) (10^{-10} to 10^{-6} M) and substance P (10^{-10} to 3×10^{-6} M) were also significantly shifted to the right by salbutamol (10^{-6} M), whereas SR 58611A and BRL 37344 (10^{-6} M) had no effect.

6 These results suggest that the stimulation of putative β_3-adrenoceptors exerts a specific prejunctional inhibitory action on NANC excitatory response induced by EFS of the isolated main bronchus of the guinea-pig. They also suggest that a β_2-adrenoceptor agonistic component may be involved in the effects of BRL 37344.

Keywords: β_3-Adrenoceptors; electrical field stimulation; guinea-pig isolated main bronchi; NANC-induced contraction

Introduction

It is now clearly recognized that stimulation of many types of receptor can influence the release of neurotransmitters from airway nerves and this is likely to be important in the physiological control of airway functions. Indeed, the stimulation of α_2- or β-adrenoceptors, muscarinic M_2, vasoactive intestinal peptide, neuropeptide Y, opioid, GABA, histamine H_3, and prostanoid EP-receptors decreases the cholinergic and/or the excitatory nonadrenergic noncholinergic responses to electrical field stimulation (EFS) of airways (Barnes, 1992). The characterization of the two components of the response to EFS as cholinergic and as nonadrenergic noncholinergic has previously been demonstrated in several studies. Tetrodotoxin abolishes both components indicating that both are neurogenic in nature. The early, transient contraction is inhibited by atropine and results from stimulation of cholinergic nerves. The late and prolonged second phase is nonadrenergic noncholinergic (NANC) in nature and is abolished or strongly reduced by antagonists of NK_2- receptors, such as SR 48968 and MEN 10,207, and partially reduced by CP-96,345, an antagonist of NK_1-receptors (Lundberg *et al.*, 1983; Maggi *et al.*, 1991; Martin *et al.*, 1992).

The β-adrenoceptor mediating the modulatory effect of catecholamines on cholinergic nerves is however disputed. In canine airways, the presence of prejunctional β_1-adrenoceptors is suggested, since metoprolol, a β_1-adrenoceptor antagonist, is effective in inhibiting the modulatory effects of catecholamines on cholinergic nerves (Danser *et al.*, 1987; Ito, 1988). In human bronchi, β_2-adrenoceptors mediate the inhibitory response of catecholamines on cholinergic nerves, since isoprenaline is more potent than adrenaline and noradrenaline, and since a β_2-selective antagonist ICI 118,551 completely blocks the modulatory effect of isoprenaline, whereas the β_1-selective antagonist, betaxolol, is ineffective (Rhoden *et al.*, 1988). Furthermore, the selective β_2-adrenoceptor agonist, procaterol, is also effective in inhibiting cholinergic neurotransmission in human airways (Aizawa *et al.*, 1991). The β_2-adrenoceptor agonists, salbutamol and formoterol, also inhibit both cholinergic and NANC excitatory bronchoconstriction in guinea-pig bronchi *in vitro*, and these effects were ascribed to a presynaptic inhibition of mediator release since these drugs were much more effective as inhibitors of the EFS than the matched contractile response induced by substance P or acetylcholine (Verleden *et al.*, 1991).

Recently, Itabashi *et al.* (1992) reported that BRL 35135 and BRL 37344, two putative β_3-adrenoceptor agonists, reduced NANC contraction of the guinea-pig bronchus induced by capsaicin or by EFS in a concentration-dependent fashion without having a significant effect on the contraction

[1] Author for correspondence.

induced by substance P and having a significant effect at concentrations above 10^{-8} M for BRL 35135 and BRL 37344 respectively, suggesting that a β_3-adrenoceptor might exert a prejunctional inhibitory action on NANC contractions.

The purpose of the present study was to investigate the modulating effects of SR 58611A, a new putative β_3-adrenoceptor agonist (Bianchetti & Manara, 1990), on neurogenic contraction of the guinea-pig isolated main bronchus induced by EFS. The effects of SR 58611A were compared with those of SR 58825A, its inactive (+)-enantiomer, and of BRL 37344, another putative β_3-adrenoceptor agonist (McLaughlin & MacDonald, 1990; Itabashi *et al.*, 1992; Webber & Stock, 1992) and of the selective β_2-adrenoceptor agonist, salbutamol.

Methods

Tissue preparation

Guinea-pig main bronchial rings were obtained from tricoloured guinea-pigs of either sex (250–350 g) anaesthetized with urethane (1.25 g kg^{-1}, i.p.) and were suspended under an initial tension of 2.0 g in Krebs solution at 37°C gassed with 95% O_2:5% CO_2. After 1 h of equilibration, resting tension was between 1.5 and 2.0 g. Under these conditions, responses to agonists were reproducible over several hours. Changes in tension were measured isometrically with Pioden strain gauges (UF-1) and amplifiers (EMKA, France), and displayed on a recorder (Linseis L65514, France). The composition of the Krebs solution was (mM): NaCl 118.0, KCl 5.4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.7.

In all experiments, after an equilibration period of 60 min, guinea-pig bronchial rings were contracted with acetylcholine 10^{-3} M and relaxed to maximal relaxation with theophylline 3×10^{-3} M, then allowed to equilibrate for a further 60 min while they were washed with Krebs solution every 15 min.

Cumulative concentration-response curves

In a first series of experiments, preparations were allowed to remain under resting tension, then SR 58611A, SR 58825A, BRL 37344 or salbutamol (10^{-9} to 10^{-6} M) cumulative concentration-response curves were obtained by addition of these compounds every 10–20 min until a plateau was reached. The relaxant effects of these drugs were expressed as percentages of the maximal relaxation induced by theophylline (3×10^{-3} M).

In a second series of experiments, the inhibitory effects of SR 58611A (10^{-6} M), BRL 37344 (10^{-6} M) or salbutamol (10^{-6} M) used as pretreatment were studied. After 30 min preincubation, cumulative concentration-response curves to acetylcholine (10^{-8} to 10^{-3} M), [Nle^{10}]NKA(4–10) (10^{10} to 10^{-6} M) or substance P (10^{-10} to 3×10^{-6} M) were obtained by addition of these compounds every 5–10 min until a plateau was reached. Spasmogen-induced contractions were expressed as a percentage of the contraction induced by control acetylcholine (10^{-3} M). The experiments with [Nle^{10}] NKA(4–10) and substance P were performed in the presence of phosphoramidon (10^{-5} M) to avoid an inhibition of their effects by metabolism (Devillier *et al.*, 1988).

Electrical field stimulation

Experiments were performed in organ baths fitted with two platinum plate electrodes (1 cm^2) placed alongside the tissue (10 mm apart) for transmural electrical field stimulation (EFS: biphasic pulse width 1 ms, constant current of 320 mA, frequency of 16 Hz for 10 s) (Szolcsanyi & Bartho, 1982; Undem *et al.*, 1990). To avoid indirect effects of prostaglandins on the neuronal responses, indomethacin 10^{-6} M was added to the Krebs solution at the beginning and for the duration of the experiment. The preparation was allowed to remain under resting tension, then was stimulated every 30–45 min with a stimulator (EMKA, France) in which the voltage output was adjusted to give a constant current of 320 mA and which produced biphasic rectangular pulses of alternating polarity. Control experiments ($n = 10$) showed no significant fading of the response to EFS during the experimental period. These stimulus parameters caused an optimal reproducible biphasic contraction which consisted of a fast contraction followed by a sustained contractile response, both abolished by tetrodotoxin. The first, fast component is inhibited by atropine and results from stimulation of cholinergic nerves. The late and prolonged second phase is NANC in nature, and is abolished or strongly reduced by antagonists of NK_2-receptors, such as SR 48968 and MEN 10,207, and partially reduced by CP-96,345, an antagonist of NK_1-receptors (Lundberg *et al.*, 1983; Maggi *et al.*, 1991; Martin *et al.*, 1992). We also have previously observed that atropine 10^{-6} M did not modify the NANC response and similarly, SR 48968 (10^{-10} to 10^{-7} M) did not modify the cholinergic response (Martin *et al.*, 1992), suggesting that in these experimental conditions no interaction between cholinergic and NANC responses was involved. These procedures were repeated in the absence or presence of SR 58611A (10^{-8} to 10^{-6} M), SR 58825A (10^{-8} to 10^{-6} M), BRL 37344 (10^{-8} to 10^{-6} M) or salbutamol (10^{-9} to 10^{-7} M) which were added to the bath 30 min before the application of EFS. To determine what type of β-adrenoceptor was involved in these responses we used propranolol (10^{-6} M), ICI 118,551 (10^{-6} M) and yohimbine (10^{-6} M) as pretreatment 30 min before the application of EFS and/or the incubation of β-adrenoceptor agonists.

Statistical analysis of results

Data are expressed as mean ± s.e.mean. Statistical analysis of the results was performed with variance analysis and Student's *t* test for paired or unpaired data, as appropriate. Probability values of $P < 0.05$ were considered significant.

Drugs

The substances used were: acetylcholine HCl (PCH, Paris); BRL 37344 (sodium-4 [2 [2-hydroxy-2(3-chlorophenyl)ethylamino]propyl]phenoxyacetate), ICI 118,551 (erythro (±)-1-(7-methylindan-4-yloxy) -3-isopropylaminobutan-2-ol-hydrochloride), SR 58611A (ethyl[(7**S**)-7-[(2**R**)-2-(3-chlorophenyl)-

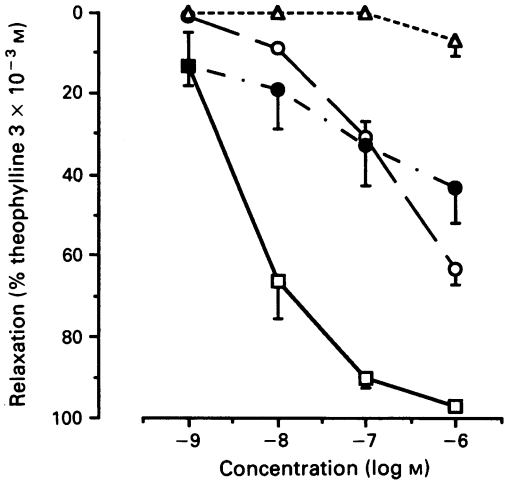


Figure 1 Cumulative relaxant concentration-response curves to salbutamol (□), BRL 37344 (○), SR 58611A (●) and SR 58825A (△) (10^{-9} to 10^{-6} M) on guinea-pig isolated main bronchi against basal tone ($n = 4–5$). Values are means ± s.e.mean.

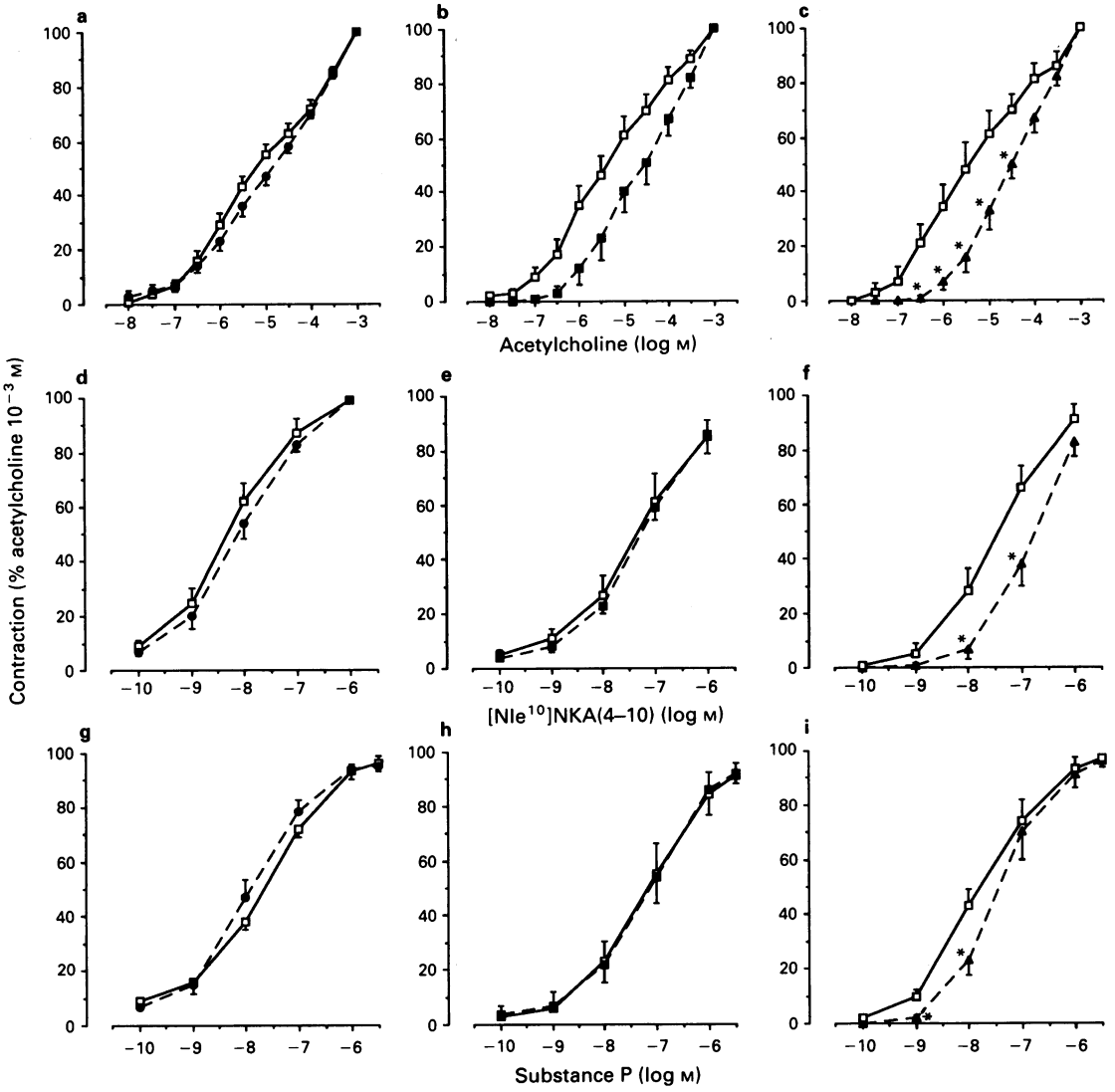


Figure 2 Cumulative contractile concentration-response curves to acetylcholine (a,b,c), [Nle^{10}]NKA(4–10) (d,e,f) and substance P (g,h,i) in the absence (control, □) or in the presence of SR 58611A (●, 10^{-6} M; a,d and g), BRL 37344 (■, 10^{-6} M; b,e and h) or salbutamol (▲, 10^{-6} M; c,f and i) in guinea-pig isolated main bronchi ($n = 4-6$). Values are means ± s.e.mean. Significant differences from control are indicated by: $*P < 0.05$.

2-hydroxyethylamino] 5,6,7,8 -tetrahydronaphthalen-2-yloxy} acetate, hydrochloride), SR 58825A (ethyl{(7**R**)-7-[(2**S**)-2-(3-chlorophenyl)-2-hydroxyethylamino] -5,6,7,8-tetrahydronaphthalen-2-yloxy} acetate, hydrochloride) (gift from Sanofi-Midy, S.p.A. Research Center, Milan, Italy); indomethacin (Merck); [Nle^{10}]NKA(4–10) (Novabiochem, Paris, France); phosphoramidon, propranolol, salbutamol, substance P, yohimbine (Sigma, St Louis, U.S.A.); theophylline sodium anisate (Bruneau, Paris, France). All drugs were dissolved in distilled water and then diluted in Krebs solution, except indomethacin which was dissolved in ethanol and then diluted in Krebs solution. The maximal amount of ethanol added to the bath (0.4%) did not alter the reactivity of the guinea-pig bronchial preparations to acetylcholine.

Results

Effects of SR 58611A, SR 58825A, BRL 37344 and salbutamol on basal tone of guinea-pig bronchi

The relaxant effects of SR 58611A, BRL 37344 and SR 58825A (10^{-9} to 10^{-6} M) were examined on preparations at basal tone. SR 58611A and BRL 37344 produced concentration-dependent falls in basal tone reaching 43% ± 9% and 63% ± 4% of maximal response to theophylline 3×10^{-3} M, respectively (10^{-6} M, $n = 4-5$) (Figure 1). In contrast, salbutamol induced a full relaxation at 10^{-6} M, with a pD_2 of 8.3 ± 0.2 ($n = 4$), whereas SR 58825A, the inactive enantiomer of SR 58611A, had no significant effect on the basal tone of guinea-pig isolated main bronchus (7% ± 4%, 10^{-6} M, $n = 4$) (Figure 1). SR 58611A and BRL 37344 were approximately 100 times less potent than salbutamol.

Effects of SR 58611A, BRL 37344 or salbutamol on cumulative concentration-response curves to acetylcholine, [Nle^{10}]NKA(4–10) and substance P on guinea-pig isolated main bronchi

SR 58611A (10^{-6} M) and BRL 37344 (10^{-6} M) did not significantly modify concentration-response curves to acetylcholine (10^{-8} to 10^{-3} M), [Nle^{10}]NKA(4–10) (10^{-10} to 10^{-6} M) or substance P (10^{-10} to 3×10^{-6} M), although BRL 37344 did cause a small apparent rightward shift of the concentration-response curve to acetylcholine, whereas salbutamol (10^{-6} M) produced significant rightward shifts of the concentration-response curves to acetylcholine, [Nle^{10}]NKA (4–10) and substance P, with no apparent depression of maximum response (Figure 2).

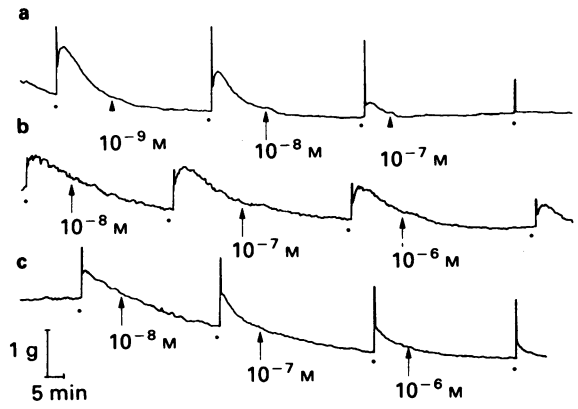


Figure 3 Representative tracings of the biphasic contraction induced by EFS of guinea-pig isolated main bronchi (●:16 Hz, 1 ms, 320 mA for 10 s; in the presence of indomethacin 10^{-6} M) and of the effects of salbutamol (a, 10^{-9} to 10^{-7} M), BRL 37344 (b, 10^{-8} to 10^{-6} M) and SR 58611A (c, 10^{-8} to 10^{-6} M).

Effects of SR 58611A, SR 58825A, BRL 37344 and salbutamol on the responses induced by electrical field stimulation (EFS)

Figure 3 shows representative recordings of the biphasic contraction induced by EFS and their modification by salbutamol, BRL 37344 and SR 58611A. SR 58611A and BRL 37344 (10^{-8} to 10^{-6} M) did not significantly modify the cholinergic response to EFS (Figure 4), but significantly inhibited the NANC contraction induced by EFS, producing 41.8% ± 10.1% ($n = 7$) and 56.8% ± 7.4% ($n = 6$) inhibition respectively (Figure 5, Table 1). Under similar conditions, salbutamol (10^{-9} to 10^{-7} M) strongly inhibited both components of the contraction induced by EFS, but the inhibition of the NANC component was greater (91.1% ± 4.2%, 10^{-7} M, $n = 7$) than the inhibition of the cholinergic component (62.0% ± 5.2%, 10^{-7} M, $n = 7$) (Figure 4, Figure 5, Table 1).

The inhibitory effect of salbutamol on both the cholinergic and the NANC components of the contraction induced by EFS (Figure 4, Figure 5) was abolished or reduced by the

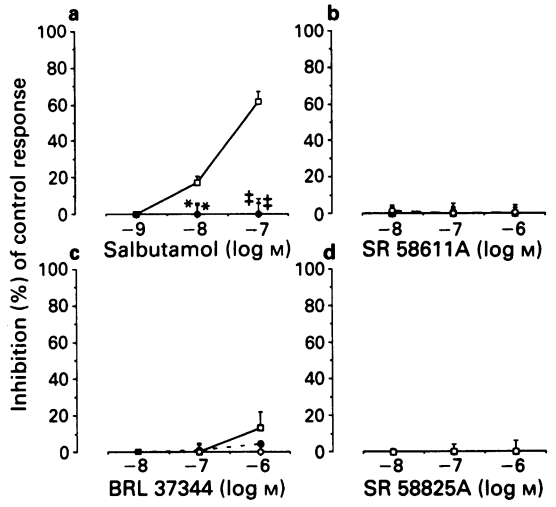


Figure 4 Concentration-response curves illustrating the inhibition (%) of the cholinergic component of the response to EFS of guinea-pig isolated main bronchi by salbutamol (a, 10^{-9} to 10^{-7} M), SR 58611A (b, 10^{-8} to 10^{-6} M), BRL 37344 (c, 10^{-8} to 10^{-6} M) and SR 58825A (d, 10^{-8} to 10^{-6} M) in the absence (□) and presence of propranolol (●, 10^{-6} M), ICI 118,551 (○, 10^{-6} M). Experiments were performed in the presence of indomethacin 10^{-6} M. Means ± s.e.mean of 5 to 7 animals are shown. Significant differences from agonists in the absence of pretreatment with β-adrenoceptor antagonists are indicated by: $*P<0.05$ and $‡P<0.001$.

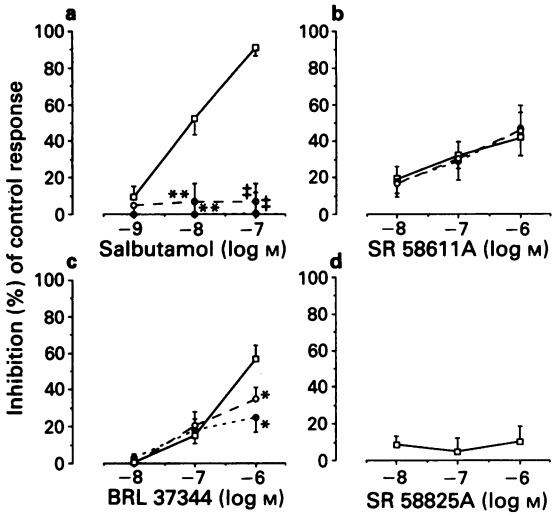


Figure 5 Concentration-response curves illustrating the inhibition (%) of the nonadrenergic noncholinergic component of the response to EFS of guinea-pig isolated main bronchi by salbutamol (a, 10^{-9} to 10^{-7} M), SR 58611A (b, 10^{-8} to 10^{-6} M), BRL 37344 (c, 10^{-8} to 10^{-6} M) and SR 58825A (d, 10^{-8} to 10^{-6} M) in the absence (□) and presence of propranolol (●, 10^{-6} M), ICI 118,551 (○, 10^{-6} M). Experiments were performed in the presence of indomethacin 10^{-6} M. Means ± s.e.mean of 5 to 7 animals are shown. Significant differences from agonists in the absence of pretreatment with β-adrenoceptor antagonists are indicated by: $*P<0.05$, $**P<0.01$, $‡P<0.001$.

Table 1 Effects of β- and α-adrenoceptor antagonists on the inhibition produced by β-adrenoceptor agonists on the NANC contraction induced by electrical field stimulation of the guinea-pig main bronchus

	Inhibition of control response (%)			
		Pretreatment		
	Without pretreatment	*Propranolol* (10^{-6} M)	*ICI 118,551* (10^{-6} M)	*Yohimbine* (10^{-6} M)
Salbutamol (10^{-7} M)	91.1 ± 4.2[c] (7)	0.64 ± 11.3** (5)	6.9 ± 9.8** (6)	70.0 ± 10.3[c] (6)
SR 58611A (10^{-6} M)	41.8 ± 10.1[b] (7)	46.0 ± 13.2[b] (6)	46.2 ± 10.2[b] (5)	30.9 ± 10.9[b] (6)
BRL 37344 (10^{-6} M)	56.8 ± 7.4[c] (6)	25.1 ± 7.2[b]* (6)	35.2 ± 6.0** (5)	71.0 ± 4.4[c] (6)

Values are means ± s.e.mean. (n) = number of guinea-pigs per group. Significant inhibitions of control response are shown as: [a] $P<0.05$; [b] $P<0.01$; [c] $P<0.001$. Significant differences from agonists in the absence of pretreatment with antagonists are shown as: $*P<0.05$; $**P<0.001$.

non-selective β-adrenoceptor antagonist, propranolol, and by the selective β_2-adrenoceptor antagonist, ICI 118,551, but not significantly modifed by the α_2-adrenoceptor antagonist, yohimbine (Figure 4, Figure 5, Table 1). Propranolol (10^{-6} M) and ICI 118,551 (10^{-6} M) slightly, but significantly, reduced the inhibition produced by BRL 37344 but not by SR 58611A on the NANC excitatory component (Figure 4, Figure 5, Table 1).

Finally, Figures 4 and 5 show that the inactive enantiomer of SR 58611A, SR 58825A, did not influence either component of the contraction induced by EFS.

Discussion

In the present study we have compared the effects of two putative β_3-adrenoceptor agonists, SR 58611A and BRL 37344, with those of salbutamol, a selective β_2-adrenoceptor agonist, on the guinea-pig isolated main bronchus.

SR 58611A is a novel β_3-adrenoceptor agonist which belongs to the family of phenylethanolaminotetralines. This drug, unlike the β-adrenoceptor agonists, isoprenaline or salbutamol, produced a half-maximal inhibition of spontaneous motility of rat isolated proximal colon at a substantially lower concentration (EC_{50} 3.5×10^{-9} M) than those inducing β_2-adrenoceptor-mediated responses (relaxation of guinea-pig isolated trachea and rat uterus), and had virtually no chronotropic action ($EC_{50} > 3 \times 10^{-5}$ M) on the guinea-pig isolated atrium (a β_1-adrenoceptor-mediated response) (Croci *et al.*, 1987; Bianchetti & Manara, 1990). Similarly, BRL 37344 relaxed guinea-pig ileum (Bond & Clarke, 1988) and gastric fundus (Coleman *et al.*, 1987), contracted by histamine and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) respectively, at concentrations which were resistant to β-adrenoceptor and α-adrenoceptor antagonists, suggesting a specific effect on a putative β_3-adrenoceptor.

Our results clearly demonstrate that SR 58611A and BRL 37344 on the one hand, and salbutamol on the other hand, exert different effects on the guinea-pig isolated main bronchus. Salbutamol induced a potent relaxant effect on basal tone of the guinea-pig bronchi and inhibited the concentration-response curves to acetylcholine, [Nle^{10}]NKA(4–10) and substance P. Furthermore, it greatly inhibited the two components of the neurally-mediated biphasic response observed following EFS of the guinea-pig isolated main bronchus. The first, fast component is inhibited by atropine and is associated with stimulation of cholinergic nerves. The late and prolonged second phase is NANC in nature, and is abolished or strongly reduced by antagonists of NK_2-receptors, such as SR 48968 and MEN 10,207, and partially reduced by CP-96,345, an antagonist of NK_1-receptors (Lundberg *et al.*, 1983; Maggi *et al.*, 1991; Martin *et al.*, 1992). Both NK_1 and NK_2-receptors are involved in the NANC component of the guinea-pig isolated main bronchus to EFS; however, NK_2 stimulation predominates, as shown by Maggi *et al.* (1991) and Martin *et al.* (1992). The effects of salbutamol were abolished or inhibited by both the non-selective β-adrenoceptor antagonist, propranolol, and by the selective β_2-adrenoceptor antagonist, ICI 118,551, showing that the effects of salbutamol are due to stimulation of β_2-adrenoceptors. The inhibitory effects of β-adrenoceptor agonists on the cholinergic response induced by EFS or vagal stimulation have previously been described for the guinea-pig isolated bronchus (Kamikawa & Shimo, 1990), the canine isolated bronchus (Cabezas *et al.*, 1971; Vermeire & Vanhoutte, 1979; Martin & Collier, 1986; Danser *et al.*, 1987; Ito, 1988) and for human bronchi (Rhoden *et al.*, 1988; Aizawa *et al.*, 1991) and they have been attributed to prejunctional inhibition because β-adrenoceptor agonists were more effective in inhibiting this response than similar contractile responses induced by exogenous acetylcholine (Vermeire & Vanhoutte, 1979; Ito, 1988; Aizawa *et al.*, 1991). Nevertheless, Martin & Collier (1986) could not demonstrate that the β-adrenoceptor agonist, noradrenaline, inhibited the release of acetylcholine from cholinergic nerves in canine airways. Other studies (Kamikawa & Shimo, 1990; Verleden *et al.*, 1991) have also previously demonstrated the inhibitory effects of noradrenaline and β_2-adrenoceptor agonists (formoterol, salbutamol) on the EFS-induced NANC contraction in guinea-pig bronchi. Our present study is consistent with a postjunctional mechanism of salbutamol since we observed an inhibitory effect of this compound on cumulative concentration-response curves to acetylcholine, substance P and [Nle^{10}]NKA(4–10), the latter compound being a selective agonist of NK_2-receptors. However, this does not rule out an additional prejunctional component.

In contrast to salbutamol SR 58611A and BRL 37344 seemed to produce an inhibition of the NANC response predominantly at a prejunctional level. Although they might exert a small direct relaxant effect on bronchial smooth muscle, as seen against a basal tone unlike salbutamol, they did not modify the first rapid and intense cholinergic component of the contraction induced by EFS, and similarly were without effect on concentration-response curves to acetylcholine, [Nle^{10}]NKA(4–10) or substance P. Likewise, Itabashi *et al.* (1992) have previously shown that BRL 37344, up to a concentration of 10^{-8} M, reduced NANC contractions without having a significant effect on similar contractions induced by exogenous substance P on the guinea-pig isolated bronchus. These results are consistent with these compounds acting on presynaptic nerve endings to inhibit the release of neuropeptides from NANC fibres. A ganglion-blocking effect seems unlikely since responses to EFS are not modified by ganglion-blocking agents in the canine bronchus (Vermeire & Vanhoutte, 1979).

The partial inhibition of the effects of BRL 37344 by propranolol or ICI 118,551 suggests that a β_2-adrenoceptor agonistic component might be implicated in the action of BRL 37344, as was suggested by Manara *et al.* (1990).

Bianchetti & Manara (1990) have reported that propranolol and alprenolol prevented the inhibition of the rat colon motility induced by SR 58611A at high concentrations (pA_2 values were around 6.5 and 7.5 respectively), whereas these drugs antagonized typical β_1- and β_2-responses at low concentrations (pA_2 values around 9.0). Our results show that propranolol 10^{-6} M caused little or no modification of the inhibitory effects of SR 58611A, and was only weakly effective against those of BRL 37344. It is difficult to explain the absence of activity of these antagonists at high concentration in the present study, but the existence of species variants of β_3-adrenoceptors might be an explanation, since Coleman *et al.* (1987) were unable to demonstrate any β_3-adrenoceptor antagonist activity with propranolol on guinea-pig fundus. Alternatively, the existence of subtypes of β_3-adrenoceptors might also explain these findings.

The absence of effects of yohimbine on the inhibitory effect of BRL 37344, SR 58611A and salbutamol enables us to exclude an α_2-adrenoceptor-mediated component in the inhibitory effect of these drugs.

Finally, it might have been expected that inhibition of the NANC response would result in some diminution of cholinergic response, since it has been demonstrated that tachykinins may lead to facilitation of the cholinergic response induced by direct vagal nerve stimulation or by electrical field stimulation of the guinea-pig airway (Watson *et al.*, 1992; Belvisi *et al.*, 1993), and capsaicin, which depletes sensory nerves, may lead to a decrease of the cholinergic response (Stretton *et al.*, 1992). This was apparently not the case in our experiments since inhibition of the NANC response induced by β-adrenoceptor agonists resulted in no modification of the cholinergic response, in agreement with our previous results (Martin *et al.*, 1992) where we demonstrated that SR 48968, a selective antagonist of NK_2 receptors, did not modify the first cholinergic response, and atropine did not modify the second NANC response. The differences between our study and those of Belvisi *et al.*

(1993), Watson et al. (1992) and Stretton et al. (1992) who demonstrated an interaction between NANC and cholinergic responses might be explained by the choice of frequency of EFS stimulation. We used 16 Hz in our experiments whereas the other authors used 2 Hz to 8 Hz in their studies, the differences of frequency involving responses of different intensity (30 to 40% of acetylcholine response in our result, 20 to 27% in their experiments). The interaction between the NANC and the cholinergic system seems to be observed for low frequencies of stimulation.

In conclusion, our findings show that the β_3-adrenoceptor agonists, SR 58611A and BRL 37344, would be expected to inhibit the release of neuropeptides from NANC fibres, while having no effect on substance P- and [Nle^{10}]NKA(4–10)-mediated bronchoconstriction. On the other hand, it is difficult to specify whether there is a prejunctional mechanism of the β_2-adrenoceptor agonist, salbutamol. Inhibition by β_3-adrenoceptor agonists of neurotransmitter release from NANC nerves in airways smooth muscle may play a role in the physiological control of airways functions.

References

AIZAWA, H., INOUE, H., IKEDA, T., HIROSE, T. & ITO, Y. (1991). Effects of procaterol, a beta$_2$-adrenoceptor stimulant, on neuroeffector transmission in human bronchial tissue. *Respir.*, **58**, 163–166.

BARNES, P.J. (1992). Modulation of neurotransmission in airways. *Physiol. Rev.*, **72**, 699–729.

BELVISI, M.G., PATTACHINI, R., BARNES, P.J. & MAGGI, C.A. (1993). Facilitatory effects of selective agonists for tachykinin receptors on cholinergic neurotransmission. *Am. Rev. Respir. Dis.*, **147**, A502.

BIANCHETTI, A. & MANARA, L. (1990). *In vitro* inhibition of intestinal mobility by phenylethanolaminotetralines: evidence of atypical β-adrenoceptors in rat colon. *Br. J. Pharmacol.*, **100**, 831–839.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95**, 723–734.

CABEZAS, G.A., GRAF, P.D. & NADEL, J.A. (1971). Sympathetic versus parasympathetic nervous regulation of airways in dogs. *J. Appl. Physiol.*, **31**, 651–655.

COLEMAN, R.A., DENYER, L.H. & SHELDRICK, K.E. (1987). β-adrenoceptors in guinea-pig gastric fundus – are they the same as the 'atypical' β-adrenoceptors in the rat adipocytes? *Br. J. Pharmacol.*, **90**, 40P.

CROCI, T., BIANCHETTI, A., POGGESI, E., BOIGERAIN, R. & MANARA, L. (1987). Gut-specific beta adrenergic agonists inhibit rat colon mobility in vitro and in vivo. *Dig. Dis. Sci.*, **32**, 900–932.

DANSER, A.H.J., ENDE, R.V.D., LORENTZ, R.R., FLAVAHAN, N.A. & VANHOUTTE, P.M. (1987). Prejunctional β_1-adrenoceptors inhibit cholinergic transmission in canine bronchi. *J. Appl. Physiol.*, **62**, 785–790.

DEVILLIER, P., ADVENIER, C., DRAPEAU, G., MARSAC, J. & REGOLI, D. (1988). Comparison of the effects of epithelium removal and of an enkephalinase inhibitor on the neurokinin-induced contractions of guinea-pig isolated trachea. *Br. J. Pharmacol.*, **94**, 675–684.

ITABASHI, S., AIKAWA, T., SEKIZAWA, K., SASAKI, H. & TAKISHIMA, T. (1992). Evidence that an atypical β-adrenoceptor mediates the prejunctional inhibition of non-adrenergic non-cholinergic contraction in guinea-pig bronchi. *Eur. J. Pharmacol.*, **218**, 187–190.

ITO, Y. (1988). Pre- and post-junctional actions of procaterol, a β_2-adrenoceptor stimulant, on dog tracheal tissue. *Br. J. Pharmacol.*, **95**, 268–274.

KAMIKAWA, Y. & SHIMO, Y. (1990). Inhibitory effects of cathecolamines on cholinergically and non-cholinergically mediated contractions of guinea-pig isolated bronchial smooth muscle. *J. Pharm. Pharmacol.*, **42**, 131–134.

LUNDBERG, J.M., SARIA, A., BRODIN, E., ROSELL, S. & FOLKERS, K. (1983). A substance P antagonist inhibits vagally induced increase in vascular permeability and bronchial smooth muscle contraction in the guinea-pig. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 1120–1124.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & SANTICIOLI, P. (1991). Tachykinin receptor and noncholinergic bronchoconstriction in the guinea-pig isolated bronchi. *Am. Rev. Respir. Dis.*, **144**, 363–367.

MANARA, L., GUZZI, U., AUREGGI, G., CROCI, T. & BIANCHETTI, A. (1990). On the selectivity of in vitro inhibition of rat colon motility by β-adrenergic substituted phenylethanolamines. *Eur. J. Pharmacol.*, **183**, 2197–2198.

MARTIN, C.A.E., NALINE, E., EMONDS-ALT, X. & ADVENIER, C. (1992). Influence of (±)-CP-96,345 and SR 48968 on electrical field stimulation of the isolated guinea-pig main bronchus. *Eur. J. Pharmacol.*, **224**, 137–143.

MARTIN, J.G. & COLLIER, B. (1986). Acetylcholine release from canine isolated airway is not modulated by norepinephrine. *J. Appl. Physiol.*, **61**, 1025–1030.

MCLAUGHLIN, D.P. & MACDONALD, A. (1990). Evidence for the existence of 'atypical' β-adrenoceptors (β_3-adrenoceptors) mediating relaxation in the rat distal colon *in vitro*. *Br. J. Pharmacol.*, **101**, 569–574.

RHODEN, K.J., MELDRUM, L.A. & BARNES, J. (1988). Inhibition of cholinergic neurotransmission in human airways by β_2-adrenoceptors. *J. Appl. Physiol.*, **65**, 700–705.

STRETTON, D., BELVISI, M.G. & BARNES, P.J. (1992). The effect of sensory nerve depletion on cholinergic neurotransmission in guinea-pig airways. *J. Pharmacol. Exp. Ther.*, **230**, 1073–1080.

SZOLCSANYI, J. & BARTHO, L. (1982). Capsaicin-sensitive non-cholinergic excitatory innervation of the guinea-pig tracheobronchial smooth muscle. *Neurosci. Lett.*, **34**, 247–251.

UNDEM, B.J., MYERS, A.C., BARTHLOW, H. & WEINREICH, D. (1990). Vagal innervation of guinea-pig bronchial smooth muscle. *J. Appl. Physiol.*, **69**, 1336–1346.

VERLEDEN, G.M., BELVISI, M.G., RABE, K. & BARNES, P.J. (1991). Inhibition of non-adrenergic, non-cholinergic neural bronchoconstriction in guinea-pig airways in vitro by β_2-adrenoceptors. *Am. Rev. Respir. Dis.*, **143**, A357.

VERMEIRE, P.A. & VANHOUTTE, P.M. (1979). Inhibitory effects of catecholamines in isolated canine bronchial smooth muscle. *J. Appl. Physiol.*, **46**, 787–791.

WATSON, N., MACLAGAN, J., BARNES, P.J. (1992). Demonstration of a facilitatory role of endogenous tachykinins in cholinergic neurotransmission in guinea-pig trachea. *Br. J. Pharmacol.*, **105**, 78P.

WEBBER, S.E. & STOCK, M.J. (1992). Evidence for an atypical, or β_3-adrenoceptor in ferret tracheal epithelium. *Br. J. Pharmacol.*, **105**, 857–862.

(Received March 5, 1993
Revised July 5, 1993
Accepted July 27, 1993)

Br. J. Pharmacol. (1993), **110**, 1317–1320 © Macmillan Press Ltd, 1993

The action of Hoe 140 on the bradykinin-induced splenic pressor reflex of the anaesthetized cat

J.B. Louttit & [1]R.A. Coleman

Department of Cardiovascular and Respiratory Pharmacology, Glaxo Group Research Ltd., Park Road, Ware, Hertfordshire SG12 0DP

1 Intrasplenic injection of bradykinin (BK) induced a dose-dependent pressor response in the anaesthetized cat, with an ED_{50} of 0.98 ± 0.43 nmol. In contrast, intrasplenic administration of desArg^{9}bradykinin (desArg^{9}BK) was without significant effect at doses of up to 200 nmol.

2 Intravenously administered BK induced a dose-dependent depressor response in the anaesthetized cat, with an ED_{50} of 0.86 ± 0.09 nmol kg^{-1}. desArg^{9}BK was again without significant effect in this system at doses of up to 200 nmol kg^{-1}.

3 Both the pressor and depressor responses to BK were antagonized to a similar degree in a dose-dependent manner by Hoe 140 (10 and 100 nmol kg^{-1}, i.v.).

4 At these doses, Hoe 140 appears to be a specific BK antagonist as it was ineffective against pressor responses to intrasplenic injection of capsaicin (5 nmol), while blocking those to an approximately equieffective dose of BK (1 nmol) in a dose-dependent fashion.

5 Both the pressor response to intrasplenic BK and the depressor response to intravenous BK in the anaesthetized cat appear to be mediated by B_2-receptors. This model may be useful in the quantitative determination of the antinociceptive potency of BK antagonists.

Keywords: Bradykinin; bradykinin antagonist; Hoe 140; cat spleen pressor reflex

Introduction

Bradykinin (BK) is among the most potent algesic substances known and when administered by close-arterial injection into the spleen of conscious dogs it produces a pseudoaffective response (Guzman *et al.*, 1962). In both the anaesthetized cat and dog, intrasplenic administration of BK induces a marked pressor reflex (Tyers & Haywood, 1979). This response to BK is thought to be due to stimulation of receptors on splenic nerves and similar pressor responses can be obtained by administration of capsaicin, which is known to stimulate C-fibres directly. Recent work in the rat carrageenin model of inflammation (Wirth *et al.*, 1991) and in BK-induced abdominal constriction in the mouse (Beresford & Birch, 1992) has suggested that B_2-receptors are involved in the mediation of BK-induced pain and oedema. The present study was undertaken to investigate the sub-type of BK-receptor responsible for mediating the splenic pressor reflex in the anaesthetized cat, using Hoe 140, a selective B_2-receptor antagonist (Hock *et al.*, 1991; Wirth *et al.*, 1991), and the selective B_1-receptor agonist, desArg^{9}BK (Rhaleb *et al.*, 1991).

Methods

Cats ($n = 18$) of either sex (2.4–3.4 kg) were anaesthetized (α-chloralose, 1 ml kg^{-1}, 8% w/v suspension and Na pentobarbitone, 9.6 mg kg^{-1}, both i.p.), the trachea was cannulated and the animal respired with room air at 20 breaths per min and a volume of 17 ml kg^{-1}. The femoral vein and artery were cannulated for administration of drugs and the measurement of arterial pressure respectively. Pressure was measured with a Gould P23XL transducer coupled to a chart recorder (Gould TA4000), and heart rate was derived electronically from the pressure signal. The spleen was exposed by an abdominal midline incision, and a branch of the splenic artery was cannulated retrogradely. The cannula was advanced until its tip lay in the junction of the branch with the main splenic artery: this allowed drugs to be administered directly into the spleen with minimal effect upon the blood supply to the organ.

BK, desArg^{9}BK (Sigma) and D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8] bradykinin (Hoe 140, Medicinal Chemistry Dept., GGR) were dissolved in saline. Capsaicin (Sigma) was dissolved in ethanol at a concentration of 1 mmol l^{-1}, and subsequent dilutions were made with saline. Dose-response curves were constructed for BK and desArg^{9}BK, administered by both the intrasplenic or the intravenous routes. Hoe 140 was given intravenously at 10 or 100 nmol kg^{-1}, and starting 10 min later, a second dose-response curve to BK was constructed by each route in the presence of the antagonist. Antagonist activity was expressed as a dose-ratio (DR), where the effective dose for half maximal response (ED_{50}) in the presence of the antagonist was divided by the ED_{50} in the absence of the antagonist. A pilot experiment showed that repeated dosing with capsaicin caused desensitization of the splenic pressor response and therefore a single dose of capsaicin (5 nmol) was used which was approximately equieffective with the ED_{50} dose of BK (1 nmol). Capsaicin (5 nmol) and BK (1 nmol) were given alternately until constant responses for each agent were obtained; these doses were then repeated in the presence of Hoe 140 (10 and 100 nmol kg^{-1}, i.v.).

Results

Intrasplenic (i.s.) administration of BK (0.05–10 nmol) caused dose-dependent pressor responses that were rapid in onset, of short duration (see Figure 1a) and had a maximum increase in the order of 80 mmHg. In contrast, when administered by the intravenous (i.v.) route, BK (0.1–10 nmol kg^{-1}) caused dose-dependent depressor responses, although at the higher doses this vasodepression was followed by an increase in blood pressure above the original resting level (see Figure 1b). Unlike BK, i.s. administration of desArg^{9}BK (5–200 nmol) was without significant effect on

[1] Author for correspondence.

blood pressure, and i.v. administration (5–200 nmol kg^{-1}) caused only a modest vasodepression (Figure 2). The ED_{50} value for the pressor response to i.s. BK was 0.98 ± 0.43 nmol, while that for the depressor response to i.v. BK was 0.86 ± 0.09 nmol kg^{-1}.

Hoe 140 (10 and 100 nmol kg^{-1}, i.v.) had no significant direct effect upon resting blood pressure or heart rate: blood pressure prior to Hoe 140 treatment was 115/76 ± 6/4 mmHg ($n = 7$), after Hoe 140 (10 nmol kg^{-1}) it was 116/75 ± 4/2 mmHg ($n = 7$), and after Hoe 140 (100 nmol kg^{-1}) it was 118/75 ± 4/2 mmHg ($n = 7$). The corresponding values for heart rate were 164 ± 10 beats min^{-1}, 166 ± 10 beats min^{-1} and 169 ± 10 beats min^{-1} respectively. However, Hoe 140 (10

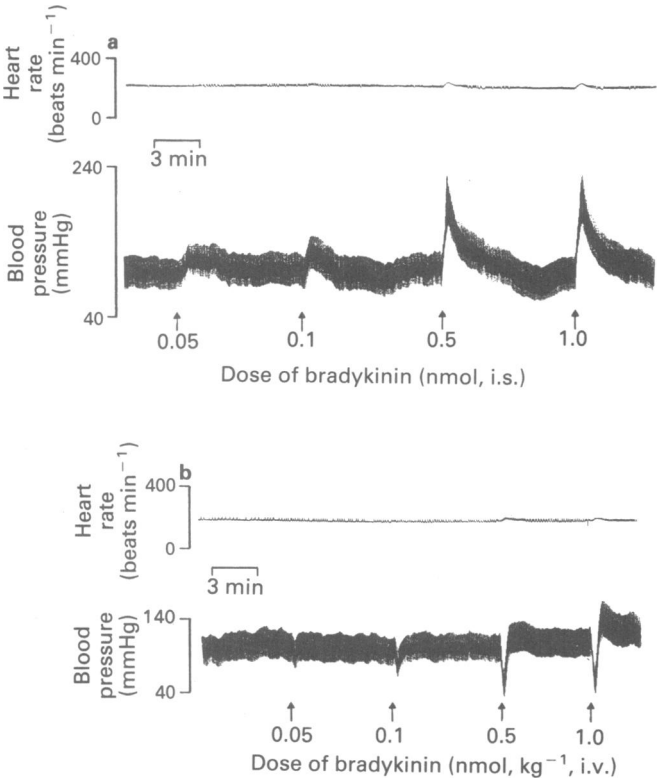


Figure 1 Effects of increasing doses of bradykinin on blood pressure in the anaesthetized cat. (a) Bradykinin given by intrasplenic (i.s.) administration, and (b) bradykinin given by intravenous administration.

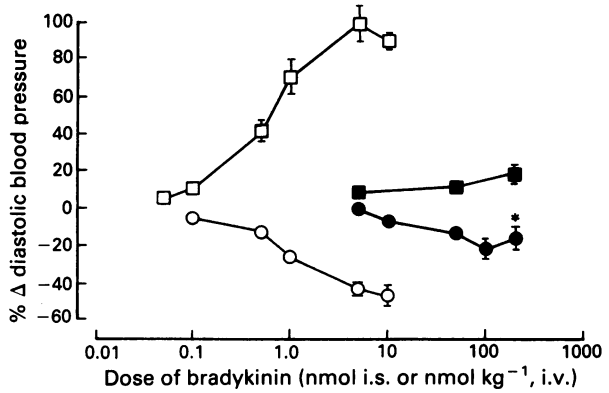


Figure 2 Mean dose-response curves to bradykinin (open symbols) and desArg^{9}bradykinin (solid symbols) on blood pressure in the anaesthetized cat. Agonists administered by the intrasplenic (squares) and intravenous (circles) routes. Each point is the mean of at least 7 determinations, and vertical bars represent s.e.mean (*mean of 4 determinations only).

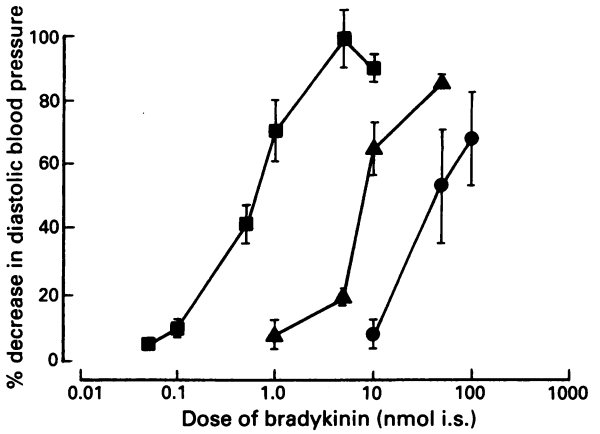


Figure 3 Mean pressor dose-response curves to bradykinin administered by the intrasplenic route to anaesthetized cats. Dose-response curves constructed before (■) and after the intravenous administration of Hoe 140 10 nmol kg^{-1} (▲) and 100 nmol kg^{-1} (●). Each point is the mean ± s.e.mean of 5–7 determinations.

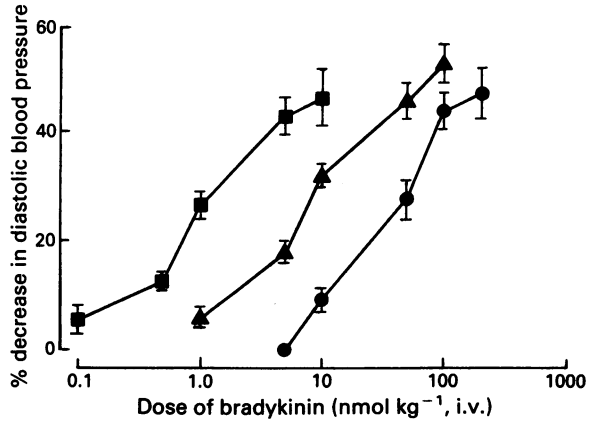


Figure 4 Mean vasodepressor dose-response curves to bradykinin administered by the intravenous route to anaesthetized cats. Dose-response curves constructed before (■) and after intravenous administration of Hoe 140 10 nmol kg^{-1} (▲) and 100 nmol kg^{-1} (●). Each point is the mean ± s.e.mean of 5–7 determinations.

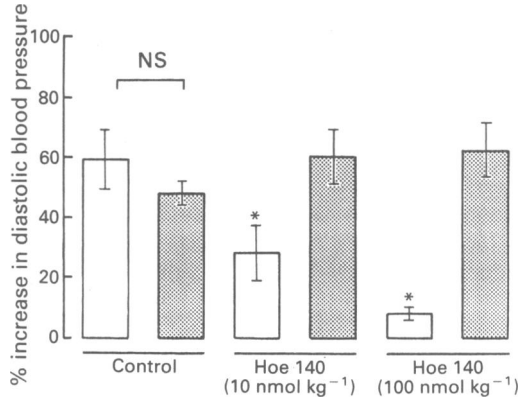


Figure 5 Mean pressor responses to bradykinin (1 nmol) (open columns) and to capsaicin (5 nmol) (hatched columns) after intrasplenic administration to anaesthetized cats. Responses determined before and after intravenous administration of Hoe 140, 10 and 100 nmol kg^{-1}. Each response is the mean ± s.e.mean of 4 determinations. Significantly different from control, $P < 0.02$; NS not significantly different.

and 100 nmol kg^{-1}, i.v. did cause dose-dependent, parallel, rightward shifts of the pressor dose-response curves to i.s. BK (Figure 3), producing dose-ratios of 8.1 ± 1.1 and 45.6 ± 8.0 respectively. Similarly, depressor dose-response curves to i.v. BK were also displaced to the right, in a parallel, dose-dependent manner by Hoe 140 (10 and 100 nmol kg^{-1}, i.v., Figure 4), producing dose-ratios of 9.8 ± 0.6 and 56.0 ± 8.3 respectively.

Capsaicin (5 nmol) by i.s. administration, like BK, produced a marked pressor response, which was approximately equi-effective with an ED_{50} dose of BK (1 nmol). However, this pressor response to capsaicin was unaffected by Hoe 140 (10 and 100 nmol kg^{-1}), while the response to BK (1 nmol) was inhibited in a dose-dependent manner (Figure 5).

Discussion

In the present study, we have confirmed the ability of BK to cause increases in systemic blood pressure when administered into the spleen of anaesthetized cats as previously reported by Tyers & Haywood (1979). This effect of BK was both marked and dose-related, but the selective B_1-receptor agonist, desArg^{9}BK, was without significant activity. When administered intravenously, BK caused a marked and dose-dependent reduction in systemic blood pressure, while again, desArg^{9}BK was only weakly active. The potency of BK in inducing these two responses suggests that specific BK-receptors are involved, and the lack of activity of desArg^{9}BK further suggests that neither the splenic pressor response, nor the direct i.v. depressor response, is mediated by B_1-receptors.

The ability of BK both to increase and decrease blood pressure is well known (see review by Regoli & Barabé, 1980), and the mechanism by which it produces these effects has received some attention. The elevation of blood pressure following i.s. administration of BK, and indeed capsaicin, probably results from a stimulation of polymodal receptors on afferent sensory nerves within the spleen, resulting in a reflex increase in sympathetic nerve activity (Calaresu *et al.*, 1984). BK-induced vasodepression is probably also indirect, involving the release of the endothelium-derived relaxing factors, prostaglandin I_2 (de Nucci *et al.*, 1988) and nitric oxide (D'Orléans-Juste *et al.*, 1985). Bellan *et al.* (1991) have suggested that in fact, nitric oxide may be primarily responsible for BK-induced vasodilatation in the cat, as they have shown that the vasodilator responses to i.v. BK in the cat hindquarters are blocked by the nitric oxide synthesis inhibitor, N^{ω}-nitro-L-arginine. Interestingly, in many species, including the cat, i.v. administration of high doses of BK has been reported to cause a biphasic response of systemic blood pressure, comprising an initial fall, followed by an increase to a level above that prior to BK administration (Lang & Pearson, 1968); this was also observed in the present study. Whether the second, pressor component of the response to i.v. BK results from a stimulation of afferent sensory nerves, as after i.s. administration, is not clear, and even if it does, the location of these nerves is not known.

In order to investigate the role of B_2-receptors in these BK-responses, we examined the effects of the selective B_2-receptor antagonist, Hoe 140. Hoe 140 caused parallel, dose-dependent rightward shifts of both the splenic pressor response and the i.v. depressor response. Thus, in common with the depressor response in conscious rats (Boa *et al.*, 1991), the direct action of i.v. BK in the anaesthetized cat appears to be mediated by B_2-receptors. Likewise, the activity of Hoe 140 on the pressor response to i.s. BK in the cat supports the hypothesis that this is also a B_2-receptor-dependent effect. The fact that the pressor response to capsaicin was unaffected by Hoe 140, suggests that the actions of this compound are specific and do not result from some non-specific functional antagonism. While these findings support a role for B_2-receptors in both responses, this conclusion must be tempered by the suggestion that there may be a third type of BK-receptor, which resembles the B_2-receptor in response to BK agonists, but is resistant to various B_2-specific antagonists. These receptors have been termed B_3-receptors (Farmer *et al.*, 1989). Interestingly, these putative B_3-receptors, like B_2-receptors, are sensitive to blockade by Hoe 140 (Field *et al.*, 1992). To date, B_3-receptors have been identified only in guinea-pig tissues, but without further information, we cannot at this point discount the possibility that such receptors are involved in either, or both, of the BK-induced responses reported in the present study.

It has been suggested that selective BK B_2-receptor antagonists may represent a novel class of analgesic drugs, as they are effective in some of the classical nociceptive test systems (Beresford & Birch, 1992). The present study has confirmed that the nociceptive splenic pressor reflex in the anaesthetized cat is mediated by Hoe 140-sensitive receptors and consequently, it may constitute a useful quantitative method for evaluating novel antinociceptive BK antagonists.

References

BELLAN, J.A., MINKES, R.K., MCNAMARA, D.B. & KADOWITZ, P.J. (1991). N^{ω}-nitro-L-arginine selectively inhibits vasodilator responses to acetylcholine and bradykinin in cats. *Am. J. Physiol.*, **260**, H1025–H1029.

BERESFORD, I.J.M. & BIRCH, P.J. (1992). Antinociceptive activity of the bradykinin antagonist HOE140 in rat and mouse. *Br. J. Pharmacol.*, **105**, 135P.

BOA, G., QADRI, F., STAUSS, B., STAUSS, H., GOHLKE, P. & UNGER, T. (1991). HOE140, a new highly potent and long-acting bradykinin antagonist in conscious rats. *Eur. J. Pharmacol.*, **200**, 179–182.

CALARESU, F.R., TOBEY, J.C., HEIDEMANN, S.R. & WEAVER, L.V. (1984). Splenic and renal sympathetic responses to stimulation of splenic receptors in cats. *Am. J. Physiol.*, **247**, R856–R865.

DENUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor-mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 2334–2338.

D'ORLÉANS-JUSTE, P., DION, S., MITZRAHI, J. & REGOLI, D. (1985). Effect of peptides and non-peptides on isolated arterial smooth muscles: role of endothelium. *Eur. J. Pharmacol.*, **114**, 9–21.

FARMER, S.G., BURCH, R.M., MEEKER, S.A. & WILKENS, D.E. (1989). Evidence for a pulmonary B_3 bradykinin receptor. *Mol. Pharmacol.*, **36**, 1–8.

FIELD, J.L., HALL, J.M. & MORTON, I.K.M. (1992). Bradykinin receptors in the guinea-pig taenia caeci are similar to the proposed BK_3 receptors in the guinea-pig trachea, and are blocked by Hoe140. *Br. J. Pharmacol.*, **105**, 293–296.

GUZMAN, F., BRAUN, C. & LIM, R.K.S. (1962). Visceral pain and the pseudoaffective response to intra-arterial injection of bradykinin and other algesic agents. *Arch. Int. Pharmacodyn.*, **136**, 353–384.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, ST., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe140 a new potent and long-acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–773.

LANG, W.J. & PEARSON, L. (1968). Studies on the pressor responses produced by bradykinin and kallidin. *Br. J. Pharmacol. Chemother.*, **32**, 330–338.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

RHALEB, N.-E., ROUISSI, N., DRAPEAU, G., JUKIC, D. & REGOLI, D. (1991). Characterization of bradykinin receptors in peripheral organs. *Can. J. Physiol. Pharmacol.*, **69**, 938–943.

TYERS, M.B. & HAYWOOD, H. (1979). Effects of prostaglandins on peripheral nociceptors in acute inflammation. In *Agents & Actions*, Supplement 6, ed. Rainsford, K.D. & Ford-Hutchinson, A.W. pp. 65–78. Basel: Birkhäuser Verlag.

WIRTH, K., HOCK, F.J., ALBUS, U., LINZ, W., ALPERMAN, H.G., ANAGNOSTOPOULOS, H., HENKE, ST., BRIEPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe140 a potent and long acting bradykinin-antagonist: *in vivo* studies. *Br. J. Pharmacol.*, **102**, 774–777.

(Received September 24, 1992
Revised July 23, 1993
Accepted July 28, 1993)

Br. J. Pharmacol. (1993), **110**, 1321–1328 © Macmillan Press Ltd, 1993

Endothelium-dependent noradrenaline-induced relaxation of rat isolated cerebral arteries: pharmacological characterization of receptor subtypes involved

[1]Ralf G. Hempelmann & Albrecht Ziegler

Department of Pharmacology, University of Kiel, Hospitalstraße 4, 24105 Kiel, Germany

1 The endothelium-dependence of catecholamine-induced relaxation of rat cerebral arteries was investigated *in vitro*.

2 In the basilar artery (BA), the maximal relaxant response was most pronounced with noradrenaline (NA), less with isoprenaline (Iso), and only very little with terbutaline. Methoxamine and the α_2-adrenoceptor selective agonists BHT 933 and clonidine, had no relaxant effect.

3 In BA, the relaxation by NA or Iso was markedly attenuated by N^{ω}-nitro-L-arginine (L-NOARG) 10^{-4} M. Short term perfusion of the vessels by Triton X 100 (1:1 000) suppressed the NA-induced relaxation.

4 The relaxation induced by NA or Iso was markedly reduced in presence of L-NOARG in the posterior, medial and anterior cerebral artery.

5 In BA, NA-induced relaxation was non-competitively inhibited by propranolol, atenolol, and the β_1- and β_2-adrenoceptor selective antagonists, CGP 20712 A and ICI 118551.

6 The relaxant NA-effect was not affected by prazosin but was non-competitively blocked by phentolamine.

7 The Iso-induced relaxation was competitively blocked by propranolol, whereas atenolol, CGP 20712 A and ICI 118551 caused a non-competitive inhibition.

8 The experiments indicate that the catecholamine-induced relaxation in rat isolated cerebral arteries depends upon the endothelium. They suggest that the NA-induced relaxation of BA is mediated by different α- and β-adrenoceptors and that the Iso-induced relaxation is mediated by different β-receptors. The findings would also be compatible with the idea of a receptor type which cannot be characterized by the pharmacological tools that we have used.

Keywords: Endothelium-dependent relaxation; rat cerebral arteries; catecholamines; β-adrenoceptor blockers; N^{ω}-nitro-L-arginine

Introduction

Since Furchgott & Zawadzki (1980) described the obligatory role of the endothelium in the acetylcholine-induced relaxation of precontracted rabbit aortic strips a multitude of vasodilator agents have been found to be endothelium-dependent (for reviews, see Vanhoutte & Miller, 1985; Furchgott, 1990; Marín & Sánchez-Ferrer, 1990; Moncada *et al.*, 1991). Catecholamine-induced vasodilatation via β-adrenoceptors was thought to be independent of an intact endothelium and mediated by receptors on the smooth muscle cells, leading to an increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Kukovetz *et al.*, 1981; Fridovich *et al.*, 1987; Heeson & De Mey, 1990). But during recent years endothelium-dependent relaxation caused by catecholamines and obviously mediated by β-receptors have been observed in different vascular beds (Kamata *et al.*, 1989; Gardiner *et al.*, 1991a,b; Gray & Marshall, 1992). These findings raised the possibility that catecholamine-induced effects might be mediated partly by endothelial NO, which is released in response to adrenoceptor activation. Recently, Gray & Marshall (1992) showed complete prevention of the vascular relaxation produced by isoprenaline, salbutamol and forskolin in rat aortic rings after removal of the endothelium. They suggested that NO is released in response to activation of endothelial β-adrenoceptors, mediated by cyclic AMP.

In cerebral arteries, a dilatation can be observed in feline (Edvinsson & Owman, 1974), bovine (Ayajiki & Toda, 1990; 1992) and rat (Winquist & Bohr, 1982) preparations in response to NA. This effect is most obvious in posterior regions and is presumably mediated by β_1-adrenoceptors.

In order to examine whether the catecholamine-induced relaxation in brain blood vessels depends on an intact endothelium and whether the adrenoceptors involved can be characterized by classical pharmacological tools, we investigated the effects of noradrenaline and isoprenaline in the rat basilar artery and in the posterior, medial and anterior cerebral artery.

Methods

General

Male Sprague-Dawley-rats (body weight 250–450 g) were stunned by a sharp blow on the cervico-thoracic spine and immediately exsanguinated by opening the carotid arteries. The brain was carefully removed and transferred to aerated buffer solution at room temperature. The basilar artery (BA) or the posterior (PCA), medial (MCA) or anterior (ACA) cerebral arteries were dissected out and cleaned of connective tissue under a dissection microscope. In some experiments the basilar artery was perfused with Triton X 100 (1:1 000 for ca. 20 s) to destroy the endothelium before further preparation. Before this procedure the distal end of the vessel was removed and served as a control. Circular arterial segments of 2 ± 0.5 mm length were mounted onto two parallel stainless steel wires with a diameter of 40 μm

[1] Author for correspondence.

avoiding any injury to the endothelium. Four arterial segments were obtained from one animal, but only two preparations from each individual were used for any particular experimental condition. The diameter of the arteries varied from 350 to 500 μm (BA) and from 200 to 400 μm (PCA, MCA, ACA). The vessel segments were mounted on a myograph described by Mulvany & Halpern (1977). The length of the segments was measured under a microscope. After the solution temperature had reached 37 ± 0.5°C, a passive diameter-tension curve was constructed as described by Mulvany & Halpern (1977) and McPherson (1992). By use of a computer model, the vessel's internal diameter and (according to the Laplace equation) the corresponding pressure could be calculated at any point on the passive diameter-tension curve. After the calibration procedure the vessel was allowed to relax to that tension which occurs at 90% of the diameter corresponding to a transmural pressure of 100 mmHg. Preliminary studies showed that at this tension the constriction caused by K^+ 120 mM was maximal. During the equilibration period of ca. 90 min, three successive constrictions to K^+ 120 mM, each lasting for 5 min were obtained. Vessels, which did not reach a tension corresponding to a transluminal pressure of 100 mmHg were discarded. The integrity of the endothelium was checked by administration of carbachol (CCh) 10^{-5} M on preconstricted vessels. Then, cumulative or single-concentration response curves to adrenoceptor agonists were established. No differences could be detected between either of these protocols. The cumulative technique was applied adding the next higher dose when a steady state of response was achieved, usually at an interval of 2 min. The response of a relaxing substance was expressed as a percentage of the tension attained by the vasoconstrictor, i.e. K^+ 40 mM or 5-hydroxytryptamine (5-HT) 3×10^{-7} M. Two control concentration-response curves were obtained before an antagonist was added. N^{ω}-nitro-L-arginine or antagonists were added to the bath solution 30 min before the agonist. Individual concentration-response curves were separated by a period of at least 20 min, during which the bath solution was changed twice and during which the antagonist was present in the corresponding concentration. Each experiment was finished by adding K^+ 120 mM to assess whether the muscular response remained constant. There was little change in the response to K^+ between the beginning and the end of each experiment. In a separate set of control experiments the reproducibility of the NA- or Iso-induced relaxation over the time period of the average experiment (4–6 h) was examined and was found essentially unaltered.

The organ bath contained 20 ml of a Tyrode buffer with the following composition (mM): Na^+ 147, K^+ 2.7, Ca^{2+} 1.8, Mg^{2+} 1.1, Cl^- 145.5, HCO_3^- 12, $H_2PO_4^-$ 0.21, glucose 5.5, EGTA 0.01 and additionally ascorbic acid 5.7 in order to prevent catalytic oxidation of NA. K^+ 40 mM was obtained by replacing 37.3 mM Na^+ by K^+. The buffer was bubbled with a mixture of 95% O_2 and 5% CO_2 to maintain a pH of 7.4, the temperature was kept at 37 ± 0.5°C.

Drugs

CGP 20712 A (2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl] phenoxy]propyl]amino] ethoxy]benzamide monomethanesulphonate) was a gift from Ciba-Geigy, Basel; ICI 118551 (*erythro*-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) was a gift from ICI Pharma, Plankstadt; and BHT 933 (2-amino-6-ethyl-4,5,7,8-tetrahydro-6*H*-oxazolo[5,4-d]azepine) and clonidine hydrochloride were contributed by Boehringer Ingelheim. BRL 37344 ((**R*R***)-(±)-4-[2′-(2-hydroxy-2-(3-chlorophenyl)ethylamino} propyl]phenoxyacetic acid sodium salt sesquihydrate) was a gift from SmithKline Beecham, London. In addition, the following drugs were used: noradrenaline hydrochloride (Hoechst Frankfurt), glycerol-trinitrate (Pohl-Boskamp-GmbH, Hohenlockstedt), carbamoylcholine chloride, indomethacin and 5-hydroxytryptamine creatinine sulphate complex (Merck Darmstadt), and isoprenaline hydrochloride, terbutaline hemisulphate, prazosin hydrochloride, methoxamine hydrochloride, phentolamine hydrochloride, (±)-propranolol hydrochloride, atenolol, N^{ω}-nitro-L-arginine (all Sigma). Stock solutions (10^{-2} M) were prepared daily. CGP 20712 A was dissolved in water, pH 5–6 (achieved by addition of hydrochloric acid); indomethacin in water, pH 8–9 (achieved by addition of sodium hydroxide); prazosin in 80% water and 20% dimethylformamide (DMF). All other drugs were dissolved in water. Further dilution was made with distilled water for all drugs. Catecholamines were protected from spontaneous oxidation by addition of ascorbic acid (100 μg l^{-1}). Solutions were protected from light before application.

Statistical analysis

Results shown in the text, tables and figures are expressed as means ± s.d.; data shown in Arunlakshana-Schild-plots are means ± s.e.mean. Statistical comparison of contractile and relaxant responses was made by Student's *t* test for paired data. Results of Triton X-treated preparations were compared with those of control preparations by Student's *t* test for unpaired data. E_{max} and EC_{50} were calculated by nonlinear regression.

Results

Relaxant actions of catecholamines on cerebral arteries

Noradrenaline (NA) concentration-dependently relaxed rat basilar arteries which were stimulated by K^+ 40 mM. The maximum relaxation (E_{max}) was 64 ± 9% of the tone induced by K^+ 40 mM and half maximum relaxation was achieved at ($-\log EC_{50}$) 6.0 ± 0.2 ($n = 42$). The maximal relaxant effect of NA on rat basilar arteries precontracted by 5-hydroxy-

Table 1 Relaxant effect of adrenoceptor agonists on isolated segments of rat basilar artery

		$-\log EC_{50}$	E_{max} [%]
Noradrenaline	($n = 42$)	6.0 ± 0.2	65 ± 9
Isoprenaline	($n = 18$)	6.9 ± 0.2	38 ± 10
Terbutaline	($n = 14$)	6.4 ± 0.4	13 ± 7
BRL 37344	($n = 5$)	Ineffective (10^{-5} M and 10^{-4} M)	
Methoxamine	($n = 8$)	Ineffective (10^{-5} M and 10^{-4} M)	
BHT 933	($n = 4$)	Ineffective (10^{-5} M and 10^{-4} M)	
Clonidine	($n = 4$)	Ineffective (10^{-5} M and 10^{-4} M)	

The preparations were precontracted with K^+ 40 mM, and dose-response curves for the relaxant effects of adrenoceptor agonists were established. Relaxation is expressed as % of tone increase induced by K^+ 40 mM. EC_{50} and E_{max} were calculated by nonlinear regression. Values represent means ± s.d.

Table 2 Effects (E_{max}) of noradrenaline, isoprenaline and carbachol on isolated segments of rat basilar artery (BA), posterior (PCA), middle (MCA) and anterior cerebral artery (ACA)

	Noradrenaline (%)	*Isoprenaline* (%)	*Carbachol* (%
BA	65 ± 9 ($n = 42$)	38 ± 10 ($n = 18$)	34 ± 15 ($n =$
PCA	31 ± 14 ($n = 6$)	21 ± 7 ($n = 7$)	41 ± 22 ($n =$
MCA	21 ± 3 ($n = 4$)	30 ± 5 ($n = 7$)	5 ± 5 ($n =$
ACA	12 ± 36 ($n = 6$)	16 ± 3 ($n = 6$)	34 ± 22 ($n =$

After precontraction with K^+ (40 mM) dose-response curve for the relaxant effects of NA, Iso or CCh were established. Relaxation is expressed as % of tone increase induced by K^+ 40 mM. Parameters were calculated by non-linear regression. Values represent means ± s.d.

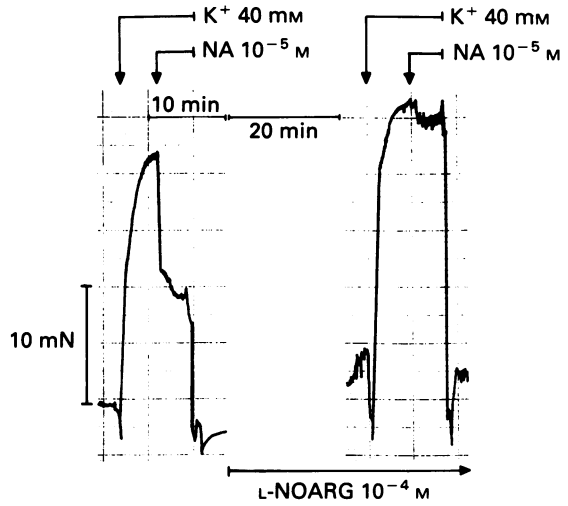


Figure 1 Tracings of responses of noradrenaline (NA)-induced relaxation of rat basilar artery precontracted by K^+ (40 mM) before and 20 min after application of N^{ω}-nitro-L-arginine (L-NOARG, 10^{-4} M).

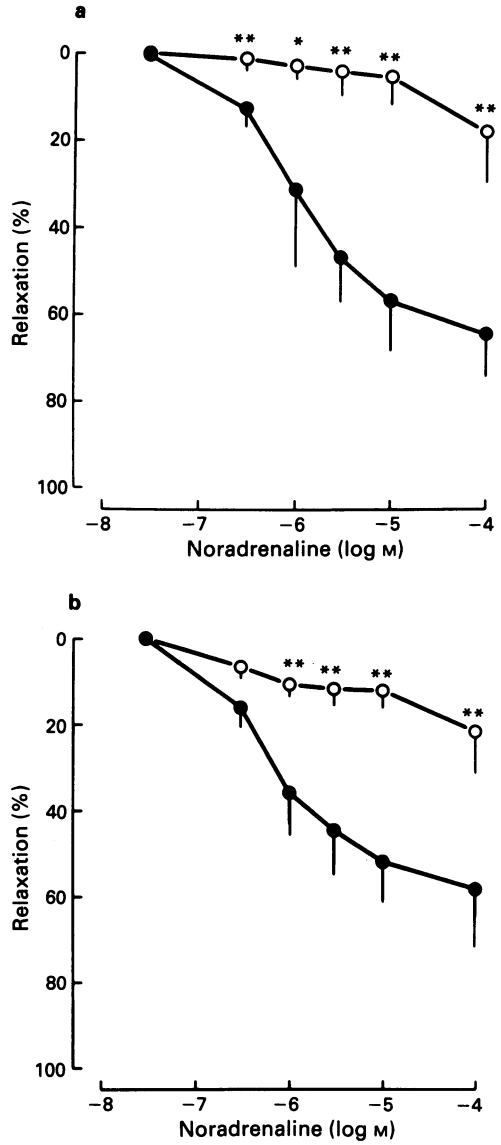


Figure 2 Noradrenaline (NA)-induced relaxation of rat basilar artery precontracted by K^+ (40 mM) under control conditions (●) and (a) in presence of N^{ω}-nitro-L-arginine (L-NOARG, 10^{-4} M) (○; $n = 9$) and (b) after short-term perfusion with Triton X 100 (1:1 000) (○; $n = 8$). Relaxation is expressed as % of tone increase induced by K^+ (40 mM). Points represent means ± s.d.; $*P < 0.01$; $**P < 0.001$.

tryptamine (5-HT) 3×10^{-7} M did not differ from the NA-effect on basilar arteries precontracted by K^+ 40 mM and was 69 ± 17% ($n = 20$, s.d., data not shown). 5-HT caused considerable myogenic activity in several preparations. In the presence of N^{ω}-nitro-L-arginine (L-NOARG) the myogenic activity induced by 5-HT was markedly enhanced. Therefore K^+ 40 mM was used to stimulate the vessels.

Neither under control conditions nor in precontracted segments of basilar arteries could a contractile effect of NA be observed. Also isoprenaline (Iso) and terbutaline concentration-dependently relaxed precontracted basilar arteries. The maximum relaxation (E_{max}), however, was markedly less pronounced than with NA and amounted to 38 ± 10% ($n = 18$, s.d.) in the case of Iso and 13 ± 7% ($n = 14$, s.d.) for terbutaline (Table 1). Methoxamine, the α_2-selective agonists BHT 933 and clonidine and the β_3-selective agonist BRL 37344 had no relaxant effect in concentrations up to 10^{-4} M (Table 1). NA also relaxed preparations obtained from the posterior, medial and anterior cerebral artery in concentrations above 3×10^{-8} M. The maximum relaxation (E_{max}) caused by NA depended on the origin of the vessel, the greatest relaxation occurring in the basilar artery and the least in the anterior cerebral artery (Table 2). The pattern of maximum relaxation (E_{max}) observed with NA in the four vascular beds differed from that elicited by carbachol (CCh). The muscarinic agonist induced a similar relaxation in the different vessel types in concentrations above 10^{-8} M with the exception of the medial cerebral artery, where the relaxation obtained was negligible (Table 2).

Catecholamine-induced relaxation after impairment of endothelial function

L-NOARG (10^{-4} M) itself caused a marked constriction of the basilar artery. The maximum constriction was achieved within 10–15 min and corresponded to 61 ± 24% ($n = 20$, s.d.) of the control stimulus by K^+ 120 mM. The tension was not maintained but declined slowly in spite of the continuous presence of L-NOARG. After about 30 min the tone corresponded to 25 ± 29% ($n = 15$, s.d.) of the maximal tone achieved with K^+ 120 mM. The new level was used as baseline for the subsequent responses. Additionally, L-NOARG produced persistent spontaneous myogenic activity.

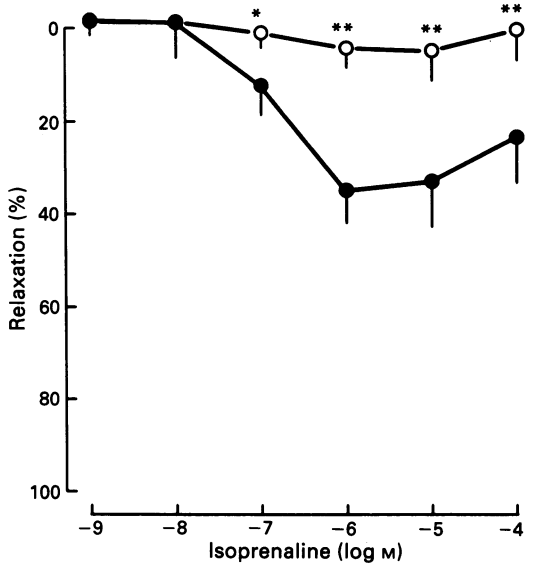


Figure 3 Isoprenaline-induced relaxation of rat basilar artery precontracted by K^+ (40 mM) under control conditions (●) and in presence of N^{ω}-nitro-L-arginine (L-NOARG, 10^{-4} M) (○). Relaxation is expressed as % of tone increase induced by 40 mM K^+. Points represent means ± s.d. of 8 experiments; $*P < 0.01$; $**P < 0.001$.

In the presence of L-NOARG the contractions induced by K^+ 40 mM developed more rapidly. The maximum contraction to K^+ 40 mM in presence of L-NOARG, however, was not significantly different from control.

Relaxations caused by NA or Iso in basilar arteries precontracted by K^+ 40 mM were markedly reduced in the presence of L-NOARG 10^{-4} M (Figures 1, 2 and 3). CCh-induced relaxations were abolished.

The relaxant effect of glycerol trinitrate (GTN) 10^{-5} M on basilar arteries was markedly enhanced in presence of L-NOARG 10^{-4} M (28 ± 6% under control conditions vs. 61 ± 14% in presence of L-NOARG, $n = 10$, s.d., $P < 0.001$, paired t test).

L-NOARG 10^{-4} M also abolished the relaxant effect of NA or Iso in the posterior, the medial and the anterior cerebral artery (results not shown).

Further evidence for the importance of the endothelium for catecholamine-induced relaxation was obtained from experiments with Triton X 100-treated vessels. We compared the drug effects on treated segments with those on untreated segments of the same arteries. In segments treated with Triton X 100 (1:1 000 for 20 s) the responses to K^+ 120 mM

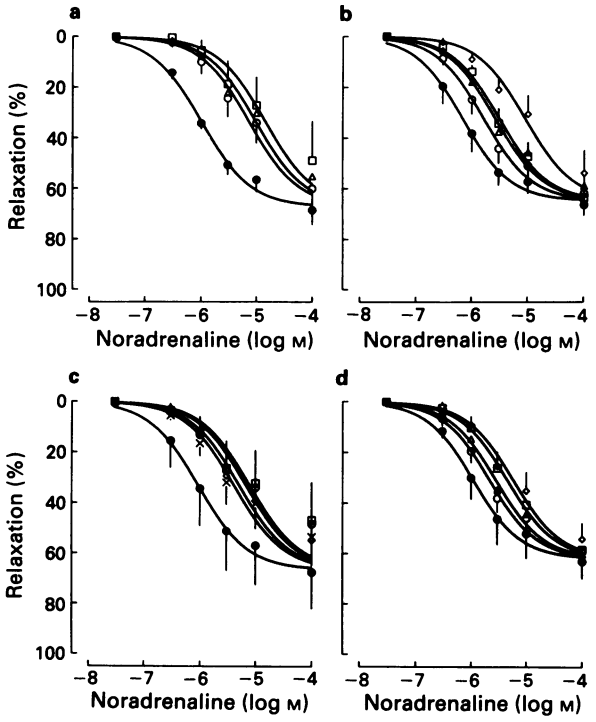


Figure 4 Noradrenaline (NA)-induced relaxation of rat basilar artery precontracted by K^+ (40 mM) (control, ●) in presence of (a) propranolol, (b) atenolol, (c) CGP 20712 A and (d) ICI 118551 (10^{-8} M, x; 10^{-7} M, ○; 3×10^{-7} M, △; 10^{-6} M, □ and 10^{-5} M, ◇). Relaxation is expressed as % of tone increase induced by 40 mM K^+. Points represent means ± s.d. of 9 experiments (a) and 8 experiments (b, c and d). All values above NA 3×10^{-8} M are significantly different ($P < 0.01$) from control.

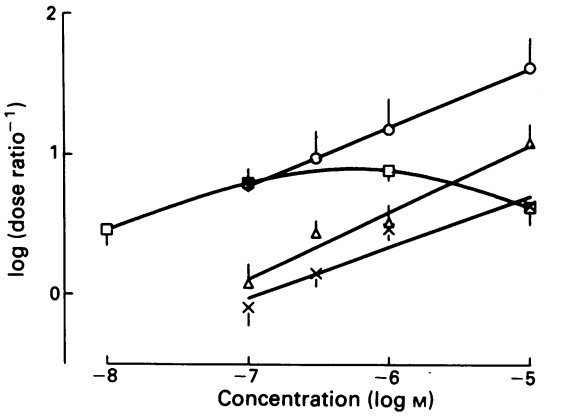


Figure 5 Arunlakshana-Schild-plot of the effects of β-receptor antagonists on the noradrenaline (NA)-induced relaxation of rat basilar artery: propranolol (○, slope = 0.42, $n = 9$), atenolol (△, slope = 0.48, $n = 8$), ICI 118551 (x, slope = 0.37, $n = 8$) and CGP 20712 A (□, $n = 8$). Experiments are the same as in Figure 4. Points represent means ± s.e.mean.

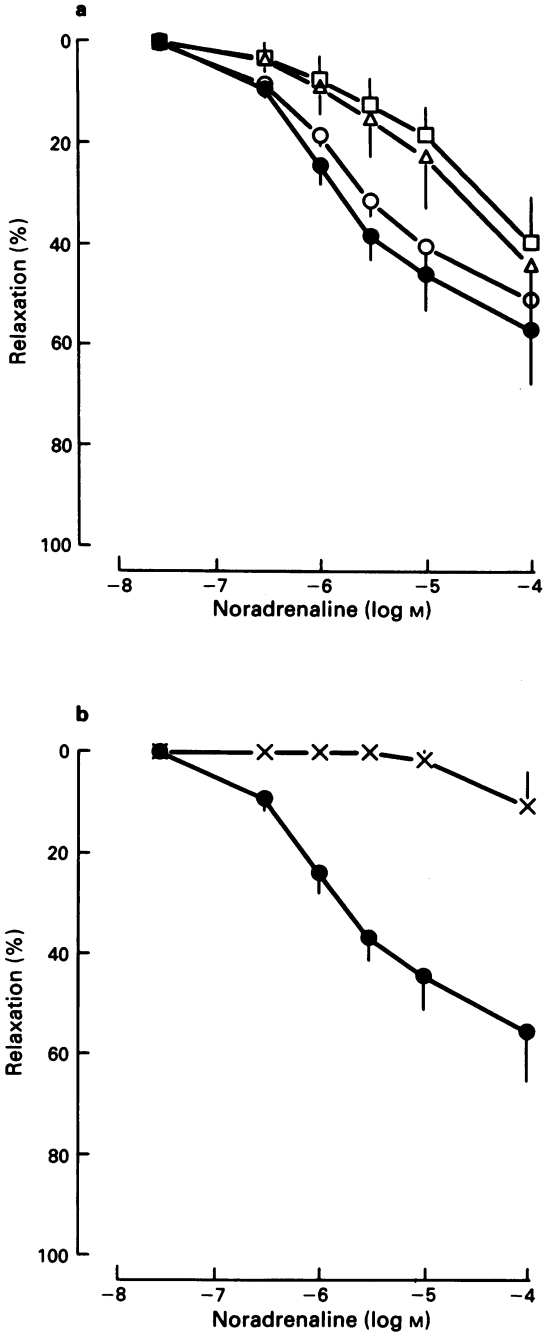


Figure 6 Noradrenaline (NA)-induced relaxation of rat basilar artery precontracted by K^+ (40 mM) (control, ●) in presence of (a) phentolamine 10^{-8} M (○); 10^{-7} M, (△) and 10^{-6} M, (□) and (b) phentolamine 10^{-6} M in combination with propranolol 10^{-6} M (x). Relaxation is expressed as % of tone increase induced by 40 mM K^+. Points represent means ± s.d. of 7 experiments (a) and 6 experiments (b). All values above NA 3×10^{-8} M and phentolamine 10^{-8} M are significantly different ($P < 0.01$) from control.

were impaired, but the vascular segments still reached a tension corresponding to a transluminal pressure of >100 mmHg (219 ± 25 mmHg in control segments vs. 141 ± 34 mmHg, in treated segments, $n = 8$, s.d.). After perfusion with the detergent, the NA-induced relaxations were markedly reduced (Figure 2) and the CCh-induced relaxations were abolished. The relaxant effect of GTN was enhanced (21 ± 7% in control segment vs. 54 ± 20% after perfusion with Triton X 100, $n = 8$, s.d., $P < 0.05$, unpaired t test). These results correspond to those found with L-NOARG.

Indomethacin (10^{-5} M) did not affect the NA-induced relaxation of the basilar artery ($n = 4$, data not shown).

Catecholamine-induced relaxation and blockade of adrenoceptors

In basilar arteries, propranolol shifted the dose-response curve for NA to the right in a non-competitive manner. The rightward shift of the dose-response curve by propranolol 10^{-6} M was about ten fold (Figure 4). Similar results were obtained with the β_1-selective antagonists atenolol and CGP 20712 A, and with the β_2-selective antagonist ICI 118551 (Figure 4). The slopes of the Arunlakshana & Schild lines were far below unity for propranolol, atenolol and ICI 118551 being 0.42 ($n = 9$), 0.48 ($n = 8$) and 0.37 ($n = 8$), respectively (Figure 5). For CGP 20712 A, the Arunlakshana-Schild-plot was curvelinear.

The NA-induced relaxation was not influenced by prazosin 10^{-6} M but was non-competitively blocked by phentolamine in concentrations above 10^{-8} M (Figure 6). A combined administration of phentolamine 10^{-6} M and propranolol 10^{-6} M abolished the NA-effect (Figure 6). Surprisingly, methoxamine, BHT 933 and clonidine were all unable to relax the basilar artery in concentrations up to 10^{-4} M.

In contrast to NA, the relaxation of the basilar artery caused by Iso was antagonized by propranolol in a competitive manner (slope of Arunlakshana-Schild-plot: 1.03; $pA_2 = 8.39$, $n = 6$, Figures 7 and 8). The Iso-induced concentration-response curve was also shifted to the right by atenolol and the β_2-selective, ICI 118551. The slopes of the Arunlakshana-Schild lines for atenolol and ICI 118551 were, however, far below unity at 0.38 ($n = 6$) and 0.66 ($n = 6$), respectively (Figure 8). The Iso-induced relaxation was also blocked by the β_1-selective CGP 20712 A, but the concentration-response curves were not shifted to the right in a parallel manner, thus indicating a non-competitive mechanism (data not shown). Phentolamine 10^{-6} M did not affect the Iso-induced relaxation of the basilar artery (data not shown).

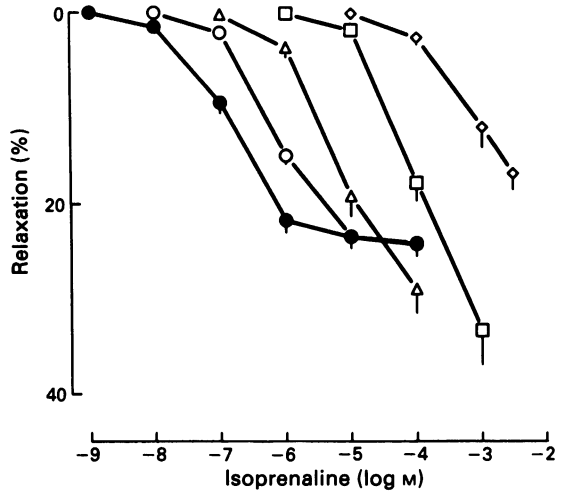


Figure 7 Isoprenaline-induced relaxation of rat basilar artery precontracted by K^+ (40 mM) (control, ●) in presence of propranolol 10^{-8} M (○), 10^{-7} M, (△), 10^{-6} M, (□) and 10^{-5} M (◇). Relaxation is expressed as % of tone increase induced by 40 mM K^+. Points represent means ± s.d. of 6 experiments.

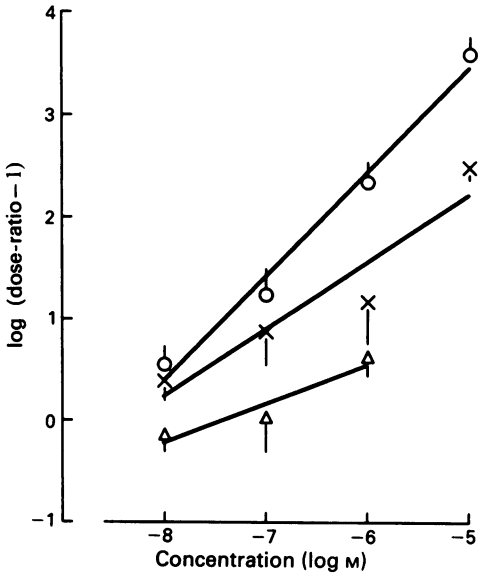


Figure 8 Arunlakshana-Schild-plot of the effects of β-receptor antagonists on the isoprenaline-induced relaxation of rat basilar artery: propranolol (○, slope = 1.03, pA_2-value = 8.39, $n = 6$), ICI 118551 (x, slope = 0.66, $n = 6$) and atenolol (△, slope = 0.38, $n = 6$). Points represent means ± s.e.mean.

Discussion

The main objective of the present study was to examine whether the relaxant effect of noradrenaline (NA) on preconstricted rat cerebral arteries was dependent on an intact endothelium. In addition, we attempted to identify the adrenoceptor involved.

General aspects

Before addressing the pharmacological results it should be mentioned that major myogenic activity did not occur during resting conditions or during contractions elicited by K^+. Spontaneous myogenic activity could have been expected from the results of McPherson & Stork (1992) in cerebral arteries from Wistar Kyoto rats and spontaneously hypertensive rats. It is tempting to speculate that the endothelial function of cerebral arteries of Sprague Dawley rats used in our experiments is different. Considerable myogenic activity occurred in our experiments only after treating the preparations with L-NOARG or with 5-hydroxytryptamine (5-HT), particularly 5-HT in the presence of L-NOARG. An obligatory role of nitric oxide (NO) for spontaneous myogenic activity, however, has been discussed for hamster aorta (Jackson *et al.*, 1991), rabbit mesenteric arteries (Omote *et al.*, 1992) and rat mesenteric arteries (Gustafsson *et al.*, 1993). Quite obviously, cerebral arteries differ from peripheral arteries in this respect, but the issue needs further investigation.

Because of the large and irregular myogenic activity induced by 5-HT in the presence of L-NOARG we decided to precontract the arteries with K^+ 40 mM. High concentrations of K^+ are known to reduce the relaxant effects of endothelium-dependent agonists (Furchgott, 1983). However, in basilar arteries contracted by K^+ (40 mM) the maximal NA-induced relaxation did not differ significantly from that in 5-HT-stimulated preparations.

Regional variations of noradrenaline effects in cerebral arteries

In cerebral arteries of the rat NA causes a complex pattern of responses. Only medial and anterior cerebral arteries were constricted by NA whereas all cerebral vessels investigated were relaxed by NA when precontracted by K^+ (40 mM). The relaxant effect was most pronounced in posterior arteries and less in anterior arteries. The regional selectivity of relaxation was quite distinct with NA and less obvious with Iso. The relaxant CCh-effect was comparable in all vessels investigated except for the medial cerebral artery, where the effect was negligible.

The relaxant effect of NA in cerebral arteries of various species is well known (Edvinsson & Owman, 1974; Winquist & Bohr, 1982; Ayajiki & Toda, 1990; 1992). Occurrence of constriction or relaxation in response to NA is thought to be determined by the relative densities of α- and β-adrenoceptors, respectively. A heterogeneous distribution of both adrenoceptor types may also be responsible for the regional selectivity of the NA-effect. A regional heterogeneity in the relative proportion of adrenoceptors mediating constriction or relaxation has been put forward in order to explain results of experiments on regional changes of cerebral blood flow (Edvinsson *et al.*, 1979) and could explain the findings of regional selectivity in vessels of cats (Harder *et al.*, 1981; Hamel *et al.*, 1988) and sheep (Gaw & Wadsworth, 1989). Ayajiki & Toda (1990; 1992) described NA-induced contractile effects in rostral and relaxant effects in caudal bovine cerebral arteries. The latter effects could be converted to contractions in the presence of propranolol. The contractions in rostral arteries could be changed into relaxations after blockade of α-receptors. It is obvious that NA causes β-receptor-mediated relaxation and α-receptor-mediated constriction in cerebral arteries of various species. In canine coronary and large peripheral arteries (Cocks & Angus, 1983; Angus *et al.*, 1986) and in rat coronary arteries (Nyborg, 1990), relaxant effects of NA were discussed as α_2-adrenoceptor-mediated. Since the α_2-selective agonists, BHT 933 and clonidine, had no relaxant effect in our experiments we suppose that α_2-adrenoceptors are of minor importance in the relaxation of precontracted rat cerebral arteries in response to NA.

Endothelium-dependence of catecholamine-induced relaxation

During the last decade several authors published observations on an endothelium-dependent dilator response to catecholamines, which seemed to be mediated through β-adrenoceptors (in conscious rats: Gardiner *et al.*, 1991a,b; in isolated rat aorta: Kamata *et al.*, 1989; Gray & Marshall, 1992; in isolated mesenteric resistance arteries: Graves & Poston, 1993). Gray & Marshall (1992) assumed an activation of endothelial adenylate cyclase as responsible for the activation of NO synthase.

In our experiments, the presence of L-NOARG (10^{-4} M), an inhibitor of NO synthase (Mülsch & Busse, 1990), abolished the relaxant effect of CCh, thus indicating a complete prevention of NO release (Faraci, 1990). The marked attenuation of the relaxant action of NA and Iso in all precontracted rat cerebral arteries examined in the presence of L-NOARG implies that the L-arginine/NO mechanism is the main signalling pathway of adrenoceptor-mediated relaxation in rat cerebral arteries.

The pronounced reduction of the relaxant NA-effect in Triton X 100-perfused basilar arteries indicates that the released NO derives from the endothelium and not from the smooth muscle cells themselves. The abolition of the CCh-induced relaxation suggests that NO release was largely suppressed. The short term perfusion of the arterial segments with Triton X 100 also seems to have affected the smooth muscle layer as indicated by a reduced response to K^+ 120 mM at the start of the experiment. A partial destruction of the smooth muscles in Triton X 100-treated preparations was verified by electron microscopy (Dr Renate Lüllmann-Rauch, Department of Anatomy, Kiel). The investigation of NA-induced relaxation in Triton X 100-treated vascular smooth muscles seems to be justified because the effect of K^+ 120 mM still exceeded a tension corresponding to a transluminal pressure of >100 mmHg and because the response to GTN was maintained and even increased.

Treating vessels by NO-synthase inhibitors sensitizes the preparations to nitrovasodilators (Busse *et al.*, 1989; Flavahan & Vanhoutte, 1989; Luscher *et al.*, 1989). This increased sensitivity also occurs *in vivo* (for review see Moncada *et al.*, 1992). The mechanism of this sensitization is, to our knowledge, not yet clarified. However, the increased response to GTN in presence of L-NOARG and after perfusion with Triton X 100 suggests that in both conditions the endothelial L-arginine-NO-pathway was blocked while the smooth muscle's ability to relax was not affected.

Our findings are at variance with the recently published results of Kitazono *et al.* (1993), who examined NA-effects on basilar artery of anaesthetized rats. The application of N^G-nitro-L-arginine methyl ester (10^{-5} M) did not impair the NA-induced vasodilatation, thus implicating an endothelium-independent relaxation. An explanation for the discrepancy could be antagonism of N^G-nitro-L-arginine methyl ester, but not L-NOARG, at muscarinic receptors, recently detected by Buxton *et al.* (1993), which may impair the acetylcholine response on the receptor level. The endothelial function, however, might have remained greatly intact.

The lack of response to indomethacin 10^{-5} M (data not shown) excludes a major contribution of metabolites of arachidonic acid to the NA-induced relaxation. This is in accordance with the findings of Kitazono *et al.* (1993).

Classification of the endothelial adrenoceptor

The α_1- and α_2-selective agonists, methoxamine, BHT 933 and clonidine, respectively, had no and terbutaline only a minor relaxant effect on precontracted basilar artery. In addition, the β_3-selective agonist BRL 37344 (Webber & Stock, 1992) also failed to relax the basilar artery. Thus, the NA-induced relaxation seems to be mediated through β_1-receptors. However, the effect of Iso was less pronounced. Therefore, we attempted to classify the endothelial receptor responsible by the use of selective β-adrenoceptor blocking agents and α-adrenoceptor antagonists.

The NA-induced relaxation of basilar artery was blocked by propranolol, the β_1-selective blockers, atenolol and CGP 20712 A and the β_2-selective blocker, ICI 118551. However, even at micromolar concentrations of all β-receptor blocking agents used the concentration-response curves were shifted only five to ten fold. The pattern of the shift was not consistent with a pure competitive antagonism. The Arunlakshana-Schild-plot (Arunlakshana & Schild, 1959) revealed a slope far below unity in the case of propranolol, atenolol and ICI 118551 and was curvelinear in case of CGP 20712 A. These results indicate that the NA-induced relaxation is not mediated via a single classical β-adrenoceptor subtype. Additionally, the NA-effect was not affected by micromolar concentrations of prazosin but was non-competitively blocked by phentolamine, although α_1- and α_2-selective agonists did not relax the basilar artery. Phentolamine (10^{-6} M) did not influence the relaxant effect of CCh ($n=4$, results not shown), indicating that the amine has no nonspecific effect on the endothelium-dependent relaxation. A combined application of phentolamine and propranolol (each 10^{-6} M) abolished the relaxation caused by NA. Thus, the relaxant NA-effect seems to be mediated by β_1- and β_2-receptor subtypes and atypical α_2-receptors, or by a receptor which cannot be identified by the pharmacological tools used.

In contrast to NA, the effect of Iso was competitively blocked by propranolol, but not influenced by phentolamine

10^{-6} M ($n = 5$, data not shown) suggesting that the Iso-effect is exclusively mediated by β-adrenoceptors. The effect was non-competitively inhibited by atenolol as well as by the β_1- and β_2-selective antagonists, CGP 20712 A and ICI 118551. Thus, the Iso-induced relaxation seems to be mediated by β_1- and β_2-receptor subtypes or by a β-receptor not belonging to the β_1- and β_2-subtypes.

Conclusions

In summary, the catecholamine-induced relaxation of rat isolated cerebral arteries depends on an intact endothelial L-arginine/NO-mechanism.

Our results do not indicate that a single endothelial adrenoceptor subtype is involved in the catecholamine-induced relaxation of the basilar artery. The findings do rather suggest that the NA-induced relaxation is mediated by β_1- and β_2-receptors and atypical α_2-receptors. The relaxant effect of Iso seems to be mediated by β_1- and β_2-receptors. The results would also be compatible with the idea of a receptor type which cannot be characaterized by classical pharmacological tools.

We thank Drs S. Herzig and H. Lüllmann for discussions and comments on the manuscript. Additionally, we are indebted to Dr Renate Lüllmann-Rauch (Department of Anatomy, Kiel) for examining the preparations by light and electron microscopy. We are grateful to Ciba-Giegy, ICI Pharma, Boehringer Ingelheim and SmithKline Beecham for the gifts of CGP 20712 A, ICI 118551, BHT 933, and BRL 37344, respectively.

References

ANGUS, J.A., COCKS, T.M. & SATOH, K. (1986). α_2-Adrenoceptors and endothelium-dependent relaxation in canine large arteries. *Br. J. Pharmacol.*, **88**, 767–777.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–59.

AYAJIKI, K. & TODA, N. (1990). Isolated bovine cerebral arteries from rostral and caudal regions: distinct responses to adrenoceptor agonists. *Eur. J. Pharmacol.*, **191**, 417–425.

AYAJIKI, K. & TODA, N. (1992). Regional difference in the response mediated by beta1-adrenoceptor subtype in bovine cerebral arteries. *J. Cereb. Blood Flow Metab.*, **12**, 507–513.

BUSSE, R., POHL, U., MÜLSCH, A. & BASSENGE, E. (1989). Modulation of the vasodilator action of SIN-1 by the endothelium. *J. Cardiovasc. Pharmacol.*, **14**, S81–S85.

BUXTON, I.L.O., CHEEK, D.J., ECKMAN, D., WESTFALL, D.P., SANDERS, K.M. & KEEF, K.D. (1993). N^G-nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.*, **72**, 387–395.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries of noradrenaline and serotonin. *Nature*, **305**, 627–630.

EDVINSSON, L., LACOMBE, P., OWMAN, C., REYNIER-REBUFFEL, A.-M. & SEYLAZ, J. (1979). Quantitative changes in regional cerebral blood flow of rats induced by alpha- and beta-adrenergic stimulants. *Acta Physiol. Scand.*, **107**, 289–296.

EDVINSSON, L. & OWMAN, C. (1974). Pharmacological characterization of adrenergic alpha and beta receptors mediating the vasomotor responses of cerebral arteries in vitro. *Circ. Res.*, **35**, 835–849.

FARACI, F.M. (1990). Role of nitric oxide in regulation of basilar artery tone in vivo. *Am. J. Physiol.*, **28**, H1216–H1221.

FLAVAHAN, N.A. & VANHOUTTE, P.M. (1989). Mechanisms underlying the inhibitory interaction between the nitrovasodilator SIN-1 and the endothelium. *J. Cardiovasc. Pharmacol.*, **14**, S86–S90.

FRIDOVICH, I., HAGEN, P.O. & MURRAY, J.J. (1987). Endothelium-derived relaxing factor: in search of the endogeneous nitroglycerin. *News Physiol. Sci.*, **2**, 61–64.

FURCHGOTT, R.F. (1983). Role of endothelium in responses of vascular smooth muscle. *Circ. Res.*, **53**, 557–573.

FURCHGOTT, R.F. (1990). Studies on endothelium-dependent vasodilation and the endothelium-derived relaxing factor. *Acta Physiol. Scand.*, **139**, 257–270.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1991a). Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to acetylcholine, 5'-N-ethylcarboxamidoadenosine or salbutamol in conscious rats. *Br. J. Pharmacol.*, **103**, 1725–1732.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1991b). Effects of N^G-nitro-L-arginine methyl ester on vasodilator responses to adrenaline or BRL 38227 in conscious rats. *Br. J. Pharmacol.*, **104**, 731–737.

GAW, A.J. & WADSWORTH, R.M. (1989). Pharmacological characterization of postjunctional α-adrenoceptors in cerebral arteries from the sheep. *Br. J. Pharmacol.*, **89**, 741–746.

GRAVES, J. & POSTON, L. (1993). β-Adrenoceptor agonist mediated relaxation of rat isolated resistance arteries: a role for the endothelium and nitric oxide. *Br. J. Pharmacol.*, **108**, 631–637.

GRAY, D.W. & MARSHALL, I. (1992). Novel signal transduction pathway mediating endothelium-dependent β-adrenoceptor vasorelaxation in rat thoracic aorta. *Br. J. Pharmacol.*, **107**, 684–690.

GUSTAFSSON, H., MULVANY, M.J. & NILSSON, H. (1993). Rhythmic contractions of isolated small arteries from rat: influence of the endothelium. *Acta Physiol. Scand.*, **148**, 153–163.

HAMEL, E., EDVINSSON, L. & MACKENZIE, E.T. (1988). Heterogeneous vasomotor responses of anatomically distinct feline cerebral arteries. *Br. J. Pharmacol.*, **94**, 423–436.

HARDER, D.R., ABEL, P.W. & HERMSMEYER, K. (1981). Membrane electrical mechanism of basilar artery constriction and pial artery dilatation by norepinephrine. *Circ. Res.*, **49**, 1237–1242.

HEESON, B.-J. & DE MEY, J.G.R. (1990). Effect of cyclic AMP-affecting agents on contractile reactivity of isolated mesenteric and renal resistance arteries of the rat. *Br. J. Pharmacol.*, **101**, 859–864.

JACKSON, W.F., MÜLSCH, A. & BUSSE, R. (1991). Rhythmic smooth muscle activity in hamster aortas is mediated by continuous release of NO from the endothelium. *Am. J. Physiol.*, **260**, H248–H253.

KAMATA, K., MIYATA, N. & KASUYA, Y. (1989). Involvement of endothelial cells in relaxation and contraction responses of aorta to isoproterenol in naive and streptotocin-induced diabetic rats. *J. Pharmacol. Exp. Ther.*, **249**, 890–894.

KITAZONO, T., FARACI, F.M. & HEISTAD, D.D. (1993). Effect of norepinephrine on rat basilar artery in vivo. *Am. J. Physiol.*, **264**, H178–H182.

KUKOVETZ, W.R., PÖCH, C. & HOLTZMANN, S. (1981). Cyclic nucleotides and relaxation of vascular smooth muscle. In *Vasodilatation*. ed. Vanhoutte, P.M. & Leusen, I. pp. 339–353. New York: Raven Press.

LUSCHER, T.F., RICHARD, V. & YANG, Z. (1989). Interaction between endothelium-derived nitric oxide and SIN-1 in human and porcine blood vessels. *J. Cardiovasc. Pharmacol.*, **14**, S76–S80.

MARÍN, J. & SÁNCHEZ-FERRER, C.F. (1990). Role of endothelium-formed nitric oxide on vascular responses. *Gen. Pharmacol.*, **21**, 575–587.

MCPHERSON, G.A. (1992). Assessing vascular reactivity of arteries in the small vessel myograph. *Clin. Exp. Pharmacol. Physiol.*, **19**, 815–825.

MCPHERSON, G.A. & STORK, A.P. (1992). The resistance of some rat cerebral arteries to the vasorelaxant effect of cromakalim and other K^+ channel openers. *Br. J. Pharmacol.*, **105**, 51–58.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MÜLSCH, A. & BUSSE, R. (1990). N^G-nitro-L-arginine (N^5-[imino-(nitroamino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. *Naunyn-Schmied. Arch. Pharmacol.*, **341**, 143–147.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.

NYBORG, N.C.B. (1990). Action of noradrenaline on isolated proximal and distal coronary arteries of rat: selective release of endothelium-derived relaxing factor in proximal arteries. *Br. J. Pharmacol.*, **100**, 552–556.

OMOTE, M., KAJIMOTO, N. & MIZUSAWA, H. (1992). The role of endothelium in the phenylephrine-induced oscillatory responses of rabbit mesenteric arteries. *Jpn. J. Pharmacol.*, **59**, 37–41.

VANHOUTTE, P.M. & MILLER, V.M. (1985). Heterogeneity of endothelium-dependent responses in mammalian blood vessels. *J. Cardiovasc. Pharmacol.*, **7** (Suppl. 3), S12–S23.

WEBBER, S.E. & STOCK, M.J. (1992). Evidence for an atypical, or β_3-adrenoceptor in ferret tracheal epithelium. *Br. J. Pharmacol.*, **105**, 857–862.

WINQUIST, R.J. & BOHR, D.F. (1982). Characterization of the rat basilar artery in vitro. *Experientia*, **38**, 1187–1188.

(Received April 26, 1993
Revised July 20, 1993
Accepted July 28, 1993)

Br. J. Pharmacol. (1993), **110**, 1329–1334 © Macmillan Press Ltd, 1993

Ca^{2+} dependency of the release of nitric oxide from non-adrenergic non-cholinergic nerves

Guy E. Boeckxstaens, Joris G. De Man, Paul A. Pelckmans, Kristel M. Cromheeke, Arnold G. Herman & [1]Yvan M. Van Maercke

Divisions of Gastroenterology and Pharmacology, Faculty of Medicine, University of Antwerp (UIA), B-2610 Antwerp-Wilrijk, Belgium

1 The role of Ca^{2+} in nitrergic neurotransmission was studied in the canine ileocolonic junction.

2 The specific N-type voltage-sensitive Ca^{2+} channel blocker ω-conotoxin GVIA (CTX, 10–100 nM) significantly reduced the electrically-evoked (2–16 Hz, 1–2 ms pulse width) non-adrenergic non-cholinergic (NANC) relaxations, preferentially affecting those to low frequency stimulation, in circular muscle strips of the ileocolonic junction. In contrast, the nerve-mediated NANC-relaxations in response to acetylcholine (30 μM), γ-aminobutyric acid (100 μM) and adenosine 5'-triphosphate (100 μM), as well as the relaxations to nitric oxide (NO) (3–10 μM) and nitroglycerin (1 μM), remained unaffected.

3 A NO-related substance (NO-R), released from the ileocolonic junction in response to NANC nerve stimulation (4 and 16 Hz, 2 ms pulse width), was assayed with a superfusion bioassay cascade. CTX (50 nM) reduced the release of NO-R induced by electrical impulses (4 Hz: from 18 ± 4% to 6 ± 4%; 16 Hz: from 33 ± 2% to 14 ± 4%, $n = 5$), but not that in response to the nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP, 0.3 mM). In Ca^{2+}-free medium, the release of NO-R evoked by electrical impulses or DMPP was inhibited. The L-type Ca^{2+} channel blockers verapamil (1–3 μM) and nifedipine (1 μM) had no effect.

4 From these results we conclude that the release of NO-R in response to NANC nerve stimulation is Ca^{2+}-dependent. The electrically-evoked release of NO-R results from Ca^{2+} entry through CTX-sensitive N-type voltage-sensitive Ca^{2+} channels, whereas that induced by nicotinic receptor activation involves CTX-insensitive Ca^{2+} channels, different from the L- or N-type.

Keywords: Bioassay; ω-conotoxin; electrical stimulation; ileocolonic junction; inhibitory innervation; nitric oxide (NO); non-adrenergic non-cholinergic nerves

Introduction

Abundant evidence has accumulated in support of our hypothesis (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990a) that nitric oxide (NO) or a NO related substance (NO-R) functions as a neurotransmitter of the inhibitory non-adrenergic non-cholinergic (NANC) innervation of the gut (for review see Sanders & Ward, 1992). Pharmacological, electrophysiological and immunocytochemical studies have all added important evidence in favour of this hypothesis, so that, at present, NO fulfils most, if not all, criteria to be considered as a neurotransmitter.

In the canine ileocolonic junction, we have previously demonstrated that NO mimicked and mediated NANC (nerve-induced) relaxations in response to electrical impulses, adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) (Boeckxstaens *et al.*, 1990a; 1991b; Bogers *et al.*, 1991). Furthermore, using a superfusion bioassay, we provided evidence indicating the release of a transferable factor in response to electrical and nicotinic receptor stimulation of the NANC nerves in this preparation (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991c). This factor behaved pharmacologically like NO: its biological activity was abolished by haemoglobin, known to bind NO avidly (Martin *et al.*, 1985) and was increased by superoxide dismutase, which prolongs the half-life of NO (Gryglewski *et al.*, 1986). In addition, the release of the factor was blocked by inhibition of NO biosynthesis and tetrodotoxin, but augmented by L-arginine (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991c), the substrate of NO biosynthesis (Palmer *et al.*, 1988). These results indicated that NO fulfilled the criteria of a putative neurotransmitter released during nerve activation (Christensen & Wingate, 1983).

In general, transmitter release from nerve terminals depends on the increase of intracellular Ca^{2+} concentration resulting from Ca^{2+} influx through Ca^{2+} channels (Augustine *et al.*, 1987; Miller, 1987). These channels can be divided into two major classes: voltage-activated channels, which respond to changes in membrane potential and which are subdivided into low threshold (T-type) and high-threshold (L, N, and P-type) channels, and receptor-operated channels, which are insensitive to depolarizing stimuli, but open when neurotransmitters, hormones or drugs combine with receptors on the cell membrane (Bertolino & Llinás, 1992; Spedding & Paoletti, 1992). ω-Conotoxin GVIA (CTX) is a potent inhibitor of the N-type voltage-sensitive Ca^{2+} channels in neurones, but not in muscle (McCleskey *et al.*, 1987; Miller, 1987). This toxin has been shown to block nerve stimulation-induced NANC relaxations in the guinea-pig taenia caeci, rat gastric fundus and rat anococcygeus muscle (De Luca *et al.*, 1990), suggesting that Ca^{2+} channels are indeed linked to neurotransmitter release in the NANC nerve terminals. However, the role of Ca^{2+} in NO release from NANC nerves has not been fully elucidated. Therefore, we have studied the effect of CTX on nitrergic relaxations in the canine ileocolonic junction and its effect, as well as that of Ca^{2+}-free medium and the L-type voltage-sensitive Ca^{2+} channel blockers, verapamil and nifedipine, on the release of the transferable factor evoked by electrical and chemical stimulation of the NANC nerves. A preliminary account of some of these results has been presented (De Man *et al.*, 1992).

[1] Author for correspondence.

Methods

Organ bath experiments

Tissue preparation Mongrel dogs of either sex (body weight 10–30 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and a laparotomy was performed. The ileum and colon were resected 10 cm above and 3 cm below the ileocolonic junction respectively. After the resected specimen was cleaned and rinsed, the mucosa was removed from the ileum and ileocolonic junction by means of sharp dissection. Circular muscle strips were cut, approximately 1.5 cm long and 0.3 cm wide, and mounted in organ baths (25 ml) (Pelckmans *et al.*, 1989) filled with a modified Krebs-Ringer solution (mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, CaEDTA 0.026 and glucose 11.1). The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

Isometric tension recording One end of each muscle strip was connected to a metal rod while the other end was attached to a strain gauge transducer (Statham UC2) for continuous recording of isometric tension. After the muscle strips were brought to the optimal point of their length-tension relationship (Pelckmans *et al.*, 1989), they were washed three times and then allowed to equilibrate for at least 45 min before experimentation.

Experimental protocols All experiments were performed on muscle strips maximally contracted with 30 μM noradrenaline and in the presence of 0.3 μM atropine. After each noradrenaline-induced contraction, the muscle strips were washed at least three times with an interval of 5 min and a cycle time of at least 20 min.

The effects of CTX (1–100 nM) were studied on the relaxations induced by electrical stimulation (2–16 Hz, 1–2 ms pulse width), ATP (100 μM), GABA (100 μM), NO (3–10 μM) and nitroglycerin (1 μM). Electrical impulses (rectangular waves, 100 mA, 9 V) were delivered by a Grass stimulator and a direct current amplifier in trains of stimuli of 10 s. CTX was added at least 20 min prior to stimulation of the tissue. The agents added remained in the organ bath until washout after the nitroglycerin-induced relaxation (Figure 1).

Superfusion bioassay cascade

Preparation of the donor tissue After resection, cleaning and rinsing of the ileocolonic junction, the mucosa and submucosa was peeled off and a circular muscle strip was prepared and mounted in a perfusion chamber, as described previously (Boeckxstaens *et al.*, 1991c). The tissue was perfused (3 ml min^{-1}) with a modified Krebs-Ringer solution maintained at 37°C. This solution contained superoxide dismutase (20 units ml^{-1}), L-arginine (50 μM) and guanethidine (3 μM) and was aerated with a mixture of 80% N_2, 15% O_2 and 5% CO_2. The perfusion chamber contained two platinum ring electrodes, through which the muscle strip was pulled. Electrical impulses (rectangular waves, 25 mA, 9 V) were delivered by a Grass stimulator and a direct current amplifier at an interval of at least 15 min, resulting in reproducible responses.

Preparation of the detector tissue New Zealand white rabbits (2000–2500 g) were killed by an overdose of sodium pentobarbitone. After a laparotomy, the abdominal aorta was removed and placed in modified Krebs-Ringer solution. Rings of abdominal aorta (3 mm wide) were cut and denuded of their endothelium by gentle rubbing. They were arranged either in a cascade, with a delay of 2–3 s between the two rings, or in parallel. The tissues were mounted under 8 g resting tension and their isometric tension was recorded. The aortic rings were superfused with the effluent of the canine ileocolonic junction and were contracted by an infusion of noradrenaline (0.1 μM). A bolus of acetylcholine (3 nmol) was injected directly into the aortic rings to verify the absence of endothelium (Furchgott & Zawadzki, 1980). Subsequently, atropine (0.3 μM) was introduced into the perfusate and the sensitivity of the bioassay tissues was standardized by a bolus injection of nitroglycerin (0.1 nmol).

Experimental protocols In a first series of experiments, the release of the transferable factor induced by electrical stimulation (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) or the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP, 0.3 mM during 1 min) was investigated in the presence of modified Krebs-Ringer solution (control) and during infusion of CTX (50 nM).

In another series, the donor tissue was first incubated in Ca^{2+}-free medium (composition in mM: NaCl 123, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, NaEDTA 0.026 and glucose 11.1) for 20 min before it was placed in the perfusion chamber. Subsequently, it was superfused with Ca^{2+}-free Krebs-Ringer solution, and after equilibration, it was stimulated with electrical impulses (8 Hz, 2 ms, pulse trains, lasting 20 s) and DMPP (0.3 mM for 1 min). The concentration of Ca^{2+} in the fluid superfusing the detector tissue was kept constant by mixing the Ca^{2+}-free effluent of the donor tissue with a 2.5 mM $CaCl_2$ solution. Next, the release in response to these stimuli was repeated after perfusion with normal Ca^{2+} (2.5 mM) containing Krebs-Ringer solution.

In a final series of experiments, the effect of verapamil (1–3 μM) and nifedipine (1 μM) was investigated on the NO-R release. Muscle strips of the canine ileocolonic junction were incubated for 20 min in Krebs-Ringer solution containing either solvent, verapamil or nifedipine. Thereafter, the donor tissue was placed into the perfusion chamber and superfused with control Krebs-Ringer solution, or Krebs-Ringer solution containing verapamil or nifedipine respectively. After equilibration (5 min), the donor tissue was stimulated with electrical impulses (4 and 16 Hz, 2 ms, pulse trains lasting 20 s) and DMPP (0.3 mM for 1 min). In order to study the effect of verapamil or nifedipine on the NO-R release within the same muscle strip, experiments were also performed in which the release of NO-R was first investigated under control conditions. Thereafter, the donor tissue was perfused with Krebs-Ringer solution containing either verapamil or nifedipine and the release of NO-R evoked by electrical impulses and DMPP was reinvestigated. Again, the effluent of the donor tissue containing verapamil or nifedipine was mixed with 2.5 mM $CaCl_2$ to maintain the tone of the detector tissue.

Drugs used

The following drugs were used: acetylcholine chloride (ACh), adenosine 5'-triphosphate (ATP), γ-aminobutyric acid (GABA), L-arginine, ω-conotoxin GVIA (CTX), 1-1-dimethyl-4-phenylpiperazinium (DMPP), nifedipine (Sigma Chemical Co., St. Louis, MO. U.S.A.), atropine sulphate (Federa, Brussels, Belgium), guanethidine monosulphate (Ciba-Geigy, Switzerland), nitroglycerin (Merck, Darmstadt, Germany), noradrenaline hydrogentartrate (Fluka AG, Buchs SG, Switzerland), verapamil (Searle Lab., Brussels, Belgium).

All drugs were administered as aqueous solutions except nifedipine, which was dissolved in dimethylsulphoxide. Ascorbic acid (0.57 mM) was added to the solution of noradrenaline. All solutions were prepared on the day of experiments, except CTX which was stored at −20°C between experiments.

NO solutions were prepared as described by Kelm *et al.* (1988). Bovine recombinant superoxide dismutase was a gift of Grünenthal GmbH (Aachen, Germany).

Presentation of results and statistical analysis

The results are expressed as percentage decrease of the noradrenaline-induced contraction of the rabbit aortic rings

or of the muscle strip of the canine ileocolonic junction.

Values are shown as mean ± s.e.mean for the number of dogs indicated. For statistical analysis, a two tailed Student's *t* test for paired or unpaired observations was used. *P* values of less than 0.05 were considered to be significant.

Results

Organ bath experiments

In the presence of atropine (0.3 μM) and guanethidine (3 μM), electrical impulses (2–16 Hz, 1–2 ms pulse width), ATP (100 μM), GABA (100 μM) and ACh (30 μM) evoked NANC nerve-induced relaxations. These relaxations were mimicked by NO (3–10 μM), whereas nitroglycerin (1 μM) induced sustained relaxations (Figure 1). CTX (10–100 nM) concentration-dependently raised the basal tension of the ileocolonic junction (10 nM: 0.2 ± 0.1 g; 100 nM; 0.5 ± 0.1g; $n = 7$) and concentration-dependently and irreversibly reduced the NANC relaxations induced by electrical impulses (Figures 1 and 2). The inhibitory effect was frequency-dependent; the lower frequencies (2–4 Hz) were preferentially reduced, whereas no effect was detected on the relaxations to 16 Hz (1 and 2 ms pulse width). The relaxations induced by ATP (control: 31 ± 3%; CTX 100 nM: 30 ± 8%, $n = 6$), GABA (control: 51 ± 6%; CTX 100 nM: 48 ± 6%, $n = 6$), ACh (control: 25 ± 4%; CTX 100 nM: 26 ± 5%, $n = 6$), NO (3 μM, control: 37 ± 7%; CTX 100 nM: 36 ± 4%, $n = 6$) and nitroglycerin (control: 80 ± 8%; CTX 100 nM: 90 ± 5%, $n = 6$) were not affected by CTX (Figure 1).

Superfusion bioassay

Effect of CTX on the release of NO-R In the presence of atropine (0.3 μM), guanethidine (3 μM), superoxide dismutase

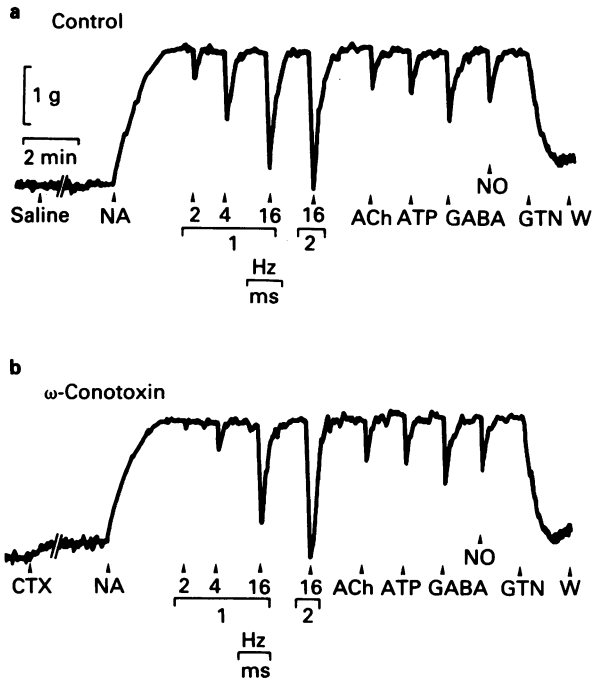


Figure 1 Typical tracing of a circular muscle strip of the canine ileocolonic junction showing the relaxations to electrical stimulation (2–16 Hz, 1–2 ms pulse width) (ACh, 30 μM), adenosine 5'-triphosphate (ATP, 100 μM), γ-aminobutyric acid (GABA, 100 μM), nitric oxide (NO, 3 μM) and nitroglycerin (GTN, 1 μM) in (a) the absence and (b) the presence of ω-conotoxin (CTX, 0.1 μM) during a noradrenaline (NA, 30 μM)-induced contraction. Similar results were obtained in at least 5 other experiments. W = wash out.

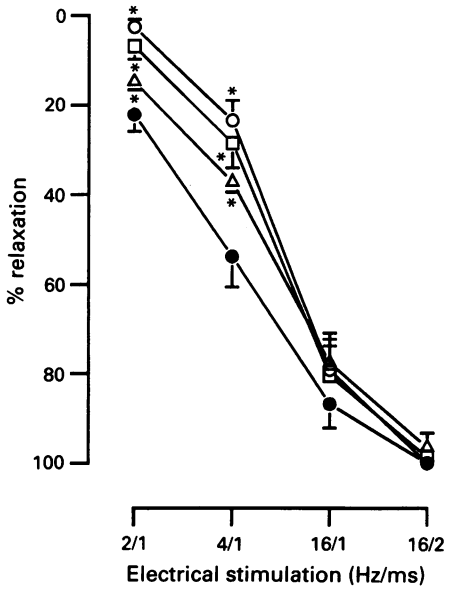


Figure 2 Effect of ω-conotoxin (CTX, △, 1 nM; □, 10 nM; ○, 100 nM) on the NANC relaxations induced by electrical impulses (●, 2–16 Hz, 1–2 ms pulse width) in the canine ileocolonic junction. Results are expressed as percentage relaxation of the noradrenaline (30 μM)-induced contraction and shown as mean ± s.e.mean for at least 6 experiments. $*P < 0.05$, significantly different from control, Student's *t* test for paired observations.

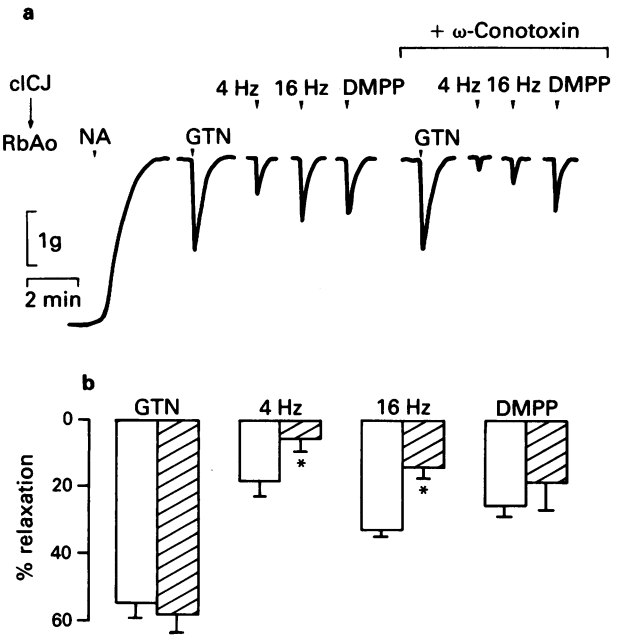


Figure 3 (a) Typical tracing of a rabbit aortic (RbAo) ring showing the effect of ω-conotoxin (CTX, 50 nM) on the relaxations to nitroglycerin (GTN, 0.1 nmol bolus injection) injected onto the cascade and on the release of a transferable factor characterized as NO or NO-R released by the canine ileocolonic junction (cICJ) in response to electrical impulses (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) and 1,1-dimethyl-4-phenylpiperazinium (DMPP, 0.3 mM 1 min). The aortic ring was precontracted by noradrenaline (NA, 0.1 μM). Breaks in the tracing represent periods of equilibration. (b) Effect of CTX (50 nm; hatched columns) on the relaxations induced by GTN (0.1 nmol bolus injection) and on the release of NO-R in response to electrical (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) and nicotinic receptor stimulation (DMPP, 0.3 mM during 1 min) (open columns). Results are expressed as percentage of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e.mean from at least 4 experiments. $*P < 0.05$, significantly different from control, Student's *t* test for paired observations.

(20 units ml^{-1}) and L-arginine (50 μM), electrical impulses (4 and 16 Hz, 2 ms pulse width) and infusion of the nicotonic agonist, DMPP (0.3 mM for 1 min) induced the release of a transferable factor from the ileocolonic junction which relaxed the bioassay tissue (rabbit aortic rings) by 18 ± 5% ($n = 5$), 33 ± 2% ($n = 6$) and 25 ± 3% ($n = 4$), respectively, of the noradrenaline-induced contraction. Infusion with CTX (50 nM) did not affect the reactivity of the rabbit aortic rings to nitroglycerin (0.1 nmol bolus injection), but reduced the release of NO-R from the ileocolonic junction induced by electrical stimulation. CTX had no effect on the release evoked by DMPP (Figure 3). As with the results obtained in the organ bath experiments, the inhibitory effect of CTX was irreversible, even after a washout period of more than 60 min.

Effect of Ca^{2+}-free medium on the release of NO-R After incubation in, and during perfusion with, Ca^{2+}-free Krebs-Ringer solution, electrical (8 Hz, 2 ms pulse width) stimulation of the ileocolonic junction failed to induce the release of NO-R, whereas DMPP (0.3 mM during 1 min) still induced a relaxation varying from 0 to 20%, resulting in a mean of 8 ± 5% ($n = 4$). Normalisation of the Ca^{2+} concentration (2.5 mM) of the perfusate resulted in a significant increase of the release of NO-R in all experiments; electrical stimulation of the ileocolonic junction now induced a relaxation of the detector tissue of 20 ± 3% ($n = 9$), whereas DMPP evoked a relaxation of 22 ± 5% ($n = 4$) of the noradrenaline-induced contraction (Figure 4). The sensitivity of the bioassay tissue to nitroglycerin (0.1 nmol bolus injected into the cascade) was not altered, since the concentration of Ca^{2+} in the Krebs-Ringer solution superfusing the detector tissue was kept constant (2.5 mM) via a $CaCl_2$ infusion.

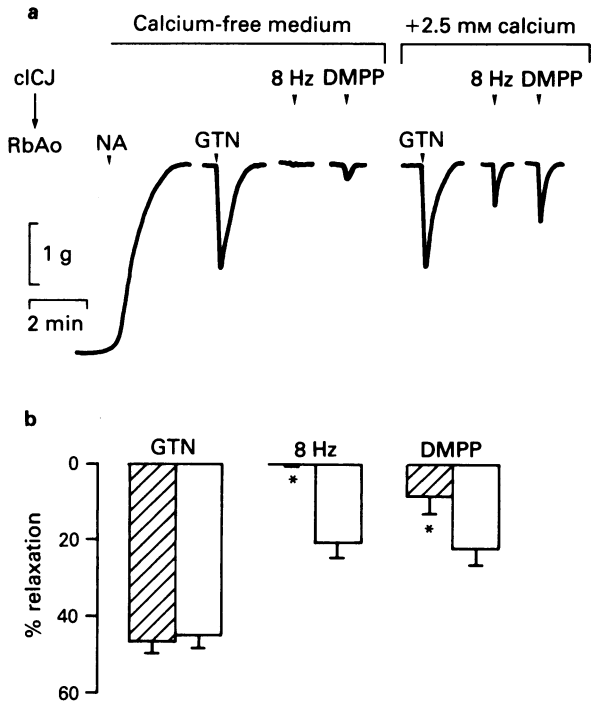


Figure 4 (a) Typical tracing of a rabbit aortic (RbAo) ring showing the relaxation to injection of nitroglycerin (GTN, 0.1 nmol bolus injection) and those in response to the release of NO-R evoked by electrical (8 Hz, 2 ms pulse width, pulse train lasting 20 s) and nicotinic receptor (1,1-dimethyl-4-phenylpiperazinium, DMPP, 0.3 mM during 1 min) stimulation of the canine ileocolonic junction (cICJ) in the absence and in the presence of Ca^{2+} (2.5 mM). The aortic ring was precontracted by noradrenaline (NA, 0.1 μM). Breaks in the tracing represent periods of equilibration. (b) Effect of Ca^{2+}-free medium (hatched columns) on the relaxations to GTN (0.1 nmol bolus injection) and on the release of NO-R by the canine ileocolonic junction (cICJ) in response to electrical impulses (8 Hz, 2 ms pulse width, pulse trains lasting 20 s) and DMPP (0.3 mM during 1 min) (open columns). Results are expressed as percentage of the noradrenaline (0.1 μM)-induced contraction and shown as mean ± s.e. mean from at least 4 experiments. $*P < 0.05$, significantly different from control, Student's t test for paired observations.

Effect of verapamil and nifedipine on the release of NO-R After incubation in Krebs-Ringer solution containing solvent, verapamil (1–3 μM) or nifedipine (1 μM), the muscle strip of the ileocolonic junction was placed in the superfusion chamber and superfused with control solution or Krebs-Ringer containing verapamil or nifedipine respectively. Although verapamil and nifedipine were used in concentrations high enough to block acetylcholine-induced contractions in the canine ileocolonic junction (unpublished results), they did not reduce the release of NO-R evoked by electrical impulses (4 and 16 Hz, 2 ms pulse width, pulse trains lasting 20 s) and DMPP (0.3 mM during 1 min) nor did they affect the relaxation of the rabbit aortic rings to nitroglycerin (0.1 nmol bolus injection) (results not shown). Similar results were obtained when the release of NO-R was first determined under control conditions, and re-evaluated in the same muscle strip after infusion with verapamil or nifedipine.

Discussion

The role of Ca^{2+} in neurotransmitter release has been well established over many years and depends on Ca^{2+} entry into the presynaptic terminal (Augustine *et al.*, 1987; Miller, 1987). CTX, a potent inhibitor of the N-type voltage-sensitive Ca^{2+} channels of neurones (McCleskey *et al.*, 1987) has been shown to be a very useful tool in evaluating the role of Ca^{2+} entry during neurotransmission (De Luca *et al.*, 1990), especially because it has no effect on the Ca^{2+} channels in the smooth muscle (McCleskey *et al.*, 1987). In several tissues, this toxin inhibits neurotransmitter release from cholinergic and adrenergic, as well as NANC nerves (Maggi *et al.*, 1988; De Luca *et al.*, 1990). Also, in the present study, the release of NO or a NO related substance, recently proposed as the inhibitory NANC neurotransmitter in the gut (Boeckxstaens *et al.*, 1990a; Bult *et al.*, 1990), was inhibited by CTX, and in Ca^{2+}-free medium, indicating a crucial role for Ca^{2+} in nitrergic neurotransmission. Indirect evidence in favour of Ca^{2+}-dependency of NANC neurotransmission was also recently reported by Stark *et al.* (1991); solutions containing low Ca^{2+} and high Mg^{2+} blocked NANC nerve-induced inhibitory junction potentials.

Indeed, in a first series of experiments performed in organ baths, CTX raised the basal tension, and inhibited the nitrergic relaxations evoked by electrical stimulation of the NANC nerves. As in previous studies (Maggi *et al.*, 1988; De Luca *et al.*, 1990), this inhibition was irreversible and frequency-dependent; mainly the relaxations to lower frequency stimulation were reduced by CTX. As CTX did not inhibit the NO-induced relaxations, its effect was presynaptic and thus most likely resulted from reduction of the NO release. This was confirmed by actually measuring the NO or NO-R release in response to nerve stimulation via a bioassay method using rabbit aortic rings without endothelium as detector tissue. These results thus confirmed the involvement of presynaptic CTX-sensitive Ca^{2+} channels in the electrically-stimulated nitrergic neurotransmission. In contrast to the organ bath experiments, however, the release of NO-R in response to 16 Hz was significantly reduced. This can be explained by differences in experimental conditions: (1) the electrical current generated in the superfusion chamber equals 25 mA whereas that in the organ baths measures 100 mA; (2) the gas mixture aerating the Krebs-Ringer solution of the bioassay contains only 15% oxygen whereas oxygen represents 95% of the gas mixture used in the organ bath experiments; (3) the release of NO-R induced by 16 Hz (2 ms) was submaximal in the bioassay experiments, whereas

the relaxation to 16 Hz (2 ms) was maximal to supramaximal in the organ bath experiments. These points may all contribute to a more pronounced effect of CTX in the bioassay experiments. Furthermore, it should also be emphasized that the bioassay cascade in fact detects the amount of NO-R that has escaped from the neuromuscular space. Once the maximal functional response has been reached, increasing the intensity of nerve stimulation, although this might result in a higher release of NO-R (detected by the bioassay), will still induce the same maximal relaxation. Conversely, at maximal to supramaximal stimulation (16 Hz, 2 ms in the organ bath experiments), CTX might reduce the output of NO-R, as shown by the bioassay; however, this amount may still be high enough to induce a maximal functional effect, resulting in a discrepancy between bioassay and tension recording data.

As the L-type Ca^{2+} channel blockers verapamil and nifedipine had no effect, these Ca^{2+} channels can be identified as N-type channels. Further, Ca^{2+} dependency was demonstrated by the finding that electrically-evoked release of NO-R was abolished in Ca^{2+}-free medium. The finding that CTX raised the basal tension of the ileocolonic junction might indicate a constant background release of NO, confirming our previous results (Boeckxstaens *et al.*, 1990a).

In contrast, drug-induced NANC neurotransmission was not affected by CTX; NANC relaxations in response to ATP, GABA or ACh, even of small amplitude, were not inhibited by CTX. Similarly, CTX did not reduce the DMPP-induced release of NO-R measured by bioassay. Experiments in Ca^{2+}-free medium however showed that the NO-R release in response to DMPP was significantly reduced. These results indicate Ca^{2+} dependency of the NO-R release and suggest that nicotinic receptor activation triggers the influx of Ca^{2+} into the NANC nerve terminals through CTX-insensitive Ca^{2+} channels. As verapamil and nifedipine were also without effect, these channels differ from the N- or L-type channels; their exact nature still remains to be determined. It thus appears that chemical stimulation of NANC nerves is coupled to activation of Ca^{2+} channels different from those activated by electrical impulses. This would imply that different stimuli can open different Ca^{2+} channels. Alternatively, different populations of NANC neurones might be activated by electrical stimuli or receptor stimulation.

Interestingly, in two of four experiments, DMPP, in contrast to electrical impulses, still induced the release of relatively large amounts of NO-R in Ca^{2+}-free medium. This might suggest mobilisation of intracellular Ca^{2+} to activate NO synthase. Alternatively, Ca^{2+}-independent NO-R synthesis might be induced by DMPP. Recently, Kostka *et al.* (1993) reported Ca^{2+}-independent NO synthase activity in myenteric synaptosomes of the canine ileum compatible with the latter possibility. Under what circumstances and to what extent these mechanisms are involved in the NO-R release from NANC nerves by DMPP cannot be determined from our experiments.

At present, two distinct types of NO synthase have been identified: a constitutive Ca^{2+}-calmodulin-dependent enzyme and an inducible Ca^{2+}-independent enzyme (for review, see Moncada *et al.*, 1991). As the enzyme present in the neurones and nerve fibres of the myenteric plexus is the constitutive isoform (Bredt *et al.*, 1990), Ca^{2+} influx into varicosities during activation thus could increase NO synthesis. Whether the Ca^{2+}-dependency of the NO release from NANC nerves results from its synthesis or its release cannot be determined from our experiments. However, based on the chemistry of NO, which implies that it is rather unlikely that NO is stored in vesicles, and the features of the neuronal NO synthase, it appears that Ca^{2+} mainly plays a role in the synthesis of the neurotransmitter.

A final and important point of discussion concerns the origin of the NO-R release. In previous studies (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1991a,c), we showed that blockade of the neuronal conductance inhibited the NO-R release. In the present study, CTX, known to inhibit neurotransmitter release via a presynaptic action (McCleskey *et al.*, 1987), also reduced the NO-R release. Based on these findings, together with the knowledge that NO synthase has been demonstrated in the nerve endings innervating gut smooth muscle, but not in the smooth muscle itself (Bredt *et al.*, 1990), we believe that the NO-R released is mainly of neuronal origin. Recently, it has been suggested that NO is released from the muscle cells by vasoactive intestinal polypeptide (VIP) in the guinea-pig gastric fundus (Grider *et al.*, 1992). However, VIP does not induce relaxations in the canine ileocolonic junction (Boeckxstaens *et al.*, 1990b), excluding this possibility. Furthermore, using a polyclonal antibody against NO synthase, morphological evidence is available demonstrating the absence of NO synthase immunoreactivity in the smooth muscle cells of the canine ileocolonic junction (Bogers *et al.*, 1993).

In conclusion, the present study demonstrates the Ca^{2+}-dependency of the release of NO-R in response to NANC nerve stimulation. Electrically-evoked NO-R release results from Ca^{2+} entry through N-type CTX-sensitive Ca^{2+} channels, whereas that induced by chemical stimulation triggers the influx of Ca^{2+} via CTX-insensitive Ca^{2+} channels, different from the N- or L-type.

This work was supported by the Belgian Fund for Medical Research (FGWO Grant 3.0014.90). The authors gratefully acknowledge F.H. Jordaens for technical assistance and Mrs L. Van de Noort for typing the manuscript.

References

AUGUSTINE, G.J., CHARLTON, M.P. & SMITH, S.J. (1987). Calcium action in synaptic transmitter release. *Annu. Rev. Neurosci.*, **10**, 633–693.

BERTOLINO, M. & LLINÁS, R.R. (1992). The central role of voltage-activated and receptor-operated calcium channels in neuronal cells. *Annu. Rev. Pharmacol. Toxicol.*, **32**, 399–421.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BOGERS, J.J., BULT, H., DE MAN, J.G., OOSTERBOSCH, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991a). Release of nitric oxide upon stimulation of non-adrenergic non-cholinergic nerves in the rat gastric fundus. *J. Pharmacol. Exp. Ther.*, **256**, 441–447.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991b). Evidence for nitrix oxide as mediator of non-adrenergic non-cholinergic relaxations induced by ATP and GABA in the canine gut. *Br. J. Pharmacol.*, **102**, 434–438.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990a). Non-adrenergic non-cholinergic relaxations mediated by nitric oxide in the canine ileocolonic junction. *Eur. J. Pharmacol.*, **190**, 239–246.

BOECKXSTAENS, G.E., PELCKMANS, P.A., RAMPART, M., VERBEUREN, T.J., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990b). NANC mechanisms in the ileocolonic function. *Arch. Int. Pharmacodyn.*, **303**, 270–281.

BOECKXSTAENS, G.E., PELCKMANS, P.A., RUYTJENS, I.F., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991c). Bioassay of nitric oxide released upon stimulation of non-adrenergic non-cholinergic nerves in the canine ileocolonic junction. *Br. J. Pharmacol.*, **103**, 1085–1091.

BOGERS, J.J., PELCKMANS, P.A., BOECKXSTAENS, G.E., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). The role of nitric oxide in serotonin-induced relaxations in the canine terminal ileum and ileocolonic junction. *Naunyn-Schmied Arch. Pharmacol.*, **344**, 716–719.

BOGERS, J.J., TIMMERMANS, J.-P., SCHEUERMANN, D.W., PELCKMANS, P.A., MAYER, B. & VAN MARCK, E. (1993). Morphological evidence for the presence of nitric oxide synthase in intrinsic neurons of the canine ileocolonic junction. *Gastroenterology*, **104**, A815.

BREDT, D.S., HWANG, P.M. & SNYDER, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature*, **347**, 768–770.

BULT, H., BOECKXSTAENS, G.E., PELCKMANS, P.A., JORDAENS, F.H., VAN MAERCKE, Y.M. & HERMAN, A.G. (1990). Nitric oxide as a possible non-adrenergic non-cholinergic neurotransmitter. *Nature*, **345**, 346–347.

CHRISTENSEN, J. & WINGATE, D.L. (1983). *A Guide to Gastrointestinal Motility*. Bristol: Wright-PSG.

DE LUCA, A., LI, C.G., RAND, M.J., REID, J.J., THAINA, P. & WONG-DUSTING, H.K. (1990). Effects of ω-conotoxin GVIA on autonomic neuroeffector transmission in various tissues. *Br. J. Pharmacol.*, **101**, 437–447.

DE MAN, J.G., BOECKXSTAENS, G.E., PELCKMANS, P.A., HERMAN, A.G. & VAN MAERCKE, Y.M. (1992). Nitric oxide release from non-adrenergic non-cholinergic nerves in the canine ileocolonic junction is Ca^{2+}-dependent. *J. Gastrointest. Motil.*, **4**, 216.

FURCHGOTT, R.F. & ZAWADSKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GRIDER, J.R., MURTHY, K.S., JIN, J.-G. & MAKHLOUF, G.M. (1992). Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am. J. Physiol.*, **262**, G774–G778.

GRYGLEWSKI, R.J., PALMER, R.M. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*, **320**, 454–456.

KELM, M., FEELISCH, M., SPAHR, R., PIPER, H.M., NOACK, E. & SCHRADER, J. (1988). Quantitative and kinetic characterization of nitric oxide and EDRF released from cultured endothelial cells. *Biochem. Biophys. Res. Commun.*, **154**, 236–244.

KOSTKA, P., ELLIOTT, J., WATSON, E.G., STEWART, J.L. & DANIEL, E.E. (1993). Nitric oxide synthase in the autonomic nervous system of canine ileum. *J. Pharmacol. Exp. Ther.*, **264**, 234–239.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., LIPPE, I.T., GUILLIANE, S., GEPPETTI, P., DEL BIANCO, E., SELLERI, S. & MELI, A. (1988). The effect of omega conotoxin GVIA, a peptide modulator of the N-type voltage sensitive calcium channels, on motor responses produced by activation of efferent and sensory nerves in mammalian smooth muscle. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 107–113.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MCCLESKEY, E.W., FOX, A.P., FELDMAN, D.H., CRUZ, L.J., OLIVERA, B.M., TSIEN, R.W. & YOSHIKAMI, D. (1987). ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 4327–4331.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. *Science*, **235**, 46–52.

MONCADA, S., PALMER, R.M.J. & GRYGLEWSKI, R.J. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

PALMER, R.M., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PELCKMANS, P.A., BOECKXSTAENS, G.E., VAN MAERCKE, Y.M., HERMAN, A.G. & VERBEUREN, T.J. (1989). Acetylcholine is an indirect inhibitory transmitter in the canine ileocolonic junction. *Eur. J. Pharmacol.*, **170**, 235–242.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262**, G379–G392.

SPEDDING, M. & PAOLETTI, R. (1992). III. Classification of calcium channels and the sites of action of drugs modifying channel function. *Pharmacol. Rev.*, **44**, 363–376.

STARK, M.E., BAUER, A.J. & SZURSZEWSKI, J.H. (1991). Effect of nitric oxide on circular muscle of the canine small intestine. *J. Physiol.*, **444**, 743–761.

(Received May 4, 1993
Revised July 22, 1993
Accepted July 29, 1993)

Br. J. Pharmacol. (1993), **110**, 1335–1340 © Macmillan Press Ltd, 1993

Nasal administration of an ACTH(4–9) peptide analogue with dimethyl-β-cyclodextrin as an absorption enhancer: pharmacokinetics and dynamics

Nicolaas G.M. Schipper, [1]J. Coos Verhoef, Larissa M. De Lannoy, Stefan G. Romeijn, *Jan H. Brakkee, *Victor M. Wiegant, *Willem Hendrik Gispen & Frans W.H.M. Merkus

Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands and *Department of Medical Pharmacology, Rudolf Magnus Institute, Medical Faculty, University of Utrecht, Vondellaan 6, 3521 GD Utrecht, The Netherlands

1 The systemic absorption and the neurotrophic effect of the metabolically stabilized ACTH (4–9) analogue, Org2766, were investigated following intranasal (i.n.) administration.

2 Without additives the nasal bioavailability of the peptide was in the order of 15 and 10% in rats and rabbits, respectively. The absorption could be improved by addition of a variety of absorption enhancers to the nasal preparation. The β-cyclodextrin derivative, dimethyl-β-cyclodextrin (DMβCD) at a concentration of 5% (w/v) improved the absorption in rats about 5 fold from 13 ± 4% (mean ± s.d.) for administration of the peptide alone to 65 ± 21%, and in rabbits 1 to 2 fold, from 10 ± 6% to 17 ± 8%.

3 The increased permeability of the rat nasal mucosa for Org2766 caused by DMβCD in rats reversed substantially within 1 h. However, the nasal absorption had not yet completely returned to the level without enhancer.

4 S.c. administered Org2766 accelerated the functional recovery from peripheral nerve damage in rats. However, the peptide did not facilitate nerve repair following i.n. administration with DMβCD, in spite of the fact that Org2766 was well absorbed. I.v. injection of Org2766 was also ineffective.

Keywords: Nasal delivery; dimethyl-β-cyclodextrin (DMβCD); ACTH(4–9) analogue (Org2766); absorption enhancer; peripheral nerve regeneration; peptide

Introduction

The metabolically stabilized ACTH(4–9) hexapeptide analogue, Org2766, stimulates the outgrowth of developing or damaged neurones (Strand *et al.*, 1991). In animal models that measure functional, electrophysiological and histological parameters, Org2766 has been shown to facilitate nerve repair following CNS as well as peripheral nerve lesions (Wolterink *et al.*, 1990; Spruyt *et al.*, 1990; Van der Zee *et al.*, 1991). Moreover, the peptide is clinically effective in cisplatin-induced neuropathies (Gerritsen Van Der Hoop *et al.*, 1990).

As in many peptide and protein drug therapies, the ACTH(4–9) analogue is administered parenterally. Parenteral injection therapy, however, is associated with social and physical discomfort, and may lead to poor patient compliance. Evidently, there is a need for alternative delivery routes. The absorption of orally administered peptides is poor mostly because of gastro-intestinal breakdown and to large first-pass elimination in the liver. Following peroral delivery, Org2766 did not improve the recovery from peripheral nerve damage in rats (Dekker *et al.*, 1987; Van der Zee *et al.*, 1988). Therefore, nasal administration may be an attractive alternative. The nasal route avoids first pass metabolism, is easily accessible and particularly suitable for self-medication. However, the bioavailability of nasally administered peptides and proteins is usually low because of poor permeability of the nasal epithelium for high molecular weight and hydrophilic compounds, enzymatic degradation in the nasal mucosa and the rapid mucociliary clearance resulting in a short residence time in the nasal cavity (Chien *et al.*, 1989). One approach to increase the absorption efficiency is the use of absorption enhancers in the nasal dosage form (Lee *et al.*, 1991).

It has recently been demonstrated that cyclodextrins and in particular α-cyclodextrin and the derivative dimethyl-β-cyclodextrin (DMβCD) are very potent enhancers of nasal insulin absorption in rats (Merkus *et al.*, 1991; Irie *et al.*, 1992).

In the present paper the nasal absorption of Org2766 was studied in rats and rabbits. The influence of DMβCD on the absorption was compared with the effect of other absorption enhancers. Moreover, the neurotrophic effect of the nasally administered ACTH(4–9) analogue/DMβCD dosage form on functional recovery from peripheral nerve damage in rats was studied in relation to the effect after s.c. and i.v. injections.

Methods

Animals

Male Wistar rats from an inbred strain with a body weight of approx. 200 g were used in the nasal absorption experiments. In the functional recovery studies the male Wistar rats weighed approx. 130–150 g at the start and 260–280 g at the end of these experiments. The animals were housed in Makrolan cages (5 rats/cage) and maintained at 12:12 h light: dark cycle. They were fed a commercial diet and had water *ad libitum*. The rats were randomized over the experimental groups prior to crush-lesioning of the sciatic nerve.

The rabbits used in absorption experiments were female New Zealand Whites with a body weight of 3.7–4.0 kg. They were obtained from Iffa Credo Broekman (Someren, The Netherlands), and housed in stainless steel cages. The animals were fed a commercial laboratory animal diet and had water *ad libitum*.

Preparation of peptide solutions

The ACTH(4–9) analogue was dissolved in a phosphate-buffered saline solution (pH = 7.4) (PBS), just prior to the

[1] Author for correspondence.

experiments. For intranasal (i.n.) application the absorption enhancing compounds were subsequently added to the peptide solutions. For i.v. s.c. and i.n. control administration, peptide solutions without enhancers were used. Blank solutions without peptide were 5% (w/v) DMβCD in PBS.

Absorption experiments

Male Wistar rats were anaesthetized with Hypnorm (1 ml kg^{-1} s.c.). Anaesthesia was maintained with additional injections of 0.5 ml kg^{-1} Hypnorm, usually every 45 min. Experimental animal procedures were as described previously (Schipper *et al.*, 1992b). Nasal solutions in a volume of 20 µl were instilled unilaterally through one of the nares with PVC-tubing connected to a microliter syringe. For s.c. administration a microlitre syringe was used to give a volume of 200 µl under the neck skin. Intravenous bolus injections of 20 µl were given through a cannulated femoral vein; directly afterwards the cannula was flushed with 200 µl PBS.

The rabbits were sedated with a s.c. injection of 0.5 ml kg^{-1} Hypnorm in the back to prevent sneezing. Nasal peptide solutions were instilled through one of the nares. PVC-tubing connected to a microlitre syringe was inserted 1.0 cm into a nostril and 50 µl drug solution was instilled. Intravenous peptide solutions were given in the right ear vein. Nasal and i.v. Org2766 formulations were given in a random order to each of the rabbits. A wash out period of 3 to 7 days was allowed between subsequent administrations.

Blood samples of approx. 300 µl were taken at regular time intervals. The samples were immediately put on ice, and were allowed to clot for 2 h. They were centrifuged at 4°C to obtain serum. The samples were stored at − 20°C until analysis.

Measurement of Org2766 serum concentrations

A radioimmunoassay procedure was used to determine the serum concentrations of the ACTH(4–9) peptide analogue (Gispen & Wiegant, 1990). A rabbit antiserum raised against bovine thyroglobulin-conjugated Org2766 was used at a final dilution of 1:4000. Aliquots of serum samples (10–50 µl) adjusted to 100 µl with buffer (PBS with 0.1% bovine serum albumin and 0.2% Tween-80), and 100 µl standard Org2766 in buffer were added to 50 µl antiserum. They were preincubated at 4°C for 16 h, followed by incubation for 24 h at 4°C with [^{125}I]-Org2766 (50 µl; 10,000 c.p.m.). The [^{125}I]-Org 2766 was prepared with a Chloramine-T labelling method. Antibody-bound and free Org2766 were separated by fractional precipitation with 20% (w/v) polyethylene glycol in PBS. Serum samples were assayed in duplicate. The sensitivity of the assay (10% displacement of tracer) was 1 pg/tube. The cross reactivity of the antiserum was (expressed on mass basis): [Met(O_2)^4,D-Lys^8,Phe^9]ACTH(4–9) (Org2766), 100%; [D-Lys^8,Phe^9]ACTH(4–9), 0.85%; [Acetyl-Met(O_2)^4,D-Lys^8, Phe^9]ACTH(4–9), 1.6%; [D-Lys^8, Phe^9] ACTH(5–9), 50%; [D-Lys^8,Phe^9]ACTH(6–9), 62%; [D-Lys^8,Phe^9]ACTH (7–9), 6.4%; ACTH(4–10), 0.001%.

Analysis of absorption data

The areas under the individual serum concentration-time curves (AUC) were calculated with the linear trapezoidal rule up to 3 h post administration. Nasal bioavailabilities were calculated according to the formula: $AUC_{i.n.}/AUC_{i.v.} \times D_{i.v.}/D_{i.n.} \times 100\%$, in which D is the administered dose. The raw rat AUC, time to peak concentration and peak concentration data were evaluated for statistical significant differences by a one way analysis of variance. The rabbit data were evaluated by a Student's *t* test for paired results. Differences were assigned to be significant for values of $P < 0.05$. All data are presented as the mean ± s.d.

Functional recovery from peripheral nerve damage

Rats were anaesthetized with Hypnorm (0.8 ml kg^{-1}). A crush-lesion was made in the right sciatic nerve as described in detail by De Koning *et al.* (1986). The nerve was crushed for 30 s with a haemostatic forceps. Nasal, i.v. and s.c. administrations of Org2766 were given immediately after the sciatic nerve was crushed, and subsequently every 48 h for 24 days. The rats were anaesthetized with 0.4 ml kg^{-1} Hypnorm just prior to nasal instillation of the drug solutions, and kept lying on their backs during and until 30 min after administration.

The return of sensorimotor function was measured by the foot reflex withdrawal test described previously by De Koning *et al.* (1986). A small electric current was applied to the sole of the rats foot. A positive response was scored when rats withdrew their feet from the electric stimulus instantaneously. A range of six brief current pulses from 0.1 to 0.6 mA was applied to determine the minimal current required to evoke a response. Rats were considered fully recovered when they retracted their paws at 0.1 mA (100% recovery). Failure to retract at 0.6 mA was taken as 0% recovery. The rats were tested on days 3 and 10 after crush-lesioning to check the effectiveness of the crush-lesion in the sciatic nerve. From days 14 to 24 the functional recovery was determined daily. On days that the functional recovery test coincided with peptide delivery, the recovery test was performed prior to peptide administration. The functional recovery experiments were performed double-blind. The data were expressed as the mean percentage recovery per day (± s.e. mean). Group differences were analysed by a Mann-Whitney test using the data before transformation to percentage recovery.

Chemicals

The ACTH(4–9) analogue, Org2766 (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH) was a gift from Organon (Oss, The Netherlands). Dimethyl-β-cyclodextrin (DMβCD) and α-cyclodextrin (α-CD) were from Avebe (Foxhol, The Netherlands). Sodium taurodihydrofusidate (STDHF) and sodium glycocholate (GC) were obtained from Sigma (St. Louis, MO, U.S.A.) and California Biotechnology (Mountain View, CA, U.S.A.), respectively. Hydroxypropyl-β-cyclodextrin (HPβ CD) and Hypnorm were from Janssen (Beerse, Belgium). Radioactive ^{125}I was obtained from Amersham (Houten, The Netherlands). All other reagents used were of standard laboratory grade.

Results

Pharmacokinetics of Org2766 after i.v., s.c. and i.n. administration in rats

The Org2766 concentrations detected in rat serum after i.n. placebo administration were very low and in the range of the detection limit of the radioimmunoassay (Table 1). Thus, the cross-reactivity of the Org2766 antibody with possibly interfering endogenous compounds was negligible and the measured peptide serum concentrations were not corrected for endogenous immunoreactivity. After an i.v. bolus injection of 75 µg kg^{-1} of the ACTH(4–9) analogue an initial rapid decline in serum concentrations was followed by a slower phase of elimination (Figure 1). After 3 h the serum levels were still elevated. I.n. delivery of the same dose of the ACTH(4–9) analogue resulted in slowly increasing serum peptide concentrations with a peak concentration of 2.5 ng ml^{-1} at approximately 1 h post-administration. The absolute bioavailability of nasally administered Org2766 was found to be 19 ± 8% (Table 1). S.c. administered Org2766 at a dose of 15 µg kg^{-1} showed absorption peak serum levels at 30 min (Figure 1).

Table 1 Bioavailabilities of Org2766 following nasal and s.c. administration to rats

Dose ($\mu g\ kg^{-1}$)	*Route*	*Additive*	AUC_{0-180} ($ng\ ml^{-1}\ min$)	*F* (%)	n
0	i.n.	–	2 ± 1	0	(4)
25	i.n.	–	55.7 ± 18.1	13 ± 4	(4)
75	i.n.	–	253.9 ± 113.3	19 ± 8	(6)
250	i.n.	–	408.8 ± 121.7	13 ± 4	(6)
25	i.n.	DMβCD 5%	269.1 ± 85.4	65 ± 21	(8)
75	i.n.	DMβCD 5%	1033.5 ± 164.7	76 ± 12	(6)
250	i.n.	DMβCD 5%	2362.6 ± 610.0	63 ± 19	(6)
25	s.c.	–	ND	ND	
75	s.c.	–	940.0 ± 158.7	69 ± 12	(6)
250	s.c.	–	3519.6 ± 854.0	108 ± 26	(4)

The areas under the serum concentration-time curves measured up to 180 min post-administration (AUC_{0-180}) and the bioavailability (F) data are given as the mean ± s.d. of the number of animals given in parentheses (*n*). ND is not determined. *Significantly different from the s.c. 75 $\mu g\ kg^{-1}$ dose ($P<0.05$).

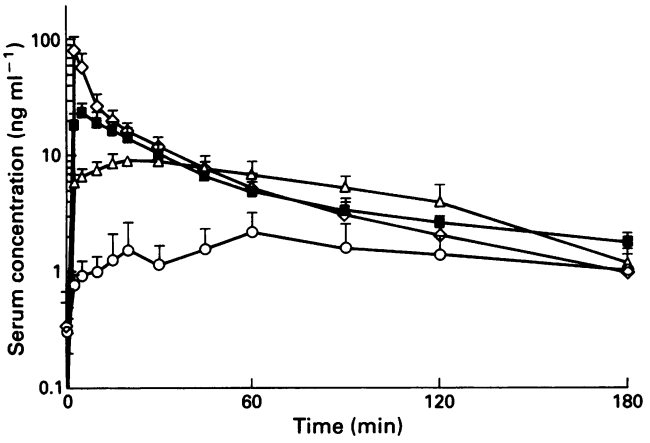


Figure 1 Mean ± s.d. serum Org2766 concentrations following administration of 75 $\mu g\ kg^{-1}$ Org2766 in rats: (◇) i.v.; (△) s.c.; (○) i.n. without enhancer; (■) i.n. with 5% (w/v) dimethyl-β-cyclodextrin. $n = 6$.

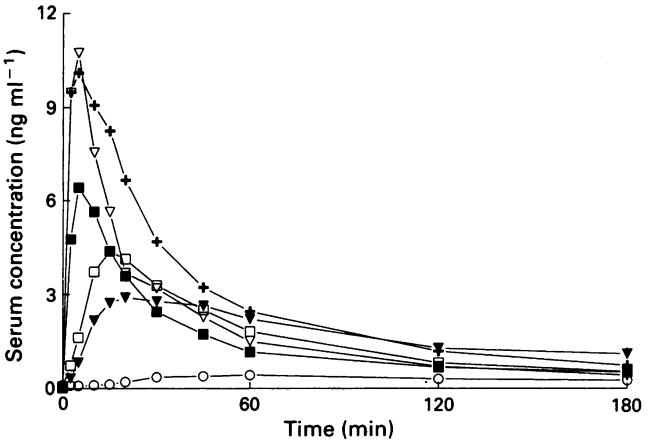


Figure 2 Mean serum Org2766 concentrations following nasal administration of a dose of 25 $\mu g\ kg^{-1}$ Org2766 with absorption enhancers in rats: (○) without enhancer; (▼) 5% α-CD; (□) 2% DMβCD; (■) 5% DMβCD; (▽) 0.5% STDHF; (+) 1% GC. See Table 2 for the number of rats used in the experiments. See text for abbreviations.

The peptide bioavailability after s.c. injection was 3 to 4 times as large as the nasal bioavailability (Table 1).

For i.n. and s.c. administered Org2766 at doses of 25 to 250 $\mu g\ kg^{-1}$ the AUC_{0-180} and the corresponding bioavailabilities are given in Table 1. The AUC's were proportional to the i.n. administered dose Org2766, indicating that the bioavailability values were not dependent on the peptide dose given. S.c. administered Org2766 was significantly better absorbed from a large 250 $\mu g\ kg^{-1}$ dose, than a smaller 75 $\mu g\ kg^{-1}$ dose.

Nasal delivery of Org2766 in rats: effect of absorption enhancers

A number of compounds were studied for their ability to improve nasal Org2766 absorption in rats. The serum concentration versus time curves obtained in these experiments are depicted in Figure 2 and the pharmacokinetic data are summarized in Table 2. Addition of HPβCD to the nasal peptide preparation (25 $\mu g\ kg^{-1}$) did not improve the absorption of the ACTH(4–9) analogue compared to administration of the peptide alone. All other compounds studied improved the nasal peptide absorption remarkably. The nasal bioavailabilities ranged from 70 ± 23% for (2% w/v) DMβCD to 118 ± 50% for (1% w/v) GC. Addition of 2 and 5% DMβCD resulted in a similar improvement in bioavailability. The times to reach the peak peptide serum concentrations were decreased by the effective enhancers. For DMβCD (5%), STDHF (0.5%) and GC (1%) the maximum peptide concentrations were already achieved at 5 min after administration. The nasal absorption was slower when α-CD (5%) and DMβCD (2%) were used as enhancers, showing times to reach the maximum serum concentration of 16 ± 2 and 27 ± 13 min (mean ± s.d.), respectively (Table 2).

Table 2 Nasal administration of Org2766 (25 $\mu g\ kg^{-1}$) with absorption enhancers in rats: pharmacokinetic parameters

Route	*Additive*	T_{max} (min)	C_{max} ($ng\ ml^{-1}$)	*F* (%)	n
i.v.	–			100 ± 31	(10)
i.n.	–	49 ± 7	0.4 ± 0.2	13 ± 4	(4)
i.n.	DMβCD 2%	16 ± 2*	4.5 ± 1.3*	70 ± 23*	(8)
i.n.	DMβCD 5%	6 ± 2*	6.8 ± 2.2*	65 ± 21*	(8)
i.n.	α-CD 5%	27 ± 13*	3.1 ± 0.4*	76 ± 17*	(4)
i.n.	HPβCD 5%	35 ± 21	1.0 ± 0.7	16 ± 4	(4)
i.n.	STDHF 0.5%	5 ± 0*	11 ± 1.1*	82 ± 24*	(4)
i.n.	GC 1%	5 ± 2*	11 ± 3.7*	118 ± 50*	(8)

The time at which the serum peak concentration was reached (T_{max}), the peak serum concentration (C_{max}), and the bioavailability (F) are given as mean ± s.d. of the number of animals given in parentheses (*n*). The statistical evaluation of the bioavailability data was performed on the raw AUC data (One way analysis of variance). *Significantly different from i.n. Org2766 without additives ($P<0.05$). For abbreviations, see text.

Reversibility of the enhancing effect of DMβCD on nasal Org2766 absorption

In order to determine the duration of the enhancing effect of DMβCD on nasal Org2766 absorption, the peptide (25 μg kg^{-1}) was given i.n. to rats 1 h after DMβCD instillation. The serum concentration versus time curves are shown in Figure 3. The serum-peptide concentrations appeared to be largely reduced compared to simultaneous administration of peptide and enhancer. The AUC values were decreased about 2 fold: $AUC_{0-60\,min}$ was 411 ± 127 ng ml^{-1} min for coadministration of peptide and enhancer, and $AUC_{0-60\,min}$ was 186 ± 94.2 ng ml^{-1} min when the peptide was given 60 min after DMβCD administration. Although the effect of DMβCD on Org2766 absorption was reversed substantially within 1 h, the nasal absorption had not yet completely returned to the level seen without any enhancer.

Nasal absorption of Org2766 with DMβCD in rabbits

In rabbits, nasal administration of 25 μg kg^{-1} Org2766 with 5% (w/v) DMβCD resulted in significantly higher Org2766 serum concentrations than administration of Org2766 alone during the first 45 min (Figure 4). Absolute bioavailability values of 10 ± 6% and 17 ± 8% were calculated for nasal administration of Org2766 without enhancer and with 5% (w/v) DMβCD, respectively (Table 3).

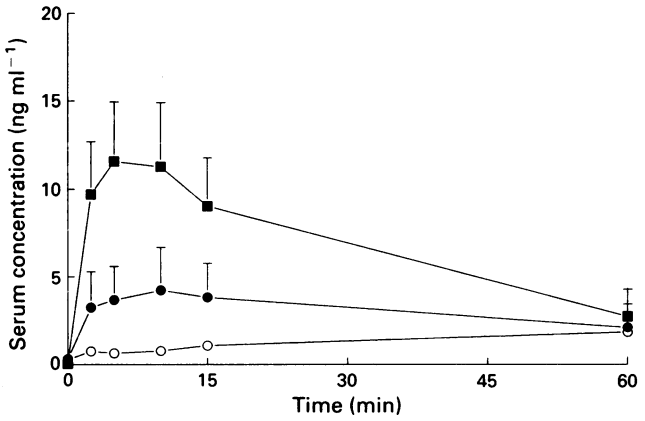


Figure 3 Mean ± s.d. serum Org2766 concentrations following nasal administration of 25 μg kg^{-1} Org2766 in rats: (○) without enhancer; (■) 5% dimethyl-β-cyclodextrin (DMβCD) administered simultaneously with Org2766; (●) 5% DMβCD given 1 h before Org2766 administration. $n = 3$ to 6 rats. The $AUC_{0-60\,min}$ following simultaneous administration of Org2766 and DMβCD was significantly different from peptide administration without enhancer and from peptide administration 1 h after DMβCD instillation ($P < 0.01$, one way analysis of variance).

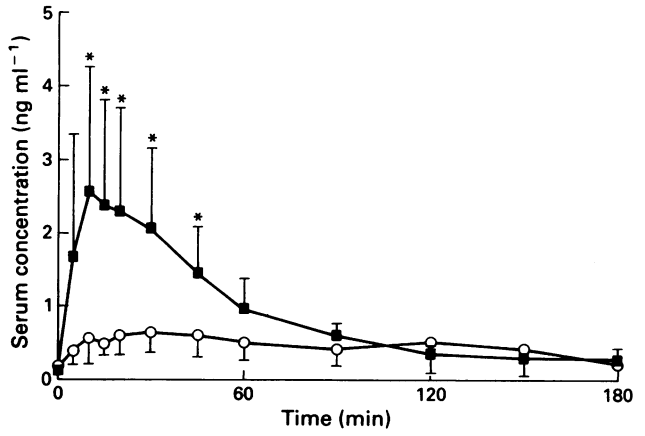


Figure 4 Mean ± s.d. serum Org2766 concentrations following nasal administration of 25 μg kg^{-1} Org2766 in rabbits: (○) without enhancer; (■) 5% dimethyl-β-cyclodextrin. $n = 7$ animals. *Significantly different from i.n. administration without enhancer ($P < 0.05$, t test for paired results).

Functional recovery from peripheral nerve damage after different routes of peptide administration

The efficacy of nasally administered Org2766 with DMβCD as an enhancer was studied in a rat model in which the functional recovery from a crush lesion in the sciatic nerve was determined with a foot reflex withdrawal test. Rats untreated with Org2766 started to respond to the electric stimulus in the withdrawal test at days 16 to 18, and were considered fully recovered at days 22 to 24. Rats receiving a s.c. dose of 75 μg kg^{-1} 48 h^{-1} Org2766 showed an accelerated recovery of nerve function compared to untreated rats (Figure 5). Since nasal dosing could only be performed reproducibly and quantitatively when the rats were anaesthetized just before peptide administration, the s.c. and i.v. treated animals were also anaesthetized. Hypnorm anaesthesia did not decrease the beneficial effect of s.c. administered Org2766 (Figure 6). I.n. and i.v. administrations of Org2766 at a dose of 75 μg kg^{-1} 48 h^{-1} were ineffective in accelerating the functional recovery from nerve damage compared to placebo treated animals. From Figure 6 it is evident that nasal peptide doses ranging from 0.75 to 375 μg kg^{-1} 48 h^{-1} did not improve the recovery rate compared to untreated rats.

Table 3 Nasal administration of Org2766 (25 μg kg^{-1}) with dimethyl-β-cyclodextrin (DMβCD, 5% w/v) as absorption enhancer in rabbits: pharmacokinetic parameters

Route	*Additive*	C_{max} (ng ml^{-1})	T_{max} (min)	*F* (%)	n
i.v.	–			100 ± 14	(7)
i.n.	–	0.8 ± 0.4	36 ± 39	10 ± 6	(7)
i.n.	DMβCD	2.9 ± 1.6*	15 ± 8	17 ± 8	(7)

The time at which the serum peak concentration was reached (T_{max}), the peak serum concentration (C_{max}) and the bioavailability (F) are given as mean ± s.d. of the number of animals given in parentheses (n). The statistical evaluation of the bioavailability data was performed on the raw AUC data (Student's t test for paired results). *Significantly different from i.n. Org2766 without additives ($P < 0.05$).

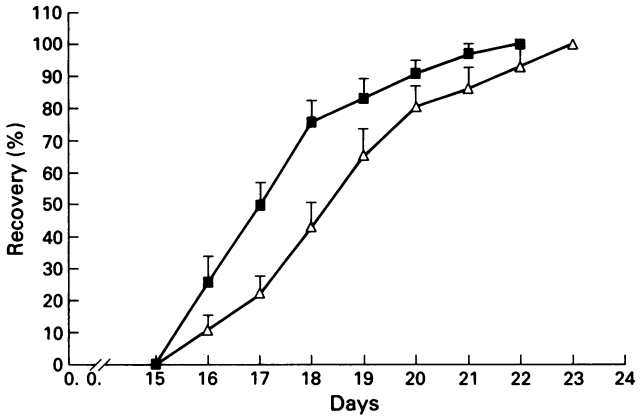


Figure 5 The effect of s.c. administered Org2766 (75 μg kg^{-1} 48 h^{-1}) on functional recovery following a crush lesion of the right sciatic nerve in rats: (△) placebo; (■) Org2766. The percentage recovery was determined by reflex withdrawal reaction on a local foot sole stimulation of 0.1–0.6 mA. The percentage recovery is plotted as the mean ± s.e.mean of 11 to 12 rats. The rats were anaesthetized.

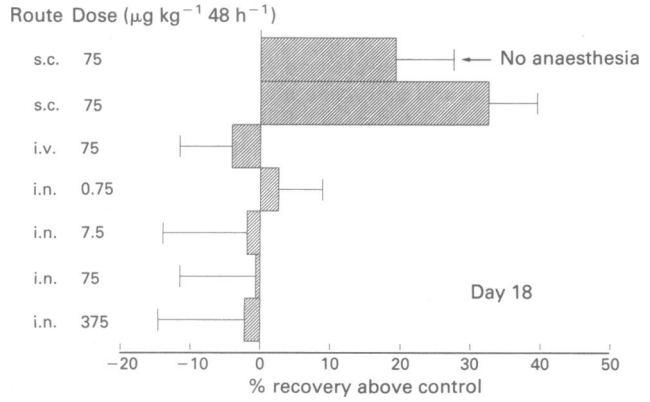


Figure 6 The effect of Org2766 on functional recovery following a crush lesion in the right sciatic nerve. The % recovery above control is the % recovery of peptide-treated animals subtracted from the % recovery of their own control group. The % recovery above control is plotted as the mean recovery ± s.e.mean of 11 to 12 rats per group on day 18 after the lesion. All rats were anaesthetized, with the exception of one s.c. dosed group. The statistical evaluation (Mann-Whitney) was performed on the raw data obtained at day 18, comparing the peptide treatment values of each experimental group with their respective saline-treatment values. Significantly enhanced absorption was only observed for s.c. administration of Org2766 with and without anaesthesia ($P<0.01$, and $P<0.05$, respectively).

Discussion

The present study showed that following nasal administration the ACTH(4–9) analogue, Org2766, was taken up in the systemic circulation in both rats and rabbits, the bioavailability being about 15% and 10%, respectively. The absorption in rats could be largely improved by addition of a number of absorption enhancers to the nasal preparation such as the cyclodextrins α-CD and DMβCD, the fusidate derivative STDHF, and the bile salt GC. Although many compounds of widely differing chemical nature have been shown to enhance nasal peptide absorption (Lee *et al.*, 1991), major problems encountered with many enhancers are their possible adverse effects on the nasal epithelium and mucociliary activity, and the occurrence or irritation. STDHF, for example, severely damages the nasal epithelium (Ennis *et al.*, 1990), decreases *in vitro* mucociliary activity at concentrations as low as 0.2% (w/v) (Gizurarson *et al.*, 1990; Hermens *et al.*, 1990), and is not well tolerated after nasal administration in man (Kissel *et al.*, 1992). GC which is an effective enhancer of many peptide and protein drugs (Pontiroli *et al.*, 1989), has less adverse effects (Ennis *et al.*, 1990; Gizurarson *et al.*, 1990; Hermens *et al.*, 1990). Previous studies in our laboratory have shown that GC and DMβCD at concentrations of 1 and 2% (w/v), respectively, had the same effect on *in vitro* ciliary activity (Schipper *et al.*, 1992a). The decrease in ciliary movement was much smaller than the influence of enhancers such as STDHF, sodium deoxycholate, sodium cholate, or laureth-9 (Hermens *et al.*, 1990). The cyclodextrin derivative DMβCD in concentrations up to 6% (w/v) was well tolerated when given intranasally as an oestradiol- or progesterone-cyclodextrin complex to female patients daily for a period of at least 6 months (Hermens *et al.*, 1992).

The high permeability of the nasal muscosa for Org2766 as caused by DMβCD returned to the normal physiological state in time. This was demonstrated by the decrease in effect of DMβCD on nasal Org2766 absorption when the peptide was not administered simultaneously with the enhancer but 1 h later. Comparable studies by Hirai *et al.* (1981) and Lee *et al.* (1988) showed that the absorption enhancer laureth-9 increased the permeability of rat nasal mucosa for a long period of time (>2 h), whereas the effects of GC and STDHF were, similar to DMβCD, limited in duration. Addition of DMβCD to the nasal peptide preparations improved the absorption in both the rat and rabbit animal model. The enhancement of absorption, however, was stronger in rats than in rabbits. Such species differences in the nasal absorption of peptides have been reported before (Baldwin *et al.*, 1990; Fisher *et al.*, 1991; Merkus *et al.*, 1993). Similar differences were also obtained for the nasal absorption of insulin with DMβCD. In rats the insulin absorption was almost complete, whereas in rabbits and man, insulin was hardly absorbed following delivery of nasal insulin/DMβCD solutions (Merkus *et al.*, 1991; Schipper *et al.*, 1993).

Several mechanisms of action by which cyclodextrins can improve nasal peptide absorption have been suggested (Irie *et al.*, 1992; Shao *et al.*, 1992). Cyclodextrins have been shown to interact with membrane lipids and proteins in the nasal epithelium, which may reduce the barrier function of the epithelium. Cyclodextrins are also able to inhibit proteolytic enzyme activity in the nasal mucosa (Irie *et al.*, 1992), and finally, they may act directly upon the peptide molecule thereby inhibiting peptide aggregation. (Shao *et al.*, 1992). In good agreement with the reported mechanisms of action, α-CD and DMβCD have been observed to influence strongly the nasal epithelial membrane barrier and enzyme activity, while HPβCD, ineffective in promoting nasal Org2766 absorption as evident from the present study, did not influence either the membrane barrier properties or the protease activities (Irie *et al.*, 1992; Shao *et al.*, 1992).

The effect of Org2766 on nerve recovery is highly dose-dependent. The dose-response relationship is of a so-called bell-shaped type. S.c. administration of Org2766 in doses of 7.5 or 75 μg kg^{-1} 48 h^{-1} facilitated peripheral nerve repair after a crush-lesion in the sciatic nerve, whereas low doses of 0.075, 0.75, and a high dose of 225 μg kg^{-1} 48 h^{-1} were completely ineffective (Gispen & Wiegant, 1990). In spite of the good absorption of nasally administered Org2766 with DMβCD, resulting in high serum peptide concentrations, the ACTH(4–9) analogue did not facilitate peripheral nerve regeneration after nasal delivery. The strong dose-dependency of the effect of Org2766, however, probably does not explain the negative results obtained with nasal administration. The serum Org2766 concentrations were in the same order of magnitude following nasal and s.c. delivery. Moreover, the dose given i.n. was varied between 0.75 and 375 μg kg^{-1} 48 h^{-1}. Thus, one would expect that at least one of these dosages would result in effective serum concentrations. Nevertheless, none of the nasal preparations used in the present study improved nerve repair. More likely, the serum concentration time-profile following nasal administration might be too rapid for the peptide to exert a beneficial effect. The serum concentration-time profile after nasal administration was quite similar to that found after i.v. injection. Although the initial serum concentration after i.v. injection was larger than the peak serum concentration obtained after i.n. delivery, they both declined very rapidly, and it is very striking that both i.n. and i.v. administration of the peptide did not result in improved functional recovery of the sciatic nerve. Following s.c. administration, which was the only effective route of delivery in the present experiments, the absorption phase was longer: the Org2766 peak serum concentrations were smaller, and did not decline as rapidly as observed with i.n. and i.v. routes of administration.

Changes in peptide binding to blood components, drug distribution and/or metabolism affected by the cyclodextrin derivative may also have influenced the amount of free serum ACTH(4–9) analogue, and, thus, the effect on peripheral nerve regeneration. This would, however, not explain the ineffectiveness of i.v. administered Org2766, since i.v. injections were performed in the absence of DMβCD. It has previously been suggested that in order to improve nerve regeneration Org2766 should be available repeatedly during a short, critical period of about 1 week following the crush (Edwards *et al.*, 1984; De Koning & Gispen, 1987; Gispen & Wiegant, 1990). The present data corroborate this view and

indicate that to facilitate nerve repair, significant amounts of the peptides must be present in the systemic circulation for an extended period of time as obtained with s.c. delivery, whereas the high but rapidly disappearing peak concentrations associated with i.v. and i.n. administration oppose the beneficial effect on peripheral nerve regeneration. The previous observation that continuous s.c. delivery of Org2766 using a depot microsphere preparation has resulted in improved nerve repair is in accordance with the present data (Gispen & Wiegant, 1990).

In conclusion, the ACTH(4–9) analogue Org2766 was absorbed following nasal administratiom in rats and rabbits. The absorption could be substantially improved by coadministration of absorption enhancers such as the cyclodextrin derivative, DMβCD. However, in spite of the large absorption, the peptide did not facilitate the functional recovery from peripheral nerve damage following nasal delivery in rats, probably due to the observed rapid absorption profile.

The technical assistance in the nerve recovery experiments of G. De Ruiter and M. Hali is greatly appreciated. We wish to thank Dr B. Naisbett for her careful evaluation of the manuscript.

References

BALDWIN, P.A., KLINGBEIL, C.K., GRIMM, C.J. & LONGENECKER, J.C. (1990). The effect of sodium tauro-24,25-dihydrofusidate on the nasal absorption of human growth hormone in three animal models. *Pharm. Res.*, **7**, 547–552.

CHIEN, Y.W., SU, K.S.E. & CHANG, S.F. (1989). *Nasal Systemic Drug Delivery*. New York: Marcel Dekker.

DEKKER, A.J.A.M., PRINCEN, M.M., DE NIJS, H., DE LEEDE, L.G.J. & BROEKKAMP, C.L.E. (1987). Acceleration of recovery from sciatic nerve damage by the ACTH(4–9) analog Org2766: different routes of administration. *Peptides*, **8**, 1057–1059.

DE KONING, P., BRAKKEE, J.H. & GISPEN, W.H. (1986). Methods for producing a reproducible crush in the sciatic and tibial nerve of the rat and rapid and precise testing of return of sensory function: beneficial effect of melanocortins. *J. Neurol. Sci.*, **74**, 237–246.

DE KONING, P. & GISPEN, W.H. (1987). Org2766 improves functional and electrophysiological aspects of regenerating sciatic nerve in the rat. *Peptides*, **8**, 415–422.

EDWARDS, P.M., VAN DER ZEE, C.E.E.M., VERHAAGEN, J., SCHOTMAN, P., JENNEKENS, F.G.I. & GISPEN, W.H. (1984). Evidence that the neurotrophic actions of α-MSH may derive from its ability to mimic actions of a peptide formed in degenerating nerve stumps. *J. Neurol. Sci.*, **64**, 333–340.

ENNIS, R.E., BORDEN, L. & LEE, W.A. (1990). The effects of permeation enhancers on the surface morphology of the rat nasal mucosa: a scanning electron microscopy study. *Pharm. Res.*, **7**, 468–475.

FISHER, A.N., FARRAJ, N.F., O'HAGAN, D.T., JABBAL-GILL, I., JOHANSEN, B.R., DAVIS, S.S. & ILLUM, L. (1991). Effect of L-α-lysophosphatidylcholine on the nasal absorption of human growth hormone in three animal species. *Int. J. Pharm.*, **74**, 147–156.

GERRITSEN VAN DER HOOP, R., VECHT, C.J., VAN DER BURG, M.E.L., ELDERSON, A., BOOGERD, W., HEIMANS, J.J., VRIES, E.P., VAN HOUWELINGEN, J.C., JENNEKENS, F.G.I., GISPEN, W.H. & NEIJT, J.P. (1990). Prevention of cis-platin neurotoxicity with an ACTH(4–9) analogue in patients with ovarian cancer. *N. Engl. J. Med.*, **322**, 89–94.

GISPEN, W.H. & WIEGANT, V.M. (1990). Melanocortins in peripheral nerve regeneration: influence of dosage forms and routes of administration. *J. Controlled Rel.*, **13**, 203–211.

GIZURARSON, S., MARRIOTT, C., MARTIN, G.P. & BECHGAARD, E. (1990). The influence of insulin and some excipients used in nasal insulin preparations on mucociliary clearance. *Int. J. Pharm.*, **65**, 243–247.

HERMENS, W.A.J.J., BELDER, C.W.J., MERKUS, C.M.W.M., HOOYMANS, P.M., VERHOEF, J. & MERKUS, F.W.H.M. (1992). Intranasal administration of estradiol in combination with progesterone to oophorectomized women: a pilot study. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **43**, 65–70.

HERMENS, W.A.J.J., HOOYMANS, P.M., VERHOEF, J. & MERKUS, F.W.H.M. (1990). Effects of absorption enhancers on human nasal tissue ciliary movement in vitro. *Pharm. Res.*, **7**, 144–146.

HIRAI, S., YASHIKI, T. & MIMA, H. (1981). Mechanisms for the enhancement of the nasal absorption of insulin by surfactants. *Int. J. Pharm.*, **9**, 173–184.

IRIE, T., WAKAMATSU, K., ARIMA, H., ARITOMI, H. & UEKAMA, K. (1992). Enhancing effects of cyclodextrins on nasal absorption of insulin in rats. *Int. J. Pharm.*, **84**, 129–139.

KISSEL, T., DREWE, J., BANTLE, S., RUMMELT, A. & BEGLINGER, C. (1992). Tolerability and absorption enhancement of intranasally administered octreotide by sodium taurodihydrofusidate in healthy subjects. *Pharm. Res.*, **9**, 52–57.

LEE, V.H.L., YAMAMOTO, A. & BHASKAR, U. (1991). Mucosal penetration enhancers for facilitation of peptide and protein drug absorption. *Crit. Rev. Ther. Drug Carrier Syst.*, **8**, 91–192.

LEE, W.A., ENNIS, R.E. & FOSTER, L.C. (1988). Histological studies of insulin absorption across the nasal mucosa in the presence of sodium taurodihydrofusidate. *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, **15**, 70–71.

MERKUS, F.W.H.M., SCHIPPER, N.G.M., ROMEIJN, S.G. & VERHOEF, J. (1993). Absorption enhancers in nasal drug delivery: efficacy and safety. *J. Controlled Rel.*, **24**, 201–208.

MERKUS, F.W.H.M., VERHOEF, J., ROMEIJN, S.G. & SCHIPPER, N.G.M. (1991). Absorption enhancing effect of cyclodextrins in intranasally administered insulin in rats. *Pharm. Res.*, **8**, 588–592.

PONTIROLI, A.E., CALDERARA, A. & POZZA, G. (1989). Intranasal drug delivery: potential advantages and limitations from a clinical pharmacokinetic perspective. *Clin. Pharmacokinet.*, **17**, 299–307.

SCHIPPER, N.G.M., DE LANNOY, L.M., ROMEIJN, S.G., VERHOEF, J. & MERKUS, F.W.H.M. (1992a). Nasal administration of an ACTH (4–9) analogue in rats. In *Minutes on Buccal and Nasal Administration as an Alternative to Parenteral Administration*. ed. Duchêne, D. pp. 297–302. Paris: Éditions de Santé.

SCHIPPER, N.G.M., ROMEIJN, S.G., VERHOEF, J. & MERKUS, F.W.H.M. (1993). Nasal insulin delivery with dimethyl-beta-cyclodextrin as an absorption enhancer in rabbits: powder more effective than liquid formulations. *Pharm. Res.*, **10**, 682–686.

SCHIPPER, N.G.M., VERHOEF, J., ROMEIJN, S.G. & MERKUS, F.W.H.M. (1992b). Absorption enhancers in nasal insulin delivery and their influence on nasal ciliary functioning. *J. Controlled Rel.*, **21**, 173–186.

SHAO, Z., KRISHNAMOORTHY, R. & MITRA, K. (1992). Cyclodextrins as nasal absorption promoters of insulin: mechanistic evaluations. *Pharm. Res.*, **9**, 1157–1163.

SPRUYT, B., PITSIKAS, N., ALGERI, S. & GISPEN, W.H. (1990). Org2766 improves performance of rats with unilateral lesions in the fimbria fornix in a spatial learning task. *Brain Res.*, **527**, 192–197.

STRAND, F.L., ROSE, K.J., ZUCARELLI, L.A., KUME, J., ALVES, S.E., ANTONAWICH, F.J. & GARETT, L.Y. (1991). Neuropeptide hormones as neurotrophic factors. *Physiol. Rev.*, **71**, 1017–1046.

VAN DER ZEE, C.E.E.M., BRAKKEE, J.H. & GISPEN, W.H. (1988). α-MSH and Org2766 in peripheral nerve regeneration: different routes of delivery. *Eur. J. Pharmacol.*, **147**, 351–357.

VAN DER ZEE, C.E.E.M., BRAKKEE, J.H. & GISPEN, W.H. (1991). Putative neurotrophic factors and functional recovery from peripheral nerve damage in the rat. *Br. J. Pharmacol.*, **103**, 1041–1046.

WOLTERINK, G., VAN ZANTEN, E., KAMSTEEG, H., RADHAKISHUN, F.S. & VAN REE, J.M. (1990). Functional recovery after destruction of dopamine systems in the nucleus accumbens of rats. II. Facilitation by the ACTH(4–9) analog Org2766. *Brain Res.*, **507**, 101–108.

(Received June 8, 1993
Revised July 28, 1993
Accepted July 30, 1993)

Br. J. Pharmacol. (1993), **110**, 1341–1346 © Macmillan Press Ltd, 1993

5-HT_3 antagonists reduce morphine self-administration in rats

[1]Siu-Chun G. Hui, *Elenita L. Sevilla & Clive W. Ogle

Department of Pharmacology, Faculty of Medicine, University of Hong Kong, Hong Kong, and *Department of Pharmacology, Cebu Institute of Medicine, Philippines

1 The effects of the 5-HT_3 receptor antagonists, ondansetron and tropisetron, on morphine consumption were studied in naive and morphine-dependent rats.

2 The administration of ondansetron (1 $\mu g\,kg^{-1}$, i.p. twice daily) 7 days prior to, and during a 21-day period of, morphine availability (increasing concentration from 0.1 to 0.4 $mg\,ml^{-1}$) in 5% sucrose solution reduced opiate intake from the 9th day of morphine treatment.

3 The administration of ondansetron (0.1 $\mu g\,kg^{-1}$, i.p. twice daily) or tropisetron (0.1 $\mu g\,kg^{-1}$, i.p. twice daily) on the 14th day of the 21-day period of morphine treatment failed to reduce opiate consumption. Administration of the larger doses of tropisetron (1 $\mu g\,kg^{-1}$) or ondansetron (1 $\mu g\,kg^{-1}$) reduced morphine consumption.

4 After receiving 21 days of treatment with morphine alone or with the ondansetron or tropisetron regimens identified above, the sucrose solutions were substituted with tap water for 7 days. These detoxified rats were then allowed a free choice of sucrose or morphine for 10 days. Animals that had received concomitant treatment with ondansetron or tropisetron showed reduced morphine intake when compared with the controls treated with morphine only or with vehicle-treated controls.

5 The administration of cyproheptadine (100 or 250 $\mu g\,kg^{-1}$, i.p. twice daily) on the 14th day of 21-day morphine treatment failed to modify morphine intake and also failed to influence the subsequent intake of the opiate in the free choice situation.

6 It is concluded that ondansetron and tropisetron can reduce morphine intake in both naive and morphine-dependent rats.

Keywords: Morphine intake; ondansetron; tropisetron; cyproheptadine; rats; self administration

Introduction

Drugs of abuse are thought to activate mesolimbic dopaminergic systems (Di Chiara & Imperato, 1988). Indeed, dopamine is considered to play an important role in the process of reward and reinforcement, and in place preference conditioning studies. It has been demonstrated that heroin reward is dependent on a dopaminergic substrate (Bozarth & Wise, 1981). Lesioning of the nucleus accumbens, using the neurotoxin 6-hydroxydopamine or kainic acid, has been shown to disrupt heroin place-preference conditioning (Spyraki *et al.*, 1983), to increase significantly intravenous morphine self-administration (Smith *et al.*, 1985) and to reduce cocaine and heroin self-administration in rats (Zito *et al.*, 1985). Evidence for 5-HT_3 receptor involvement in the regulation of limbic dopamine function has come from both behavioural and biochemical studies. Thus, the locomoter hyperactivity induced by the injection or infusion of dopamine or amphetamine into the nucleus accumbens or amygdala is exacerbated by the 5-HT_3 receptor agonist, 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) and inhibited by the 5-HT_3 receptor antagonist, ondansetron (Costall *et al.*, 1987). Discrete activation of midbrain dopamine cells also causes an increase in activity and release of forebrain dopamine which is antagonized by ondansetron or tropisetron (Hagan *et al.*, 1987; 1990). Such evidence indicates that activation of 5-HT_3 receptors will enhance dopamine function; limbic dopamine release is indeed reported to be increased following activation of 5-HT_3 receptors (Jiang *et al.*, 1990; Chan *et al.*, 1991). The ability of 5-HT_3 receptors to influence dopamine release suggests that 5-HT_3 receptor antagonists may be useful in the treatment of some aspects of drug abuse in man.

Recent evidence has shown that ondansetron, tropisetron and MDL 72222 are able to prevent the induction of place preference to nicotine or morphine (Carboni *et al.*, 1988; Koob & Goeders, 1989; Higgins *et al.*, 1991). Furthermore, ondansetron has been shown to reduce alcohol consumption in the rat and marmoset (Oakley *et al.*, 1988; Sellers *et al.*, 1988) and to prevent the exacerbation in behavioural response to an aversive situation following withdrawal from alcohol, cocaine, nicotine or diazepam (Costall *et al.*, 1990). The aim of the present study was to investigate the influence of ondansetron and tropisetron on morphine intake in naive and morphine-dependent rats.

Methods

General

Male Sprague-Dawley rats (weighing 140–160 g) were used. The animals were kept in groups of 6 per cage in a rat battery. Room temperature was maintained at 22 ± 1°C and relative humidity at 60–70%. The rats were exposed to a daily cycle of 12 h light and 12 h darkness, with lights on at 06 h 00 min to 18 h 00 min. They were allowed free access to a standard balanced laboratory diet of rat pellets (Ralston Purina Co., U.S.A.) and drinking water containing either 5% sucrose (Taikoo, Hong Kong) (w/v) or morphine sulphate (Macfarlan Smith Ltd., U.K.) prepared in a 5% sucrose solution. The drinking solutions were freshly prepared daily. Morphine was given in increasing concentrations of 0.1, 0.2, 0.3 and, finally 0.4 $mg\,ml^{-1}$ at 48 h intervals. The final concentration of morphine was maintained until the end of the 3 week induction period. All animals were permitted to drink *ad libitum*.

Experimental design

Rats were randomly divided into a control sucrose-treated group and a morphine-treated group which received the treatments for 21 days. The sucrose or morphine solutions were then withdrawn for 7 days, the drinking solutions being

[1] Author for correspondence.

replaced by tap water. For the following 10 days, both groups of rats were allowed a free choice between drinking morphine or sucrose solutions. During the entire experimental period (38 days), the body weight of each animal and the volume of the drinking fluid consumed were recorded daily. Although the total volume measured daily was for 6 rats in the cage, the actual amount drunk by each animal was calculated in proportion to its daily body weight. The daily volume consumed by each rat was, therefore, calculated on an individual basis and not derived by dividing the total consumed volume by the 6 animals. In order to ensure that tolerance and dependence had developed in these animals given 21 day morphine treatment, the naloxone-precipitated withdrawal syndrome (wet-dog shakes, head shakes, diarrhoea, ptosis, chattering teeth, writhing, chewing, paw tremor and irritability to touch and handling) and responses to the tail-flick test were evaluated as described previously (Dai *et al.*, 1984); both assessments were carried throughout the 21 day induction period. These tests were also used to demonstrate that morphine-treated rats after withdrawal for 7 days were detoxified and did not respond to naloxone; the naloxone-precipitated syndrome was absent and tail-flick times were normal in these animals. A similar regimen of tests for addiction was repeated in other groups of sucrose-treated or morphine-treated rats given ondansetron (Glaxo) (0.1 or 1 $\mu g\,kg^{-1}$, i.p. twice daily), tropisetron (Sandoz) (0.1 or 1 $\mu g\,kg^{-1}$, i.p. twice daily) or cyproheptadine (Sigma) (100 or 250 $\mu g\,kg^{-1}$, i.p. twice daily) 2 weeks after sucrose or opiate treatment. A higher dose of ondansetron (1 $\mu g\,kg^{-1}$, i.p. twice daily) was also given to other groups of animals 7 days before sucrose or opiate treatment. All injections were made twice daily at 09 h 00 min and 16 h 00 min and were continued until the end of the experiment (day 38).

Drugs

All drugs were freshly prepared before use. Morphine sulphate, expressed as its salt, was dissolved in 5% sucrose solution. Ondansetron, tropisetron or cyproheptadine hydrochloride, expressed as the base, was dissolved in a solution of 0.9% NaCl.

Statistical analysis

Values are given as means ± s.e.means. Where relevant, statistical analyses were carried out using Student's unpaired *t* test. Differences were considered significant at the 0.05 level.

Results

General observations

Throughout the 3 week induction period, the daily intake of morphine solution was similar to that of sucrose (Figure 1). The body weights of the rats in both groups increased steadily during the first 13 days, with no significant differences between the two treatments. Measurements taken on days 17, 19 and 21 indicated that the body weights of the morphine-treated rats were slightly but significantly less than those of the sucrose-treated groups (Figure 1). The morphine-treated rats exhibited naloxone-precipitated withdrawal changes (wet-dog shakes, head shakes, diarrhoea, ptosis, chattering teeth, writhing, chewing, paw tremor and irritability to touch and handling) and prolonged reaction times in the tail-flick test starting from the 7th day of opiate treatment and persisting up to day 21 (data not shown). When the drinking solution was replaced by tap water, these morphine-dependent animals no longer responded to the naloxone and tail-flick tests by the 7th day of opiate withdrawal (data not shown).

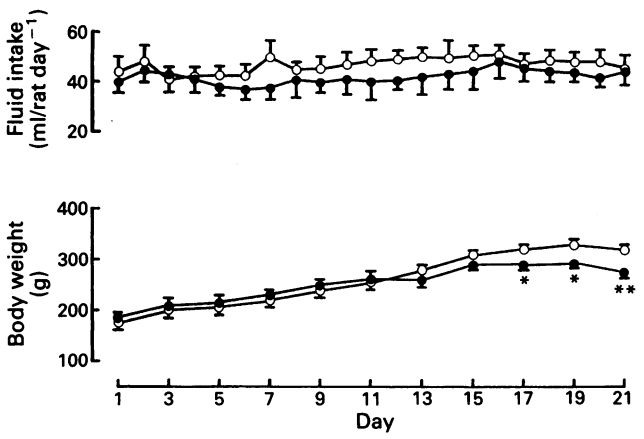


Figure 1 Daily fluid consumption and body weights of rats receiving sucrose (○) or morphine (●) (0.1 mg^{-1} days 1 and 2, 0.2 mg ml^{-1} days 3 and 4, 0.3 mg ml^{-1} days 5 and 6, 0.4 mg ml^{-1} days 7 to 21) for 21 days. Each value is the mean ± s.e.mean of 24 animals. $*P<0.05$; $**P<0.001$, compared to sucrose-treated controls.

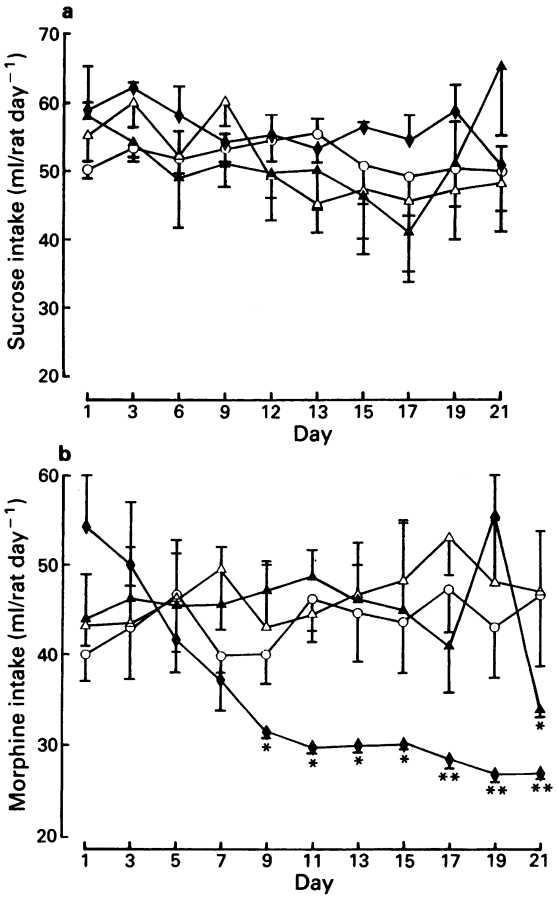


Figure 2 (a) Sucrose intake during the 21 day treatment period in rats treated with sucrose only (○); sucrose + ondansetron 0.1 $\mu g\,kg^{-1}$, i.p. twice daily (△) and sucrose + ondansetron 1 $\mu g\,kg^{-1}$, i.p. twice daily (▲) when given from the 14th day of sucrose administration, or with sucrose + ondansetron 1 $\mu g\,kg^{-1}$, i.p. twice daily given 7 days prior to sucrose treatment (◆). Each value is the mean of 24 rats. (b) Morphine intake during the 21 day treatment period in rats treated with morphine only (○); morphine + ondansetron 0.1 $\mu g\,kg^{-1}$, i.p., twice daily (△) and morphine + ondansetron 1 $\mu g\,kg^{-1}$, i.p., twice daily (▲) when given from the 14th day of morphine administration, or with morphine + ondansetron 1 $\mu g\,kg^{-1}$, i.p. twice daily given 7 days prior to morphine treatment (◆). Each value is the mean ± s.e.mean of 24–36 rats. $*P<0.05$; $**P<0.02$, compared to morphine-treated controls.

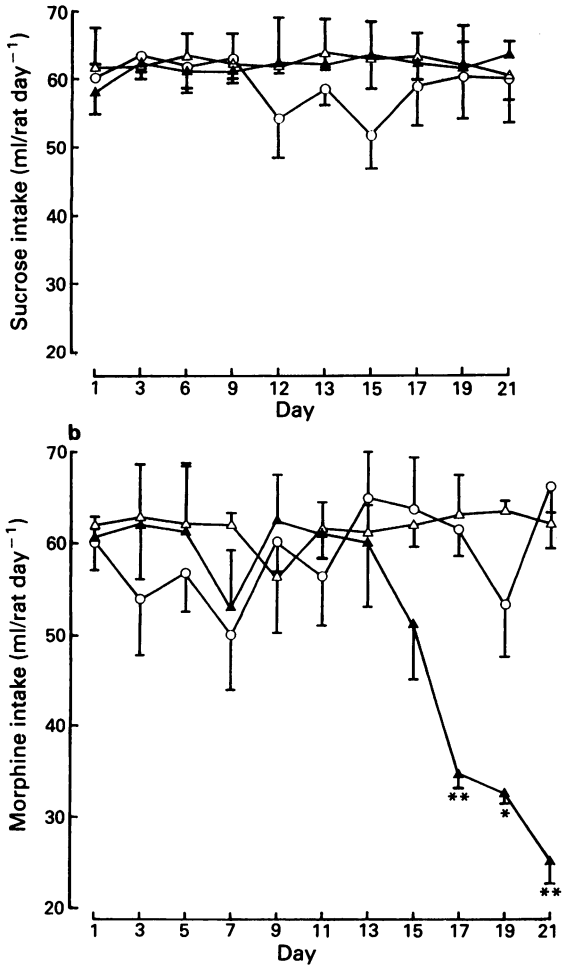


Figure 3 (a) Sucrose intake during the 21 day treatment period in rats treated with sucrose only (○); sucrose + tropisetron 0.1 μg kg^{-1}, i.p. twice daily (△) and sucrose + tropisetron 1 μg kg^{-1}, i.p. twice daily (▲), given from the 14th day of sucrose administration. (b) Morphine intake during the 21 day treatment period in rats treated with morphine only (○); morphine + tropisetron 0.1 μg kg^{-1}, i.p., twice daily (△) and morphine + tropisetron 1 μg kg^{-1}, i.p., twice daily (▲), given from the 14th day of morphine administration. Each value is the mean ± s.e.mean of 24 rats. $*P<0.05$; $**P<0.001$, compared to morphine-treated controls.

Effects of ondansetron or tropisetron on sucrose or morphine intake in rats during 21 day sucrose- or morphine-treatment

Ondansetron (0.1 or 1 μg kg^{-1}, i.p.) given twice daily, commencing either 7 days prior to or 14 days after starting sucrose treatment did not have any significant effect on sucrose intake (Figure 2a). Similarly, ondansetron (0.1 or 1 μg kg^{-1}), starting on day 14 of morphine treatment, did not affect opiate consumption in morphine-treated rats during days 14 to 20. However, at day 21, the higher dose of ondansetron (1 μg kg^{-1}) significantly reduced morphine intake (Figure 2b). When ondansetron treatment was started 7 days before the morphine regimen, there was a significant reduction in morphine consumption from the 9th day which persisted until the 21st day of the opiate-treatment period (Figure 2b).

The administration of tropisetron (0.1 or 1 μg kg^{-1}, i.p. twice daily) starting at day 14 of sucrose treatment did not change the volume of sucrose intake (Figure 3a). However, the administration of the higher dose of tropisetron (1 μg kg^{-1}), starting on the 14th day of morphine treatment, significantly reduced morphine intake from days 17 to 21 of

Table 1 Morphine consumption (ml/rat day^{-1}) in previously sucrose- or morphine-treated rats during the 10 day drinking preference period

	Day of drinking preference									
	1	2	3	4	5	6	7	8	9	
Previously sucrose-treated	3.6 ± 1.5	4.5 ± 0.7	5.3 ± 0.9	7.8 ± 3.1	7.3 ± 3.4	5.2 ± 1.1	4.8 ± 0.4	5.3 ± 1.3	4.1 ± 0.5	
Previously morphine-treated	23.8 ± 0.4*	26.1 ± 2.3**	27.4 ± 1.7**	27.7 ± 2.0**	31.7 ± 2.2**	28.1 ± 1.9**	29.3 ± 1.7**	32.0 ± 2.0**	28.2 ± 2.2**	

Each value is the mean ± s.e.mean of 24 rats. $*P<0.01$; $**P<0.001$, compared to the corresponding previously sucrose-treated controls.

Table 2 Effect of ondansetron, tropisetron or cyproheptadine on sucrose or morphine consumption in rats observed for 10 days after 7 day withdrawal from sucrose or opiate treatment

Treatment	*Morphine intake* (ml/rat day^{-1})	*Sucrose intake* (ml/rat day^{-1})
Previously sucrose-treated rats		
Saline 2 ml kg^{-1}, i.p. b.d.	5.6 ± 0.7	30.1 ± 0.2
Ondansetron given from 14th day of treatment		
0.1 μg kg^{-1}, i.p. b.d.	3.1 ± 0.7*	28.7 ± 2.1
1.0 μg kg^{-1}, i.p. b.d.	3.3 ± 0.3*	30.6 ± 0.5
Ondansetron given 7 days prior to treatment		
1.0 μg kg^{-1}, i.p. b.d.	3.0 ± 0.5*	31.2 ± 0.2
Tropisetron given from 14th day of treatment		
0.1 μg kg^{-1}, i.p. b.d.	4.7 ± 0.8	32.3 ± 3.4
1.0 μg kg^{-1}, i.p. b.d.	1.2 ± 0.2**	30.7 ± 0.2
Cyproheptadine given from 14th day of treatment		
100 μg kg^{-1}, i.p. b.d.	7.1 ± 2.4	31.0 ± 1.2
250 μg kg^{-1}, i.p. b.d.	7.0 ± 2.9	29.0 ± 2.5
Previously morphine-treated rats		
Saline 2 ml kg^{-1}, i.p. b.d.	30.8 ± 1.0†	25.1 ± 3.1
Ondansetron given from 14th day of treatment		
0.1 μg kg^{-1}, i.p. b.d.	21.2 ± 2.4*	29.2 ± 1.0
1.0 μg kg^{-1}, i.p. b.d.	17.4 ± 4.3**	28.4 ± 1.4
Ondansetron given 7 days prior to treatment		
1.0 μg kg^{-1}, i.p. b.d.	17.0 ± 4.3**	25.4 ± 2.6
Tropisetron given from 14th day of treatment		
1.0 μg kg^{-1}, i.p. b.d.	20.5 ± 3.3**	26.8 ± 3.0
1.0 μg kg^{-1}, i.p. b.d.	20.4 ± 3.0	25.3 ± 4.3
Cyproheptadine given from 14th day of treatment		
100 μg kg^{-1}, i.p. b.d.	33.5 ± 1.9	31.4 ± 3.4
250 μg kg^{-1}, i.p. b.d.	29.1 ± 1.2	32.1 ± 1.1

Each value is the mean ± s.e.mean derived from the total 10 day consumption of 24 rats in each group. *$P<0.05$; **$P<0.02$, compared to their own sucrose- or morphine-treated controls. †$P<0.001$, compared to previously sucrose-treated saline controls.

treatment. The lower dose of tropisetron (0.1 μg kg^{-1}) was without effect on morphine consumption (Figure 3b).

Effect of ondansetron or tropisetron on sucrose or morphine intake in rats previously treated for 21 days with sucrose or morphine

Table 1 shows the morphine intake in rats which had previously received 21 day treatment with morphine or sucrose and had been withdrawn for 7 days; they were then allowed access for 10 days to a choice between sucrose or morphine. Throughout the 10 day drinking preference period, rats that were previously morphine-dependent showed a clear preference for morphine intake which was significant from the 1st day until the last day of the free choice period (Table 1). The volume of opiate solution intake in the previously morphine-dependent rats observed for 10 days following opiate withdrawal was approximately five fold higher than that of their previously sucrose-treated controls (Table 2). In contrast, the volume of sucrose solution intake was similar in both groups. This marked increase in morphine intake was significantly reduced in the groups of rats that had received ondansetron (0.1 or 1 μg kg^{-1}, i.p., twice daily) or tropisetron (0.1 or 1 μg kg^{-1}, i.p., twice daily) from day 14 of the morphine regimen and in the group which had received ondansetron 7 days before and during the 21 day period of morphine administration (Table 2).

In rats that had previously received sucrose, the intake of morphine was reduced in those animals that had received concomitant ondansetron (0.1 or 1 μg kg^{-1} from day 14 of treatment or 7 days before and during treatment) or tropisetron (1 μg kg^{-1} from day 14 of treatment) (Table 2). The sucrose solution intake during the 10 day period was similar in the previously sucrose- or morphine-treated groups given ondansetron or tropisetron.

Effect of cyproheptadine on sucrose or morphine intake in rats previously treated for 21 days with sucrose or morphine

During the 21 day treatment with sucrose or morphine, the administration of cyproheptadine (100 or 250 μg kg^{-1}, i.p., twice daily) given from day 14 of treatment, did not affect the volume of sucrose or opiate solution intake in either group of animals. Sucrose intake during the 21 day sucrose treatment period before and after cyproheptadine treatment was 54.2 ± 5.9–63.8 ± 3.3 and 54.0 ± 6.3–60.2 ± 5.7 ml/rat day^{-1}, respectively. Morphine intake during the 21 day morphine treatment period before and after cyproheptadine treatment was found to be 43.3 ± 5.6–50.9 ± 8.7 and 41.2 ± 5.3–55.2 ± 6.9 ml/rat day^{-1}, respectively.

During the 10 day drinking preference period after 21 day treatment with sucrose or morphine, the administration of cyproheptadine (100 or 250 μg kg^{-1}, i.p. twice daily) failed to modify either sucrose or morphine intake when compared to control values (Table 2).

Discussion

Previous studies in our laboratory have shown that a gradual increase in the concentration of morphine in sucrose drinking water can produce physical dependence and a relapse after successful opiate withdrawal and detoxification (Dai *et al.*, 1984). Preliminary data have also indicated that morphine-dependence, as manifested by the naloxone-precipitated withdrawal signs and morphine tolerance as well as by the reaction time in response to pain using the tail-immersion test, starts to develop at the 7th day after morphine treatment. These effects are found to be fully established from the 14th day of opiate treatment and persist until the end of the 21 day treatment period (data not shown in this paper). In order to

study the effect of 5-HT$_3$ antagonists on morphine consumption in morphine-dependent rats, administration of 5-HT$_3$ antagonists was, therefore, started on day 14 of morphine treatment when the animals had already been shown to have developed dependence and tolerance to the opiate. The purpose of using the other dosage regimen in which the administration of 5-HT$_3$ antagonists was started 7 days before morphine treatment, was to investigate whether 5-HT$_3$ antagonists would affect morphine consumption in rats before they received opiate treatment and became opiate-dependent. Naloxone treatment had no effect on the volume of sucrose drunk daily by the sucrose-drinking controls given ondansetron or tropisetron (Figures 2a, 3a), nor did it influence the volume consumed daily by morphine-addicted rats which were subsequently not given the 5-HT$_3$ receptor blockers (Figures 2b, 3b). Bradley *et al.* (1986) have illustrated the pharmacological characteristics of the 5-HT$_3$ receptor site and have indicated that one of the criteria is that the 5-HT$_3$ receptor should be blocked selectively by low concentrations of MDL 72222, ICS 205-930, granisetron or ondansetron. Hence, in the present study, two relatively low doses (0.1 and 1 $\mu g\,kg^{-1}$) of ondansetron and tropisetron were selected in order to verify whether the effect of these 5-HT$_3$ antagonists on morphine consumption could be mediated via the 5-HT$_3$ receptors. Indeed, the two doses of ondansetron and tropisetron used in this study have been demonstrated by other researchers to be effective in inhibiting the increased behavioural suppression following withdrawal from treatment with diazepam and other drugs of abuse (Costall *et al.*, 1989; 1990), and in reducing raised mesolimbic dopaminergic activity in the rat and marmoset (Costall *et al.*, 1987). Using such a dosage regimen in the present study, a dependency to morphine was established over a 21 day period, and 7 days after successful opiate withdrawal these detoxified rats showed a clear preference for morphine intake in a free choice situation. The total fluid intake in the detoxified rats was about 57% greater than their controls; the main reason for this difference was the increased intake of morphine solution, with only a small decrease in sucrose solution consumption. There is no clear explanation for this at present. It is possible that animals showing a relapse, under the experimental conditions studied, may tend to drink more morphine solution without a commensurate decrease in the volume of the non-drug solution which they also consume. Further detailed studies are needed. The major finding of the study was that ondansetron or tropisetron when administered prior to and during morphine intake significantly reduced the amount of morphine consumed. The effects of such treatments using ondansetron or tropisetron were also evident after discontinuing the drug regimens. Thus, in a free choice situation the animals that had previously received ondansetron or tropisetron showed a reduced intake of morphine when compared to those that had previously received morphine alone. The common pharmacological action of ondansetron and tropisetron is 5-HT$_3$ receptor blockade (Richardson *et al.*, 1985; Butler *et al.*, 1987). Since low doses of both compounds were effective in antagonizing morphine intake, their effects can reasonably be attributed to 5-HT$_3$ receptor antagonism.

Evidence which indicates an influence of morphine-like drugs and a role for opioid peptides in positive reinforcement effects comes from the phenomenon of opiate self-administration as exemplified by the present data, opiate-induced place preference and facilitation of operant self-stimulation behaviour (see reviews by Carr *et al.*, 1989; Bradshaw & Szabadi, 1989; Koob & Goeders, 1989). Whereas no single neural mechanism has been found to be responsible for the control of behaviour by positive reinforcers, evidence of limbic dopamine involvement comes from the reinforcement effects of psychostimulants and their antagonism by neuroleptic agents as well as lesions of the dopamine pathways (see Carr *et al.*, 1989; Bradshaw & Szabadi, 1989). Dopamine may also play a role in mediating the reinforcing effects of morphine because conditioned place preference is inhibited by neuroleptics, intraventricular 6-hydroxydopamine lesions and catecholamine synthesis inhibition (Schwartz & Marchok, 1974; Smith *et al.*, 1985; Di Chiara & Imperato, 1988; see also Carr *et al.*, 1989). However, there are other studies in which neuroleptic treatment or dopamine neurone destruction has no effect on morphine self-administration (Ettenberg *et al.*, 1982; Pettit *et al.*, 1984). In all such experiments the treatment, in order to be successful, must overcome both rewarding and conditioning effects.

It is unlikely that the rewarding and conditioning effects are mediated via a single brain system. Thus, morphine and other opiate peptides cause self-administration/conditioned place preference following injection into the hypothalamus (Stein & Olds, 1977), the nucleus accumbens (Olds, 1982; Goeders *et al.*, 1984; 1985) and ventral tegmental area (Bozarth, 1983). Kainic acid lesions of the nucleus accumbens of intra-accumbens injection of the opiate receptor antagonist methyl naloxinium inhibits opiate self-administration (Zito *et al.*, 1985; Vaccarino *et al.*, 1985) to indicate the importance of this limbic area. Within the nucleus accumbens, drugs abused by man, including morphine, are known to increase dopamine concentrations in freely moving rats (Di Chiara & Imperato, 1988) and to decrease dopamine release following drug withdrawal (Rossetti *et al.*, 1992). Thus, a raised limbic dopamine function is likely to be an important factor in reinforcement effects, but other systems may play a major or modulatory role. The intraventricular or intra-accumbens injection of neurotoxins selective for the 5-HT neurone, attenuate morphine-conditioned place preference (Schwartz & Marchok, 1974; Nomikos *et al.*, 1986), electrolesions of the raphe nuclei shift the dose-effect function for morphine to the right (Glick & Cox, 1977) and lesioning of the 5-HT projection to the nucleus accumbens increases self-administration of morphine in dependent rats (Smith *et al.*, 1987).

From the above perspectives, it can be hypothesized that the ability of ondansetron and tropisetron to reduce morphine intake involves 5-HT$_3$ receptor antagonism and, therefore, an inhibition of 5-HT function causing the known reduction in limbic dopamine activity (Costall *et al.*, 1987; Hagan *et al.*, 1990). This is supported by the ability of tropisetron to reduce dose-dependently dopamine release in the nucleus accumbens induced by morphine, nicotine or ethanol (Carboni *et al.*, 1989). The ability of the 5-HT$_3$ receptor antagonists to prevent the exacerbation in aversive responses following withdrawal from treatment with drugs of abuse may contribute to the effects. Therefore, the reported ability of ondansetron to reduce the intake of alcohol (Oakley *et al.*, 1988; Sellers *et al.*, 1988) has been extended to the control of morphine intake, tropisetron being shown to have a similar effect. However, the site(s) of action of the 5-HT$_3$ receptor antagonists that reduce morphine self-administration remains to be investigated.

The authors wish to thank Glaxo Group Research Ltd. and Sandoz Pharmaceutical Ltd. for their generous gifts of ondansetron and tropisetron (ICS 205-930), respectively. The excellent technical help of Mr W. Leung is gratefully acknowledged.

References

BOZARTH, M.A. (1983). Opiate reward mechanisms mapped by intracranial self-administration. In *The Neurobiology of Opiate Reward Processes*. ed. Smith, J.E. & Lane, J.D. pp. 331–359. Amsterdam: Elsevier Press.

BOZARTH, M.A. & WISE, R.A. (1981). Heroin reward is dependent on a dopaminergic substrate. *Life Sci.*, **29**, 1881–1886.

BRADLEY, P.B., ENGEL, G., FENIUK, W., FOZARD, J.R., HUMPHREY, P.P.A., MIDDLEMISS, D.N., MYELCHARANE, E.J., RICHARDSON, B.P. & SAXENA, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology*, **25**, 563–576.

BRADSHAW, C.M. & SZABADI, E. (1989). Central neurotransmitter systems and the control of operant behaviour by natural positive reinforcers. In *The Neuropharmacological Basis of Reward*. ed. Liebman, J.M. & Cooper, S.J. pp. 320–376. Oxford: Science Publications.

BUTLER, A., HILL, J.M., IRELAND, S.J., JORDAN, C.C. & TYERS, M.B. (1988). Pharmacological properties of GR 38032F, a novel antagonist at 5-HT_3 receptors. *Br. J. Pharmacol.*, **94**, 397–412.

CARBONI, E., ACQUAS, E., LEONE, P., PEREZZANI, L. & DI CHAIRA, G. (1988). 5-HT_3 receptor antagonists block morphine- and nicotine-induced place preference conditioning. *Eur. J. Pharmacol.*, **151**, 159–160.

CARBONI, E., ACQUAS, E., FRAU, R. & DI CHIARA, G. (1989). Differential inhibitory effects of 5-HT_3 antagonist on drug-induced stimulation of dopamine release. *Eur. J. Pharmacol.*, **164**, 515–519.

CARR, G.D., FIBIGER, H.C. & PHILLIPS, A.G. (1989). Conditioned place preference as a measure of drug reward. In *The Neuropharmacological Basis of Reward*. ed. Liebman, J.M. & Cooper, S.J. pp. 264–319. Oxford: Science Publications.

CHAN, J., VAN PRAAG, H.M. & GARDNER, E.L. (1991). Activation of 5-HT_3 receptor by 1-phenylbignamide increases dopamine release in the rat nucleus accumbens. *Brain Res.*, **543**, 354–357.

COSTALL, B., DOMENERY, A.M., KELLY, M.E., NAYLOR, R.J. & TYERS, M.B. (1987). Effects of the 5-HT_3 receptor antagonist, GR 38032F, on raised dopaminergic activity in the mesolimbic system of the rat and marmoset. *Br. J. Pharmacol.*, **92**, 881–894.

COSTALL, B., JONES, B.J., KELLY, M.E., NAYLOR, R.J., OAKLEY, N.R., ONAIVI, E.S. & TYERS, M.B. (1989). The effects of ondansetron (GR 38032F) in rats and mice treated subchronically with diazepam. *Pharmacol. Biochem. Behav.*, **34**, 769–778.

COSTALL, B., JONES, B.J., KELLY, M.E., NAYLOR, R.J., ONAIVI, E.S. & TYERS, M.B. (1990). Ondansetron inhibits a behavioural consequence of withdrawing from drugs of abuse. *Pharmacol. Biochem. Behav.*, **36**, 339–344.

DAI, S., HUI, S.-C.G. & OGLE, C.W. (1984). Morphine preference in rats previously morphine-dependent. *Pharmacol. Res. Commun.*, **16**, 495–512.

DI CHIARA, G. & IMPERATO, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 5274–5278

ETTENBERG, A., PETTIT, H.O., BLOOM, F.E. & KOOB, G.F. (1982). Heroin and cocaine intravenous self-administration in rats: mediation by separate neuronal systems. *Psychopharmacology*, **78**, 204–209.

GLICK, S.D. & COX, R.D. (1977). Changes in morphine self-administration after brainstem lesions in rats. *Pyschopharmacology*, **52**, 151–156.

GOEDERS, N.E., LANE, J.D. & SMITH, J.E. (1984). Intracranial self-administration of methionine enkephalin into the nucleus accumbens. *Pharmacol. Biochem. Behav.*, **20**, 451–455.

GOEDERS, N.E., GUERIN, G.F., DWORKIN, S.I. & SMITH, J.E. (1985). Reinforcing stimulus properties of endocoids. In *Endocoids*. ed. Lal, H., Labella, F. & Lane, J. pp. 63–69, New York: Alan R. Liss.

HAGAN, R.M., BUTLER, J.M., HILL, J.C., JORDAN, C.C., IRELAND, S.J. & TYERS, M.B. (1987). Effect of the 5-HT_3 receptor antagonist, GR 38032F, on responses to injection of a neurokinin agonist into the ventral tegmental area of the rat brain. *Eur. J. Pharmacol.*, **138**, 303–305.

HAGAN, R.M., JONES, A.B.J., JORDAN, C.C. & TYERS, M.B. (1990). 5-HT_3 receptor antagonist on responses to selective activation of mesolimbic dopaminergic pathways in the rat. *Br. J. Pharmacol.*, **99**, 227–232.

HIGGINS, G.A., JOHARCHI, N., NGUYEN, P. & SELLERS, E.M. (1991). Effects of 5-HT_3 receptor antagonists MDL 72222 and ondansetron on morphine place conditioning. *Psychopharmacology*, **106**, 315–320.

JIANG, L.H., ASHBY, C.R., KASSER, R.J. & WANG, R.Y. (1990). The effect of intraventricular administration of the 5-HT_3 receptor agonist 2-methyl serotonin on the release of dopamine in the nucleus accumbens: an in vivo chronoconlometric study. *Brain Res.*, **513**, 156–159.

KOOB, G.F. & GOEDERS, N.E. (1989). Neuroanatomical substance of drug self-administration. In *Neuropharmacological Basis of Reward*. ed. Liebman, J.M. & Cooper, S.J. pp. 214–263. Oxford: Science Publications.

NOMIKOS, G., SPYRAKI, C., GALANOPOULON, P. & PAPADOPULU, Z. (1986). Amphetamine and morphine induced place preference in rats with 5,7 dihydroxytryptamine lesions of the nucleus accumens. *Psychopharmacology*, **89**, S26.

OAKLEY, N.R., JONES, B.J., TYERS, M.B., COSTALL, B. & DOMENEY, A.M. (1988). The effect of GR 38032F on alcohol consumption in the marmoset. *Br. J. Pharmacol.*, **95**, 870p.

OLDS, M.E. (1982). Reinforcing effects of morphine in the nucleus accumbens. *Brain Res.*, **237**, 429–440.

PETTIT, H.O., ETTENBERG, A., BLOOM, F.E. & KOOB, G.F. (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. *Psychopharmacology*, **84**, 167–173.

RICHARDSON, B.P., ENGEL, G., DONATSCH, P. & STADLER, O.A. (1985). Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. *Nature*, **316**, 126–131.

ROSETTI, Z.L., HONAIDAN, Y. & GESSA, G.L. (1992). Marked inhibition of mesolimbic dopamine release: a common feature of ethanol, morphine, cocaine and amphetamine abstinence in rats. *Eur. J. Pharmacol.*, **221**, 227–234.

SCHWARTZ, A.S. & MARCHOK, P.L. (1974). Depression of morphine seeking behaviour by dopamine inhibition. *Nature*, **248**, 257.

SELLERS, E.M., KAPLAN, H.L., LOWRIN, M.O., SOMER, G., NARANJO, C.A. & FRECKER, R.C. (1988). The 5-HT_3 receptor antagonist GR38032F decreases alcohol consumption in rats. *Proc. Soc. Neurosci.*, 18th Ann. Meeting, Toronto, Abs. 21.4.

SMITH, J.E., GUERIN, C.F., CO, C., BARR, T.S. & LANE, J.D. (1985). Effects of 6-OHDA lesions of the central medial nucleus accumbens on rat intravenous morphine self-administration. *Pharmacol. Biochem. Behav.*, **23**, 843–849.

SMITH, J.E., SHULTZ, K., CO, C., GOEDERS, N.E. & DWORKIN, S.I. (1987). Effects of 5,7-dihydroxytryptamine lesions of the nucleus accumbens on rat intravenous morphine self-administration. *Pharmacol. Biochem. Behav.*, **26**, 607–612.

SPYRAKI, C., FIBIGER, H.C. & PHILLIPS, A.G. (1983). Attenuation of heroin reward in rats by disruption of the mesolimbic dopamine system. *Psychopharmacology*, **79**, 278–283.

STEIN, E.A. & OLDS, J. (1977). Direct intracerebral self-administration of opiates in the rat. *Neurosci. Abs.*, **3**, 302.

VACCARINO, F.J., BLOOM, F.E. & KOOB, G.F. (1985). Blockade of nucleus accumbens opiate receptors attenuates intravenous heroin reward in the rat. *Psychopharmacology*, **85**, 37–42.

ZITO, K.A., VICKERS, G. & ROBERTS, D.C.S. (1985). Disruption of cocaine and heroin self-administration following kainic acid lesions of the nucleus accumbens. *Pharmacol. Biochem. Behav.*, **23**, 1029–1036.

(Received January 18, 1993
Revised July 22, 1993
Accepted August 2, 1993)

Br. J. Pharmacol. (1993), **110**, 1347–1352 © Macmillan Press Ltd, 1993

Modulation by nitric oxide of platelet-activating factor-induced albumin extravasation in the conscious rat

[1]János G. Filep & Éva Földes-Filep

Research Center, Maisonneuve-Rosemont Hospital, Department of Medicine, University of Montréal, Montréal, Québec, Canada H1T 2M4

1 The objective of this study was to assess whether or not endogenous nitric oxide (NO) could mediate the hypotensive response to platelet-activating factor (PAF) and modulate PAF-induced microvascular albumin leakage in the conscious rat.

2 PAF (0.19 and 1.9 nmol kg^{-1}, i.v.) evoked dose-dependent hypotension and significantly enhanced albumin extravasation in the large airways, pancreas, stomach and duodenum 15 min after its administration. Inhibition of NO synthesis by N^G-nitro-L-arginine methyl ester (L-NAME, 0.125–2 mg kg^{-1}, i.v.) produced marked dose-dependent increases in albumin accumulation (up to 290%) in large airways, liver, spleen, pancreas, kidney, stomach and duodenum as measured by the extravasation of Evans blue dye. L-NAME (2 mg kg^{-1}) treatment markedly potentiated PAF (1.9 nmol kg^{-1})-induced albumin extravasation in these tissues, whereas it did not modify the hypotensive response to PAF.

3 Maintenance of mean arterial blood pressure at the level observed following 2 mg kg^{-1} L-NAME by infusion of noradrenaline (620–790 ng kg^{-1} min^{-1}) neither affected significantly albumin extravasation nor potentiated the permeability effect of PAF in the vascular beds studied with the exception of large airways, where noradrenaline mimicked the effects of L-NAME.

4 These results indicate that inhibition of endogenous NO formation leads to an increase in albumin extravasation and to potentiation of the vascular permeability effect of PAF, whereas the hypotensive action of PAF seems to be independent of NO formation in the conscious rat. These data suggest an important role for NO in the regulation of albumin extravasation.

Keywords: Nitric oxide, N^G-nitro-L-arginine methyl ester; N^G-monomethyl-L-arginine; albumin extravasation; hypotension; PAF; airways; gastrointestinal tract; kidney

Introduction

The non-thrombogenic and high solute and water permeability properties of the vascular endothelium have long been recognized as essential components of normal microvascular integrity. Endothelial cells are also an active site for the production of a large number of potent vasoactive substances essential in the regulation of vascular tone and reactivity. One of these substances, endothelium-derived relaxing factor (EDRF) has been identified as nitric oxide (NO) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987) or a closely related nitroso compound (Myers *et al.*, 1989). NO acts as an endogenous nitrovasodilator via activation of vascular smooth muscle cytosolic guanylate cyclase (Murad, 1986; Furchgott & Vanhoutte, 1989). NO may also modulate the interactions between inflammatory cells and vascular endothelium. This notion is based on the fact that analogues of L-arginine, including N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME), which inhibit NO synthesis (Rees *et al.*, 1990) also promote platelet aggregation (Radomski *et al.*, 1987a) as well as adhesion of platelets and neutrophil granulocytes to vascular endothelium (Radomski *et al.*, 1987b; Kubes *et al.*, 1991). These latter events would lead to endothelial dysfunction (Lefer *et al.*, 1991).

Platelet-activating factor (PAF) has been implicated as an important regulator of the cardiovascular system in various pathological conditions, including ischaemia-reperfusion (Filep *et al.*, 1989; Montrucchio *et al.*, 1989) and endotoxin shock (Doebber *et al.*, 1985; Chang *et al.*, 1987). Impaired vascular relaxation (Lefer *et al.*, 1991) and enhanced vascular permeability (cf. Filep *et al.*, 1991a) are characteristic features of these conditions. Since endothelial dysfunction associating ischaemia-reperfusion can be attenuated by organic NO donors (Siegfried *et al.*, 1992), it is conceivable that reduced release or action of NO may, in part, account for endothelial dysfunction (Lefer *et al.*, 1991). The purposes of the present experiments were to examine the role of endogenous NO in mediating the hypotensive action of PAF and to study whether or not inhibition of NO biosynthesis compromises PAF-induced increases in microvascular albumin extravasation in selected vascular beds of the rat.

Methods

The experiments were performed on conscious, chronically catheterized male Wistar rats weighing 230–290 g (Charles River Canada Inc., St. Constant, Québec, Canada). The animals were housed in individual metabolic cages and were prepared as described previously (Filep *et al.*, 1987). Briefly, under anaesthesia (ketamine, 75 mg kg^{-1} and sodium pentobarbitone, 15 mg kg^{-1}) catheters were implanted into the abdominal aorta and vena cava through the central tail artery and left femoral vein, respectively. The venous catheter was led subcutaneously to the root of the tail. The catheters emerging from the tail were protected by an acrylic cuff glued to the tail. The cuff was connected to a stainless steel spiral and was fed through the loop of the metabolic cage. The animals were allowed to recover completely for at least 4 days following the surgical procedures. On the day of the experiment, following an equilibration period of 2 h, basal cardiovascular parameters were measured for 20 min before drug administration. During the experiments the animals could move freely and had free access to food and water. Mean arterial blood pressure was monitored continuously with an electromanometer using a COBE CDX III pressure transducer (Digi-Med, Louisville, KY, U.S.A.). Protein extravasation was estimated by measuring tissue accumulation of Evans blue dye, which binds to plasma albumin (Rawson,

[1] Author for correspondence.

1943). Previous studies have demonstrated close correlation for the exudation of Evans blue dye and radioiodinated serum albumin (Udaka *et al.*, 1970; Rogers *et al.*, 1989). All procedures followed were in accordance with the recommendations of the Canadian Council of Animal Care.

Experimental protocols

Initial experiments showed that L-NMMA and L-NAME at a dose of 25 and 2 mg kg^{-1}, respectively, evoked near maximal (93–97%) increases in mean arterial blood pressure. Therefore, these doses of L-arginine analogues, unless otherwise stated, were used in subsequent experiments. In the second series of experiments, the ability of L-NMMA and L-NAME to inhibit acetylcholine-induced decrease in mean arterial blood pressure was examined. The hypotensive responses to graded doses of acetylcholine (1–8 nmol kg^{-1}, injected i.v. at 3 to 5 min intervals) were compared before and after treatment with L-arginine analogues. In some animals, mean arterial blood pressure was maintained at the level observed following L-arginine analogues by the continuous infusion of noradrenaline (620–790 ng kg^{-1} min^{-1}, 6.9 µl min^{-1}) for 10 min before generation of a dose-response curve. Each animal served as its own control. The effects of L-NAME on protein extravasation were studied in additional experiments. The animals were treated with 0.125, 0.5 or 2 mg kg^{-1} L-NAME or its vehicle (0.9% NaCl) for 10 min, then Evans blue dye (20 mg kg^{-1}, 25 mg ml^{-1} in 0.9% NaCl) was injected i.v. In the last series of experiments, Evans blue dye was injected together with PAF (0.9 or 1.9 nmol kg^{-1}) or its vehicle in the absence and presence of L-NAME (2 mg kg^{-1}, 10 min before injection of PAF). In order to compare directly the responses to PAF before and after NO blockade, the effects of PAF were studied in animals receiving noradrenaline infusion. Ten minutes after injection of PAF or its vehicle, the animals were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.v.) and were perfused with 40 ml 0.9% NaCl through a catheter inserted into the abdominal aorta. The perfusion was completed within 5 min (i.e. 15 min after injection of PAF). Portions of pulmonary tissues, liver, spleen, pancreas, kidney, stomach, duodenum and dorsal skin were excised and weighed. Tissue Evans blue dye content was measured by spectrophotometry following extraction with formamide as described previously (Filep *et al.*, 1991b). Evans blue content of each sample was expressed as µg per g dry tissue weight to avoid underestimation of changes due to plasma fluid extravasation.

Drugs

Platelet-activating factor (1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphocholine) was purchased from Bachem (Bubendorf, Switzerland), L-NMMA was from Research Biochemicals Inc. (Natick, MA, U.S.A.), noradrenaline hydrochloride, L-NAME and Evans blue dye were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All drugs were dissolved in 0.9% NaCl immediately before use.

Statistics

Results are expressed as means ± s.e.mean. Statistical evaluation of the data was performed by Dunn's multiple contrast hypothesis test (Dunn, 1964) when various treatments were compared to the same control or by Wilcoxon signed rank test and Mann-Whitney U test for paired and unpaired observations, respectively. A level of $P<0.05$ was considered significant for all tests.

Results

Effects of L-NAME and L-NMMA on the depressor response to PAF

Intravenous bolus administration of L-NMMA (25 mg kg^{-1}) or L-NAME (2 mg kg^{-1}) increased mean arterial blood pressure from 108 ± 3 to 157 ± 5 mmHg ($n=8$) and from 106 ± 3 to 158 ± 4 mmHg ($n=8$), respectively. The pressor effect of L-NMMA was a transient response, returning to baseline by 45–50 min. L-NAME produced a sustained pressor effect, mean arterial blood pressure remained elevated by 50 mmHg for up to 90 min. Acetylcholine evoked similar depressor responses in control and L-NMMA or L-NAME-treated animals (Figure 1) in spite of differences in the prein-

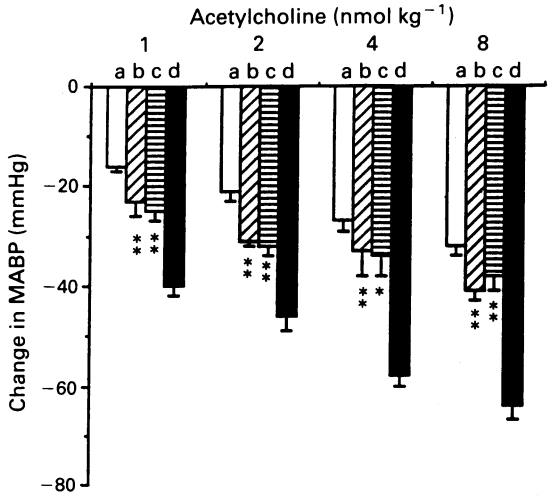


Figure 1 Hypotensive effects of acetylcholine in conscious rats before and after treatment with N^G-nitro-L-arginine methyl ester (L-NAME, 2 mg kg^{-1}), N^G-monomethyl-L-arginine (L-NMMA, 25 mg kg^{-1}) or noradrenaline (NA, 620–790 ng kg^{-1} min^{-1}). Mean arterial blood pressure was 107 ± 2 mmHg ($n=9$), 158 ± 4 mmHg ($n=8$), 151 ± 8 mmHg ($n=6$) and 156 ± 5 mmHg ($n=10$) before injection of acetylcholine in control (a), L-NAME (b), L-NMMA (c) and noradrenaline-treated (d) groups, respectively. Values are means ± s.e.mean. $^*P<0.05$; $^{**}P<0.01$ (compared to noradrenaline-treated group).

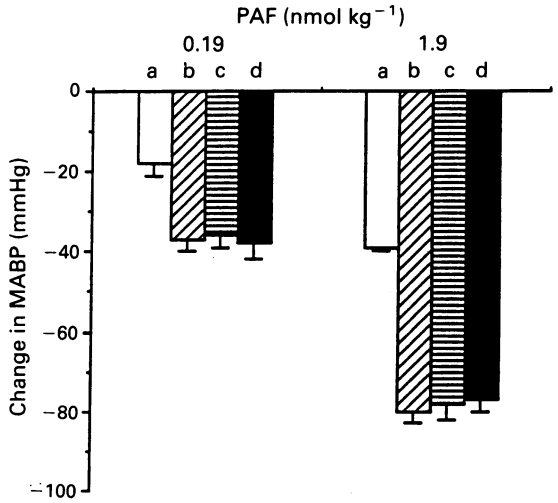


Figure 2 Hypotensive response to PAF in conscious rats following administration of 0.9% NaCl (control, a), N^G-nitro-L-arginine (L-NAME, 2 mg kg^{-1}, b), N^G-monomethyl-L-arginine (L-NMMA, 25 mg kg^{-1}, c) or noradrenaline (NA, 620–790 ng kg^{-1} min^{-1}, d). Mean arterial blood pressure was 108 ± 3 mmHg ($n=6$), 151 ± 4 mmHg ($n=6$), 150 ± 3 mmHg ($n=6$) and 151 ± 2 mmHg ($n=6$) before injection of PAF in control, L-NAME, L-NMMA and noradrenaline-treated animals, respectively. Values are means ± s.e. mean.

jection mean arterial blood pressure levels. To circumvent these differences, mean arterial blood pressure was elevated by infusion of noradrenaline to comparable levels (156 ± 5 mmHg, $n = 10$) as seen after L-arginine analogues. In this paradigm, the hypotensive effects of acetylcholine were significantly reduced by both L-NMMA and L-NAME (Figure 1). The inhibition ranged from 30 to 41%. In contrast, the maximum depressor responses to PAF were identical in L-NMMA and L-NAME-treated animals and during noradrenaline infusion (Figure 2).

Effects of L-NAME on protein extravasation

In agreement with previous studies, intravenous bolus injection of L-NAME (0.125–2 mg kg^{-1}) elicited a dose-dependent pressor effect (Table 1). This was accompanied by a dose-dependent increase in tissue Evans blue content in all vascular beds studied with the exception of the pulmonary parenchyma (Table 1). However, the sensitivity of various vascular beds to L-NAME treatment showed considerable variation. For example, significantly enhanced protein extravasation was detected in the stomach following L-NAME at a dose as low as 0.125 mg kg^{-1}, whereas a 16 fold higher dose was required to evoke significant increases in Evans blue content in the spleen (Table 1).

Effects of L-NAME on PAF-induced albumin extravasation

Injection of L-NAME (2 mg kg^{-1}) evoked increases of 120 to 190% in tissue Evans blue dye content in the bronchus, pancreas, kidney, stomach and duodenum. L-NAME increased protein extravasation by 74, 108 and 66% in the trachea, liver and spleen, respectively (Table 2). No significant changes in Evans blue content were detected in the pulmonary parenchyma and skin (Table 2). Maintenance of mean arterial blood pressure at the level observed following L-NAME administration by infusion of noradrenaline did not enhance albumin extravasation, except in the pulmonary circulation. Evans blue content of pulmonary tissues did not differ significantly following L-NAME and noradrenaline treatments (Table 2).

As expected, PAF produced a dose-dependent increase in albumin extravasation in most vascular beds studied (Table 2). When PAF (1.9 nmol kg^{-1}) was injected following L-NAME, protein extravasation was markedly enhanced in the large airways, stomach and duodenum (Table 2). A significant increase in Evans blue content was observed even in the pulmonary parenchyma, where neither PAF nor L-NAME by itself enhanced albumin extravasation (Table 2). In the pancreas, combined administration of L-NAME and PAF produced smaller, but still significant increases in tissue Evans

Table 1 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) on mean arterial blood pressure (MABP) and protein extravasation in conscious rats

	Con ($n = 5$)	*L-NAME* (mg kg^{-1}) 0.125 ($n = 4$)	0.5 ($n = 4$)	2 ($n = 5$)
Basal MABP (mmHg)	105 ± 2	104 ± 3	108 ± 3	105 ± 3
Maximum increase in MABP (mmHg)	1 ± 1	16 ± 1*	24 ± 4*	45 ± 3**
Protein extravasation (μg Evans blue dye per g dry tissue weight)				
Trachea	52 ± 2	66 ± 4	73 ± 4*	95 ± 8**
Bronchus	44 ± 4	75 ± 12	78 ± 14**	93 ± 17**
Pulmonary parenchyma	73 ± 7	92 ± 13	101 ± 17	106 ± 12
Liver	47 ± 5	54 ± 7	74 ± 10*	102 ± 9**
Spleen	90 ± 11	86 ± 16	113 ± 10	148 ± 23*
Pancreas	32 ± 5	38 ± 6	93 ± 12*	91 ± 10**
Kidney	51 ± 4	55 ± 6	84 ± 16*	123 ± 32**
Stomach	64 ± 6	95 ± 7*	112 ± 10*	158 ± 24**
Duodenum	74 ± 10	88 ± 11	172 ± 15*	216 ± 18**

Values are means ± s.e.mean. The animals were treated with L-NAME (0.125–2 mg kg^{-1} i.v. in a volume of 10 μl 0.9% NaCl per 100 g body weight) or its vehicle (Con, control) for 10 min, then Evans blue dye (20 mg kg^{-1}) was injected i.v. The rats were anaesthetized and perfused with 0.9% NaCl 10 min after injection of the dye. *$P<0.05$; **$P<0.01$ compared to control by Dunn's multiple contrast hypothesis test.

Table 2 Effects of platelet-activating factor, N^G-nitro-L-arginine methyl ester (L-NAME) and noradrenaline (NA) on albumin extravasation in selected organs in conscious rats

	Con	*PAF* (0.19 nmol kg^{-1})	*PAF* (1.9 nmol kg^{-1})	*L-NAME*	*L-NAME plus PAF* (1.9 nmol kg^{-1})	*NA*	*NA plus PAF* (1.9 nmol kg^{-1})
Trachea	62 ± 6	99 ± 4	116 ± 10*	108 ± 12*	152 ± 13**	118 ± 21*	161 ± 17**
Bronchus	47 ± 6	63 ± 6	112 ± 10*	104 ± 9 *	227 ± 38**	162 ± 28*	215 ± 15**
Pulmonary parenchyma	81 ± 7	74 ± 5	92 ± 12	110 ± 8	171 ± 27*	135 ± 13*	145 ± 27*
Liver	53 ± 5	52 ± 2	53 ± 4	110 ± 10*†	111 ± 20*‡	58 ± 4	61 ± 6
Spleen	118 ± 8	139 ± 8	131 ± 8	195 ± 23**††	212 ± 24**‡‡	113 ± 8	138 ± 10
Pancreas	38 ± 5	61 ± 6	186 ± 19**	100 ± 12*†	89 ± 9*	43 ± 5	103 ± 20*
Kidney	55 ± 8	64 ± 6	71 ± 6	136 ± 20**†	121 ± 14**‡‡	70 ± 6	74 ± 7
Stomach	71 ± 5	68 ± 7	140 ± 5*	155 ± 9 *††	316 ± 48**‡‡	77 ± 6	163 ± 19*
Duodenum	90 ± 9	123 ± 4	196 ± 7*	252 ± 21**††	360 ± 46**‡	102 ± 16	212 ± 17*
Skin	32 ± 5	34 ± 8	35 ± 9	43 ± 4	39 ± 8	41 ± 3	38 ± 7

Values are expressed as Evans blue dye, μg g^{-1} of tissue (dry weight) mean ± s.e.mean for 6 experiments. The animals were given L-NAME (2 mg kg^{-1}), noradrenaline (620–790 ng kg^{-1} min^{-1}) or 0.9% NaCl (Con, control) for 10 min before i.v. bolus injection of platelet-activating factor (PAF, 0.19 or 1.9 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The rats were anaesthetized and perfused with 0.9% NaCl 10 min after the injection of PAF. The permeability measurement was made 15 min after injection of PAF. Organ distribution of Evans blue dye was determined as described in Methods. *$P<0.05$; **$P<0.01$ (compared to control by Dunn's multiple contrast hypothesis test); †$P<0.05$; ††$P<0.01$ (compared to noradrenaline); ‡$P<0.05$; ‡‡$P<0.01$ (compared to noradrenaline plus PAF).

blue content than either L-NAME or PAF by itself (Table 2). Injection of PAF during noradrenaline infusion evoked similar changes in tissue Evans blue dye content in the lung to those observed when PAF was administered to L-NAME-treated animals (Table 2). On the other hand, noradrenaline infusion did not potentiate albumin extravasation elicited by PAF in the other vascular beds studied, whereas a decreased response to PAF was detected in the pancreas in these animals (Table 2).

Discussion

The present results show that inhibition of endogenous NO synthesis does not affect the hypotensive response to PAF, whereas it leads to potentiation of albumin extravasation elicited by PAF in selected vascular beds of conscious rats.

In confirmation of earlier observations (Whittle *et al.*, 1989; Furchgott & Vanhoutte, 1989; Rees *et al.*, 1990; Vargas *et al.*, 1991), the present study also demonstrates that L-arginine analogues induce large increases in mean arterial blood pressure in the normotensive rat. At a dose of 25 and 2 mg kg^{-1} respectively, both L-NMMA and L-NAME yielded near maximal pressor effect. This is widely regarded as an indicator of inhibition of endogenous NO production. However, depressor responses to acetylcholine, which exerts its *in vitro* vasorelaxant action through liberation of EDRF/NO (cf. Furchgott & Vanhoutte, 1989) were similar in control rats and in animals treated with L-arginine analogues. To circumvent the influence of the level of inherent vascular tone (i.e. normal or elevated blood pressure) on responses to acetylcholine, mean arterial blood pressure was adjusted by noradrenaline infusion to levels similar to those observed following L-NMMA or L-NAME treatment. In this paradigm, significant attenuation, but not complete inhibition of the acetylcholine-induced depressor response could be observed. Previous studies investigating the effect of L-arginine analogues on the peak hypotensive effect of acetylcholine in the rat resulted in controversial results as inhibition (Whittle *et al.*, 1989), significant attenuation (Vargas *et al.*, 1991) and lack of effect (Gardiner *et al.*, 1990; Van Gelderen *et al.*, 1991) have been reported. Although the reasons for these conflicting results are not clear, the present results indicate that endogenous NO partially mediates the hypotensive action of acetylcholine in conscious rats. These observations also suggest that i.v. injection of acetylcholine does not provide a simple means of quantifying the degree of NO blockade. Unlike acetylcholine, under no conditions did we find that the absolute falls in mean arterial blood pressure elicited by PAF were attenuated in the presence of NO synthesis inhibitors. These observations are consistent with those of Boughton-Smith *et al.* (1992) who reported that L-NMMA does not attenuate PAF (100 nmol kg^{-1} min^{-1})-induced fall in mean arterial blood pressure in anaesthetized rats. Therefore, it is feasible that the depressor action of PAF is independent of NO.

Administration of L-NAME resulted in a dose-dependent increase in albumin extravasation in the large airways, liver, spleen, pancreas, kidney, stomach and duodenum, but not in the pulmonary parenchyma and skin. Recently, it has been reported that L-NAME (10 mg kg^{-1}) or L-NMMA (50 mg kg^{-1}) have no significant effect on the vascular leakage in the guinea-pig trachea and main bronchi (Kuo *et al.*, 1992) and rat gastrointestinal tract (Boughton-Smith *et al.*, 1992), respectively. Although the reasons for the different results are not known at present, it might be related to differences in the action of L-NAME on the airways in different species and to differences in the metabolism of L-NMMA and L-NAME in the rat. Indeed, L-NMMA, but not L-NAME is rapidly metabolized by endothelial cells to L-citrulline, and subsequently to L-arginine (Hecker *et al.*, 1990), the precursor of NO. The enhanced albumin accumulation cannot be attributed to an elevation in mean arterial blood pressure and consequently to an increase in perfusion pressure, since no significant changes in tissue Evans blue dye content were observed following noradrenaline infusion in all vascular beds studied except the pulmonary circulation. Furthermore, L-NAME has been reported to increase transcapillary protein fluxes without elevating capillary hydrostatic pressure in the cat ileum (Kubes & Granger, 1992). These findings are consistent with the notion that capillary hydrostatic pressure is not the primary determinant of microvascular protein extravasation (Grega *et al.*, 1986). An increase in the capillary surface area by L-NAME is also unlikely as vasoconstrictors generally cause derecruitment of capillaries (Grega *et al.*, 1986; Kubes & Granger, 1992). Therefore, it is plausible to assume that L-NAME treatment leads to interendothelial cell gap formation and consequently to opening of the large-pore system, the dominant macromolecular pathway operant in inflammation (Grega *et al.*, 1986). In the pulmonary circulation, a rapid increase in perfusion pressure (over 40 cmH_2O) would lead to widening and disruption of endothelial junctions (Tsukimoto *et al.*, 1990). Thus, it is likely that enhanced pulmonary protein extravasation is a consequence of endothelial damage due to either L-NAME or noradrenaline.

Inhibition of NO production by L-NAME resulted in enhanced tissue Evans blue dye content 15 min after administration of PAF in the large airways, spleen, stomach and duodenum. In contrast, no further elevation in plasma leakage by L-NMMA was reported in the gastrointestinal tract following infusion of PAF in anaesthetized rats (Boughton-Smith *et al.*, 1992). However, interpretation of results obtained with L-NMMA is complicated by the fact that L-NMMA is rapidly metabolized to L-arginine (see above), which, in turn, could reverse its inhibitory action on NO synthase. Furthermore, differences in the experimental protocols used by Boughton-Smith *et al.* (1992) and in the present study (i.e. anaesthetized versus conscious rats, infusion versus bolus injection of PAF) might also be responsible for the conflicting results. Interestingly, combined administration of L-NAME and PAF evoked a significant increase in albumin extravasation in the pulmonary parenchyma, where neither PAF nor L-NAME by itself produced significant changes. Noradrenaline infusion did not mimic the effect of L-NAME on albumin extravasation elicited by PAF in all but the pulmonary vascular beds studied. These observations suggest that with the exception of the pulmonary circulation, the potentiating effect of L-NAME on PAF-induced albumin accumulation cannot be attributed to an increase in perfusion pressure. In the lung, endothelial damage caused by high perfusion pressure may potentiate albumin accumulation evoked by PAF. On the other hand, the vasoconstrictor effect of L-NAME could mask any oedema-inducing effect of PAF, as was observed in the pancreas. Similarly, the potent vasoconstrictor action of PAF in the renal circulation (Sherf *et al.*, 1986) may be responsible for the slightly decreased albumin extravasation seen following L-NAME plus PAF administration compared to the effect of L-NAME.

Although high perfusion pressure-induced structural changes in the capillaries may explain the permeability enhancing effect of L-NAME in the pulmonary circulation, the mechanism by which inhibition of NO production increases protein extravasation in other vascular beds remains unclear. Inhibition of NO formation promotes adhesion of platelets and neutrophil granulocytes to the vascular endothelium (Radomski *et al.*, 1987b; Kubes *et al.*, 1991), an event that is known to induce endothelial dysfunction (Lefer *et al.*, 1991). The findings that a monoclonal antibody directed against the common β-subunit (CD18) of the leukocyte integrins significantly attenuated L-NAME-induced protein fluxes in the cat ileum (Kubes & Granger, 1992) give further support to this hypothesis. Another possibility is that local accumulation of superoxide and/or hydrogen peroxide released by endothelial cells (Zweier *et al.*, 1988) or by neutrophil granulocytes activated by PAF (O'Flaherty *et al.*,

1981; Shaw *et al.*, 1981) may account for the increased permeability (Del Maestro *et al.*, 1981). Since NO may scavenge small amounts of superoxide (Rubanyi & Vanhoutte, 1986; Gryglewski *et al.*, 1986), inhibition of its production would result in accumulation of free radicals.

In conclusion, the present findings show that L-NMMA and L-NAME do not affect the hypotensive response to PAF, whereas they markedly augment PAF-induced albumin extravasation in selected vascular beds of conscious rats. These findings suggest an important role for NO in the regulation of albumin extravasation.

This study was supported by the Foundation of the Maisonneuve-Rosemont Hospital. J.G.F. and É.F.F. were recipients of Fellowships from the Medical Research Council of Canada. Parts of this work were presented at the Fourth International Congress on PAF and Related Lipid Mediators, Snowbird, Utah, U.S.A., 1992.

References

BOUGHTON-SMITH, N.K., DEAKIN, A.M. & WHITTLE, B.J.R. (1992). Actions of nitric oxide on the acute gastrointestinal damage induced by PAF in the rat. *Agents Actions, Special Conference Issue*, C3-C9.

CHANG, S.W.V., FEDDERSEN, C.O., HENSON, P.M. & VOELKEL, N.F. (1987). Platelet-activating factor mediates hemodynamic changes and lung injury in endotoxin treated rats. *J. Clin Invest.*, **79**, 1498–1509.

DEL MAESTRO, R.F., BJORK, J. & ARFORS, K.E. (1981). Increase in microvascular permeability induced by enzymatically generated free radicals. I. In vivo study. *Microvasc. Res.*, **22**, 239–254.

DOEBBER, T.W., WU, M.S., ROBBINS, J.C., CHOY, B.M., CHANG, M.N. & SHEN, T.Y. (1985). Platelet-activating factor (PAF) involvement in endotoxin-induced hypotension in rats. Studies with PAF receptor antagonist kadsurenone. *Biochem. Biophys. Res. Commun.*, **129**, 799–808.

DUNN, O.J. (1964). Multiple comparisons using rank sums. *Technometrics*, **6**, 241–252.

FILEP, J., BRAQUET, P. & MOZES, T. (1991a). Significance of platelet-activating factor in mesenteric ischemia-reperfusion. *Lipids*, **26**, 1336–1339.

FILEP, J., FÖLDES-FILEP, E. & FRÖLICH, J.C. (1987). Vascular responses to leukotrienes B_4, C_4 and D_4 following FPL 55712, indomethacin, saralasin, phentolamine and verapamil in the conscious rat. *Br. J. Pharmacol.*, **90**, 431–439.

FILEP, J., HERMÁN, F., BRAQUET, P. & MOZES, T. (1989). Enhanced levels of platelet-activating factor in blood following intestinal ischemia in the dog. *Biochem. Biophys. Res. Commun.*, **158**, 353–359.

FILEP, J.G., SIROIS, M.G., ROUSSEAU, A., FOURNIER, A. & SIROIS, P. (1991b). Effect of endothelin-1 on vascular permeability in the conscious rat: interactions with platelet-activating factor. *Br. J. Pharmacol.*, **104**, 797–804.

FURCHGOTT, R.F. & VANHOUTTE, P.M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007–2018.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990). Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effect of N^G-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **101**, 632–639.

GREGA, G.J., ADAMSKI, S.W. & DOBBINS, D.E. (1986). Physiological and pharmacological evidence for the regulation of permeability. *Fed. Proc.*, **45**, 96–100.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*, **320**, 454–456.

HECKER, M., MITCHELL, J.A., HARRIS, H.J., KATSURA, M., THIEMERMANN, C. & VANE, J.R. (1990). Endothelial cells metabolize N^G-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. *Biochem. Biophys. Res. Commun.*, **167**, 1037–1040.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9265–9269.

KUBES, P. & GRANGER, D.N. (1992). Nitric oxide modulates microvascular permeability. *Am. J. Physiol.*, **262**, H611–H615.

KUBES, P., SUZUKI, N. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4651–4655.

KUO, H.P., LIU, S. & BARNES, P.J. (1992). The effect of endogenous nitric oxide on neurogenic plasma exudation in guinea-pig airways. *Eur. J. Pharmacol.*, **221**, 385–388.

LEFER, A.M., TSAO, P.S., LEFER, D.J. & MA, X.L. (1991). Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. *FASEB J.*, **5**, 2029–2034.

MONTRUOCCHIO, G., ALLOATTI, G., TETTA, C., DE LUCA, R., SAUNDERS, R.N., EMANUELLI, G. & CAMUSSI, G. (1989). Release of platelet-activating factor from ischemic-reperfused rabbit heart. *Am. J. Physiol.*, **256**, H1236–H1246.

MURAD, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. *J. Clin. Invest.*, **78**, 1–5.

MYERS, P.R., GUERRA, R. Jr. & HARRISON, D.G. (1989). Release of NO and EDRF from cultured bovine aortic endothelial cells. *Am. J. Physiol.*, **256**, H1030–H1037.

O'FLAHERTY, J.T., MILLER, C.H., LEWIS, J.C., WYNKLE, R.L., BASS, D.A., MCCALL, C.E., WAITE, M. & DE CHATELET, L.R. (1981). Neutrophil responses to platelet-activating factor. *Inflammation*, **5**, 193–201.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987a). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.*, **92**, 181–187.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987b). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet*, **ii**, 1057–1058.

RAWSON, R.A. (1943). The binding of T-1824 and structurally related diazo dyes by the plasma proteins. *Am. J. Physiol.*, **138**, 708–717.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

ROGERS, D.F., BOSCHETTO, P. & BARNES, P.J. (1989). Plasma exudation. Correlation between Evans blue dye and radiolabeled albumin in guinea pig airways in vivo. *J. Pharmacol. Met.*, **21**, 309–315.

RUBANYI, G.M. & VANHOUTTE, P.M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am. J. Physiol.*, **250**, H822–H827.

SHAW, J.O., PINCKARD, R.N., FERRIGNI, K.S., MCMANUS, L.M. & HANAHAN, D.J. (1981). Activation of human neutrophils with 1-O-hexadecyl/octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (platelet-activating factor). *J. Immunol.*, **127**, 1250–1255.

SHERF, H., NIES, A., SCHWERTSCHLAG, U., HUGHES, M. & GERBER, J.G. (1986). Hemodynamic effect of platelet-activating factor in the kidney in vivo. *Hypertension*, **8**, 737–741.

SIEGFRIED, M.R., ERHARDT, J., RIDER, T., MA, X.L. & LEFER, A.M. (1992). Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion. *J. Pharmacol. Exp. Ther.*, **260**, 668–675.

TSUKIMOTO, K., MATHIEU-CASTELLO, D., PREDILETTO, R. & WEST, J.B. (1990). Structural basis of increased permeability of pulmonary capillaries with high transmural pressures. *Am. Rev. Respir. Dis.*, **141**, A297.

UDAKA, K., TAKEUCHI, Y. & MOVAT, H.Z. (1970). Simple method for quantitation of enhanced vascular permeability. *Proc. Soc. Exp. Biol. Med.*, **133**, 1384–1387.

VAN GELDEREN, E.M., HEILIGERS, J.P.C. & SAXENA, P.R. (1991). Haemodynamic changes and acetylcholine-induced hypotensive responses after N^G-nitro-L-arginine methyl ester in rats and cats. *Br. J. Pharmacol.*, **103**, 1899–1904.

VARGAS, H.M., CUEVAS, J.M., IGNARRO, L.J. & CHAUDHURI, G. (1991). Comparison of the inhibitory potencies of N^G-methyl, N^G-nitro- and N^G-amino-L-arginine on EDRF function in the rat: evidence for continuous basal EDRF release. *J. Pharmacol. Exp. Ther.*, **257**, 1208–1215.

WHITTLE, B.J.R.. LOPEZ-BELMONTE, J. & REES, D. (1989). Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.*, **98**, 646–652.

ZWEIER, J.L., KUPPUSAMY, P. & LUTTY, G.A. (1988). Measurement of endothelial cell free radical generation: Evidence for a central mechanism of free radical injury in postischemic tissues. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 4046–4050.

(Received March 11, 1993
Revised July 5, 1993
Accepted August 3, 1993)

Br. J. Pharmacol. (1993), **110**, 1353–1358 © Macmillan Press Ltd, 1993

$[Ca^{2+}]_i$-sensitive, IP_3-independent Ca^{2+} influx in smooth muscle of rat vas deferens revealed by procaine

Mohammad A. Khoyi, Hugh H. Dalziel, Lubo Zhang, Richard A. Bjur, William T. Gerthoffer, Iain L.O. Buxton & [1]David P. Westfall

Department of Pharmacology, Howard Medical Sciences Building, University of Nevada School of Medicine, Reno, Nevada 89557, U.S.A.

1 The actions of procaine (10 mM) on noradrenaline-induced effects on ^{45}Ca-influx, ^{45}Ca-efflux, ^{45}Ca-content, total inositol phosphates, inositol-1,4,5-trisphosphate, and contractile status of the rat vas deferens were examined.

2 Noradrenaline alone had no effect on ^{45}Ca-influx or ^{45}Ca-content, but released Ca^{2+} from intracellular stores as indicated by an increased ^{45}Ca-efflux and increased total inositol phosphates, specifically inositol-1,4,5-trisphosphate, leading to contraction of the rat vas deferens.

3 Noradrenaline, in the presence of 10 mM procaine, increased ^{45}Ca-influx and ^{45}Ca-content. Procaine blocked the noradrenaline-induced ^{45}Ca-efflux, the increase in total inositol phosphates, the increase in inositol-1,4,5-trisphosphate, and contraction.

4 The noradrenaline-induced increase in ^{45}Ca influx which was observed in the presence of procaine was abolished by phentolamine and nifedipine but was not altered significantly by propranolol suggesting that, in the presence of procaine, noradrenaline activates dihydropyridine-sensitive calcium channels through α-adrenoceptors.

5 These findings indicate that, in the rat vas deferens, noradrenaline induces contraction by releasing intracellularly stored Ca^{2+}. The effects of procaine appear to be due to its ability to block the release of Ca^{2+} from intracellular stores. Furthermore, the simultaneous increase in ^{45}Ca influx and inhibition of inositol-1,4,5-trisphosphate formation in tissues treated with procaine plus noradrenaline indicates that Ca^{2+} influx is independent of inositol-1,4,5-trisphosphate formation.

Keywords: Smooth muscle; vas deferens; inositol trisphosphate; IP_3; calcium influx; calcium efflux; procaine

Introduction

In the rat vas deferens depolarizing concentrations of K^+ increase Ca^{2+} influx and exchangeable Ca^{2+} cor ent (Hay & Wadsworth, 1984; Khoyi *et al.*, 1988). In contrast, noradrenaline (NA) does not stimulate Ca^{2+} influx but increases its efflux into both normal physiological salt solution and Ca^{2+}-free medium, resulting in decreased Ca^{2+} content of the muscle (Khoyi *et al.*, 1988). These results suggest that NA-induced contractions in the rat vas deferens primarily involve intracellular calcium. An apparent inconsistency with this hypothesis is the observation that contractions of vas deferens induced by NA are inhibited by the organic Ca^{2+} channel blocker, nifedipine (Feddersen *et al.*, 1988; Vesperinas *et al.*, 1989; personal observations). One possible explanation for this paradoxical observation is that NA initially produces a nifedipine-sensitive Ca^{2+} influx, which then causes a secondary release of Ca^{2+} from intracellular stores via a Ca^{2+}-induced Ca^{2+} release mechanism. The subsequent increase in intracellular Ca^{2+} might inactivate ^{45}Ca influx in a manner similar to the inactivation of potential-dependent calcium currents by intracellular Ca^{2+} (Hagiwara & Byerly, 1981; Tsien, 1983; Chad *et al.*, 1984; Pacaud *et al.*, 1987) and help to explain previous observations that NA did not stimulate Ca^{2+} influx (Khoyi *et al.*, 1988).

If this hypothesis is correct, agents capable of preventing the release of Ca^{2+} from the sarcoplasmic reticulum should prevent the inactivation of ^{45}Ca influx resulting in an increase in ^{45}Ca influx upon application of NA. Procaine, at a concentration (10 mM) which is known to prevent the release of Ca^{2+} from the sarcoplasmic reticulum (Itoh *et al.*, 1981; 1982; Imai *et al.*, 1984; Ahn & Karaki, 1988) would be expected to have this effect. Therefore, the effects of procaine on ^{45}Ca fluxes, generation of inositol phosphates and the development of tension were examined in the rat vas deferens.

[1] Author for correspondence.

Methods

Male albino rats, weighing 250–350 g, were killed by exsanguination following CO_2 anaesthesia. Vasa deferentia were removed and cleaned of vascular and connective tissue. The luminal contents of the vasa were removed by flushing with physiological salt solution (PSS). For some studies, animals were injected with reserpine 48 h (5 mg kg^{-1}) and 24 h (2.5 mg kg^{-1}) before each experiment to deplete the vas deferens of endogenous NA and reduce the potential confounding effects related to release of endogenous NA by agents such as procaine (see below). For similar reasons some studies were conducted with vasa deferentia which were sympathetically denervated by a modification of the 6-hydroxydopamine (6-OHDA) method of Aprigliano & Hermsmeyer (1976). Briefly, tissues were incubated with 6-OHDA (250 μg ml^{-1}) and glutathione (20 μM) in buffer-free PSS (pH 4.9) for two 15 min periods separated with an interval of 10 min in normal PSS (Khoyi *et al.*, 1988). Tissues were then washed thoroughly and kept at room temperature overnight in oxygenated PSS at pH 7.4. The next day the vasa deferentia were warmed to 37°C in oxygenated PSS and used in one of the experimental protocols as described below. A lack of contraction in response to electrical field stimulation (EFS), using parameters known to excite sympathetic nerves in this tissue was accepted as evidence of denervation.

Contraction studies

For contraction studies, tissues were mounted in isolated organ chambers and isometric contractions were recorded by

conventional methods that have been described previously (Khoyi *et al.*, 1987). Krebs buffer was the PSS used in these studies and was of the following composition (mM): NaCl 110, KCl 4.6, $CaCl_2$ 2.5, KH_2PO_4 1.15, $MgSO_4$ 1.15, $NaHCO_3$ 24.5, glucose 5.5. Tissues were equilibrated for 60 min at a resting tension of 1 g prior to challenge with NA (10 μM). Tissues were then washed several times and allowed to re-equilibrate in drug-free PSS for 15 min before a 15 min incubation period with procaine (10 mM). The tissues were then rechallenged with NA. For the studies which utilized Ca^{2+}-free PSS the $CaCl_2$ was omitted and EGTA (50 μM) was included. In these experiments the control response to NA (10 μM) was obtained after a 15 min incubation in Ca^{2+}-free medium.

Subsequently, Ca^{2+}-containing medium was introduced for 15 min (to reload intracellular stores). Next the tissues were re-incubated in Ca^{2+}-free medium plus procaine (10 mM) for 15 min before the tissue was rechallenged with NA.

Measurement of NA overflow

Tissues were incubated in an organ chamber and PSS solution was changed every 3 min. Samples of the PSS solution (3.5 ml) were transferred every 3 min to ice-cold tubes containing 0.7 ml of a solution containing 0.4 M perchloric acid, 1.3 mM Na_2EDTA and 5.3 mM $Na_2S_2O_5$ to stabilize catecholamines. The samples were then extracted over alumina to concentrate catecholamines and the NA content measured by high performance liquid chromatography (h.p.l.c.) with electrochemical detection as described previously (Shinozuka *et al.*, 1988). The effect of drugs on the overflow of NA from the tissue was monitored in this manner.

Extraction, separation and measurement of inositol phosphates

This was done according to the method of Zhang & Buxton (1991). Briefly, prior to experiments, tissues were incubated in an oxygenated labelling buffer containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 0.6, NaH_2PO_4 0.6, $MgCl_2$ 1.2, glucose 5, $CaCl_2$ 0.5, HEPES 10, pH = 7.4 and $NaHCO_3$ 5 along with [^{3}H]-*myo*-inositol (200 μCi ml^{-1}) and LiCl (10 mM) for 3 h at 35°C. After 2 min exposure to NA (10 μM) in the presence or absence of procaine (10 mM for 15 min), tissues were homogenized in $CHCl_3$:CH_3OH:HCl (66:33:1, v/v), clarified by centrifugation at 1 000 *g* for 10 min at room temperature and the aqueous phase lyophilized to dryness. The pellet was resuspended in 500 μl of H_2O and filtered (0.45 μm nylon) for h.p.l.c. analysis. Separation of inositol phosphates was accomplished using a gradient chromatographic system consisting of H_2O and 2 M $NH_4H_2PO_4$ (pH 3.8) run over a strong anion exchange column (Whatman Partisil 5-SAX, 10 cm) at a flow rate of 0.8 ml min^{-1} (100% H_2O to 100% 2 M $NH_4H_2PO_4$ over 30 min). Radioactivity was monitored continuously with a Radiomatic flow detector at 12–15% efficiency for ^{3}H. Inositol phosphate standards were well resolved and used to identify unknowns. Data were determined as area under the h.p.l.c. peak in counts per min (c.p.m.) for inositol-1,4,5-trisphosphate (IP_3) and each of the inositol phosphate isomers. For total inositol phosphates, c.p.m. from individual peaks were added together.

Measurement of ^{45}Ca influx

Influx of ^{45}Ca was measured by the method of Meisheri *et al.* (1981) as described previously (Khoyi *et al.*, 1988). Briefly, a PSS of the following composition (in mM) was used: NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, HEPES 5 and glucose 10. The PSS was maintained at pH 7.4 and gassed with 100% O_2. After 90 min of equilibration, the tissues were exposed to PSS containing ^{45}Ca (0.6–0.8 μCi ml^{-1}) and the drugs being studied. For experiments in which the effects of procaine were examined, this agent was added to the bathing solution during the last 20 min of the equilibration period. For determination of ^{45}Ca influx, the exposure of ^{45}Ca was limited to 2 min. After incubation in ^{45}Ca-labelling solution, the tissues were immediately transferred to 10 ml of ice-cold (0.5°C) Ca^{2+}-free oxygenated PSS containing 2 mM EGTA (pH 7.2) for 2 min. All tissues were then immersed in 500 ml of this same solution for 48 min to remove extracellular ^{45}Ca. It has been shown that even during the first minutes of washing some of the intracellular ^{45}Ca is lost to the washing solution (Aaronson, 1989). However, several investigators have observed that the remaining intracellular ^{45}Ca after washout is proportional to the amount of intracellular ^{45}Ca present at the end of the labelling period (see Loutzenhiser *et al.*, 1985). The duration of the wash period in Ca^{2+}-free, ice-cold solution was determined by preliminary desaturation experiments. These experiments showed that the tissues lost approximately 25% of intracellular ^{45}Ca in the 50 min wash period. The tissues were then placed on filter paper and the vasa deferentia bisected into epididymal and prostatic segments. Each segment was then cut into small pieces (2–3 mm), blotted, and put in preweighed vials to determine wet weight. Three ml of EDTA solution (5 mM) was added to each vial which was then left overnight at room temperature. Finally, 3.5 ml scintillation cocktail (ScintiVerse I) was added to each vial and radioactivity was determined in a Beckman liquid scintillation counter. Tissue counts were converted to tissue Ca^{2+} content by using the specific activity of ^{45}Ca in the incubation medium, and influx was expressed as micromol ^{45}Ca kg^{-1} wet tissue weight.

Measurement of ^{45}Ca content

Tissues were equilibrated in PSS at 37°C for 90 min and then exposed to ^{45}Ca containing PSS for an additional 2 h to label the exchangeable intracellular Ca^{2+} pool. The tissues were then exposed to NA (10 μM) and procaine (10 mM) in the last 10 and 20 min of incubation, respectively. The ^{45}Ca content was then determined as described above.

Measurment of ^{45}Ca efflux

Tissues were equilibrated in PSS (37°C) and then exposed to ^{45}Ca containing medium for up to 3 h to achieve steady-state labelling of the exchangeable intracellular Ca^{2+} pool. Tissues were then passed at 2-min intervals through a series of test tubes that contained calcium-free medium to which EGTA (0.05 mM) had been added. Procaine (10 mM) was added to the experimental group at the 70th min of rinsing until the end of the experiment and NA (100 μM) was added to both groups during the 80th–90th min. At the end of each experiment, the radioactivity in each test tube and that of the tissue was determined as described above. The rate of ^{45}Ca efflux was then calculated as the fraction of radioactivity present in the tissue at the time of sampling. From this, an efflux curve was generated. The efflux data were obtained at 20°C rather than at 37°C to slow ^{45}Ca efflux and thereby assess more accurately the influence of NA on the rate of ^{45}Ca efflux.

Statistical analysis

All data are presented as means ± s.e. Student's *t* test was used for paired or unpaired data as appropriate. Analysis of variance was performed with Duncan's multiple range test for multiple comparisons between means. A $P<0.05$ was considered significant.

Drugs used

Drugs used were phentolamine mesylate supplied by Ciba-Geigy, Summit, NJ, U.S.A., nifedipine supplied by Pfizer, New York, NY and noradrenaline (NA), propranolol, reserpine, 6-hydroxydopamine (6-OHDA), procaine and tetraethylammonium (TEA) supplied by Sigma Chemical, St. Louis, MO, U.S.A.

Results

Effect of procaine on the overflow of NA from rat vas deferens

Incubation of vas deferens with procaine (10 mM) caused a significant and maintained increase in the overflow of NA from the tissue for a period of 60 min (not shown). Therefore, unless otherwise stated, the experiments described below were carried out with denervated or reserpine-treated tissues. These procedures abolished the procaine-induced overflow of NA and eliminated complications due to release of endogenous NA.

Effects of procaine on the contractile response of rat vas deferens to NA

Figure 1 shows a representative example of the effect of procaine (10 mM) on the contractile response to NA (10 μM) in normal PSS (2.5 mM Ca^{2+}) and Ca^{2+}-free PSS (0 mM Ca^{2+} + 50 μM EGTA, see Methods section for protocol). In the presence of Ca^{2+}, procaine (10 mM) reduced the contractile response of vasa deferentia to NA (10 μM) such that after procaine only 2.8 ± 1.2% of the response remained ($P<0.05$, compared to control, $n=9$). Figure 1 also shows that the contraction to NA was markedly reduced in Ca^{2+}-free PSS compared to that observed in normal PSS. The initial phasic component was reduced and the secondary maintained phase was abolished. Subsequent addition of procaine completely abolished the contractile response to NA ($n=6$).

Effects of procaine on NA stimulated [^{3}H]-inositol phosphate accumulation

Stimulation by NA produced a large increase in IP_3 accumulation (1.9 ± 0.05 fold, Figure 2) which was mirrored by similar increases in total inositol phosphates (2.26 ± 0.22 fold). Procaine (10 mM) at a concentration which blocked NA-induced contraction, abolished the effect of NA on inositol phosphates (Figure 2).

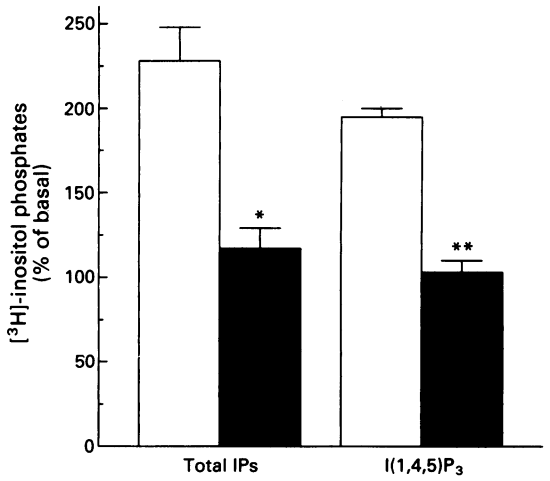


Figure 2 Effects of procaine (10 mM for 15 min) on accumulation of [^{3}H]-inositol phosphates stimulated by noradrenaline (NA, 10 μM for 2 min) in rat vas deferens. Pretreatment of the tissue with 10 mM procaine showed no effect on basal phosphoinositide metabolism (total IP from 4172 ± 1260 to 4183 ± 1335 c.p.m. mg^{-1} tissue wet weight, IP_3 from 272 ± 107 to 300 ± 118 c.p.m. mg^{-1} tissue wet weight). Open columns represent the effect of NA (10 μM) in the absence of procaine and solid columns represent the levels in the presence of procaine as % of basal levels. Procaine completely blocked NA-induced generation of total inositol phosphates and IP_3. Values shown are mean ± s.d. of 3 experiments. Significant difference from control group: $*P<0.05$; $**P<0.001$.

Effect of procaine and NA on ^{45}Ca influx

In reserpine-treated tissues, as previously shown (Khoyi *et al.*, 1988), NA had no effect on the rate of ^{45}Ca influx (Figure 3). However, when reserpine-treated tissues were incubated with procaine, exposure to NA significantly increased the rate of ^{45}Ca influx (Figure 3). This increased influx of ^{45}Ca was prevented by phentolamine but was not changed significantly by propranolol (data not shown). Procaine alone did not significantly increase ^{45}Ca influx in reserpine-treated tissues.

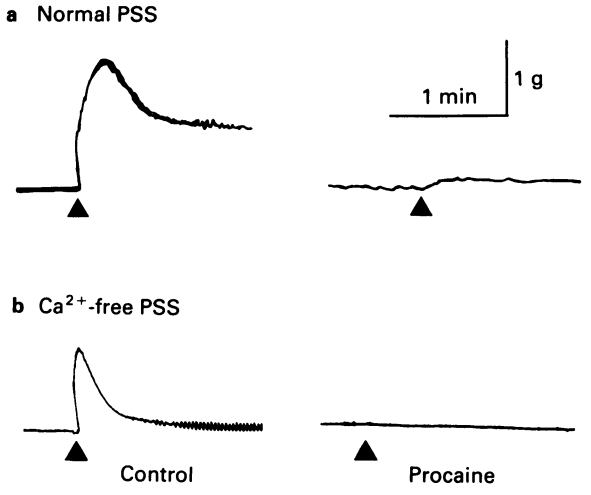


Figure 1 Representative examples of 6–9 experiments examining the effect of procaine on noradrenaline (NA)-induced contraction of rat vas deferens in Ca^{2+}-containing Krebs solution (a) and in Ca^{2+}free solution (b, see Methods). The first trace in each panel shows the control contraction to NA (10 μM) and the second trace the contraction to NA in the presence of procaine (10 mM for 15 min).

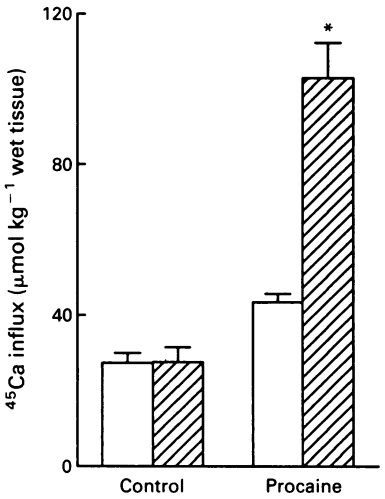


Figure 3 Effect of noradrenaline (NA, 10 μM) and procaine (10 mM) on ^{45}Ca influx in vasa deferentia from reserpinized rats. The concentration of Ca^{2+} in the medium was 1.5 mM. Open columns represent basal levels of ^{45}Ca influx and hatched columns represent stimulation with NA. Note that NA significantly increased ^{45}Ca influx in the presence ($P<0.05$, $n=6$) but not in the absence ($n=6$) of procaine.

Effect of nifedipine on ^{45}Ca influx produced by NA in the presence of procaine

Nifedipine blocked the increase in Ca^{2+} influx which is observed in tissues exposed to procaine plus NA. The combination of nifedipine and NA, without procaine, was no different from NA alone (Figure 4). In tissues that were not stimulated with NA, neither procaine, nifedipine nor a combination of the two had any significant effect on Ca^{2+} influx ($n = 4-8$, data not shown).

Effect of tetraethylammonium (TEA) on Ca^{2+} flux

TEA (20 mM) alone caused an increase in Ca^{2+} influx from 27.3 ± 2.42 μmol kg^{-1} wet weight to 47.75 ± 2.32 μmol kg^{-1} wet weight ($P < 0.05$, $n = 5$). In the presence of TEA, NA significantly reduced Ca^{2+} influx to 40.34 ± 1.21 μmol kg^{-1} wet weight ($P < 0.025$, $n = 5$).

Effect of procaine on the ^{45}Ca content of vas deferens

The exchangeable ^{45}Ca content of vasa deferentia was measured after a 2 h incubation in PSS with ^{45}Ca. For this purpose, the rats were pretreated with reserpine and phentolamine (1 μM) was added to the incubation medium when tissues were not to be exposed to exogenous NA. Procaine (10 mM) and NA (10 μM), when present, were added during the last 20 and 10 min of the ^{45}Ca labelling period, respectively. The ^{45}Ca content of these tissues was increased moderately by procaine. The ^{45}Ca content of vasa deferentia was significantly increased by NA in the presence of procaine (Figure 5).

Effects of procaine on ^{45}Ca efflux

For ^{45}Ca efflux experiments, vasa deferentia from reserpine pretreated rats were utilized. After equilibration and incubation with ^{45}Ca containing medium, vasa deferentia were rinsed with Ca^{2+}-free medium in the absence or presence of 10 mM procaine for the entire period of the ^{45}Ca efflux experiment and NA (100 μM) was added for 10 min during the 80th–90th min of rinsing. Procaine did not change the rate of ^{45}Ca efflux in unstimulated tissues but completely abolished the stimulatory effect of NA on ^{45}Ca efflux (Figure 6).

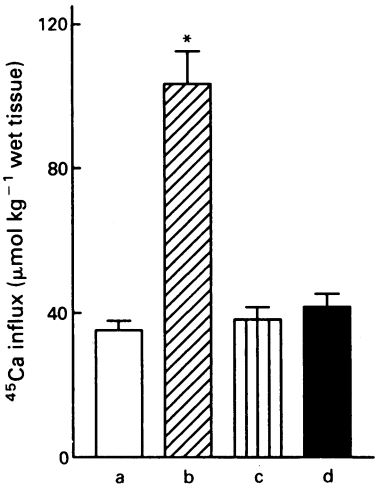


Figure 4 Effect of nifedipine on ^{45}Ca influx induced by noradrenaline (NA) in the presence of procaine. Columns indicate tissues stimulated with 10 μM NA (column a), 10 μM NA + 10 mM procaine (column b), 10 μM NA + 1 μM nifedipine (column c) and 10 μM NA + 10 mM procaine + 1 μM nifedipine (column d). Note that nifedipine completely blocked the increase in ^{45}Ca influx induced by NA + procaine. The combination of nifedipine plus NA, without procaine, was no different from NA alone ($n = 4-7$).

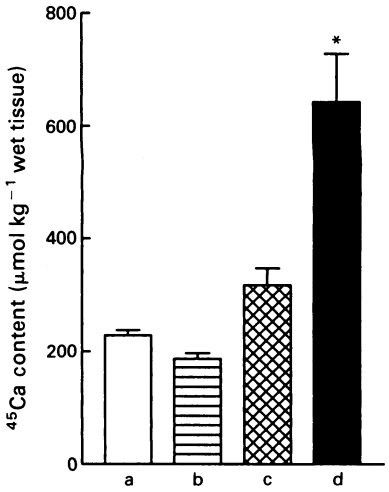


Figure 5 ^{45}Ca-content of rat vasa deferentia under unstimulated conditions (column a), and in tissues exposed to noradrenaline (10 μM, column b), procaine (10 mM, column c) and the combination of NA plus procaine (column d). Note that the ^{45}Ca content of the vasa deferentia was increased by NA in the presence ($P < 0.05$), but not in the absence of procaine. Each column is the mean ± s.e.mean of 6–9 experiments.

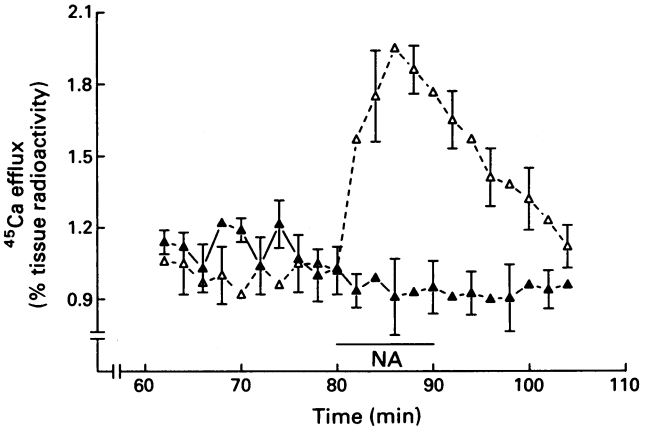


Figure 6 Effect of procaine (10 mM) on ^{45}Ca efflux induced by noradrenaline (NA, 100 μM) from vasa deferentia of reserpine pretreated rats. In tissues preincubated with procaine (▲) the NA-induced increase in ^{45}Ca efflux which was seen in control tissues (△) was blocked ($n = 6$).

Discussion and conclusions

The results of the present study suggest that; (i) NA-induced contraction of the rat vas deferens is dependent on Ca^{2+} release from the sarcoplasmic reticulum and not on Ca^{2+} influx, because block of release of Ca^{2+} from sarcoplasmic reticulum by procaine results in inhibition of contraction; (ii) NA-induced Ca^{2+} influx is only apparent when IP_3 generation and release of Ca^{2+} from the sarcoplasmic reticulum are blocked by procaine, suggesting that in the absence of procaine, elevated cytosolic Ca^{2+} concentrations cause inactivation of Ca^{2+} influx; (iii) IP_3 generation is not necessary for the occurrence of Ca^{2+} influx during exposure of the tissue to procaine plus NA, because this combination blocked the generation of IP_3 but increased Ca^{2+} influx.

In the present study, NA contracted the vas deferens but increased neither ^{45}Ca influx nor ^{45}Ca content. However, it caused release of Ca^{2+} from intracellular stores, increased ^{45}Ca efflux and increased IP_3 generation. These findings are in agreement with other data (Khoyi *et al.*, 1988) which indicate

(i) that in the vas deferens, as in dog coronary artery (Imai *et al.*, 1984), NA utilizes intracellularly stored Ca^{2+} for contraction, and (ii) that Ca^{2+} entry through the cell membrane seems to be unnecessary to provide free Ca^{2+} to activate the contractile protein. After exposure of the vas deferens to NA, either IP_3-, or Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum, or both, may increase the intracellular Ca^{2+} concentration to a level which activates the contractile apparatus. However, a paradox exists in that while NA appears to translocate ^{45}Ca only from intracellular stores within the vas deferens, contractions induced by NA have been shown to be inhibited by nifedipine (Feddersen *et al.*, 1988; Vesperinas *et al.*, 1989). This organic calcium channel antagonist blocks voltage-sensitive Ca^{2+} channels without affecting Ca^{2+} gradient between intra- and extra-cellular mediums. It is possible that the method used in this study is not sensitive enough to detect a nifedipine-sensitive Ca^{2+} influx which may then act as a trigger for Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum, as has been suggested previously (Itoh *et al.*, 1986). It is also possible that nifedipine inhibits the contraction of vas deferens to NA through mechanisms other than the blockade of calcium channels (Rinaldi *et al.*, 1987; Jaquemond & Rougier, 1988; Xuan *et al.*, 1989). However, the present observation that NA increases ^{45}Ca influx in vasa deferentia that were preincubated with procaine and that this increased Ca^{2+} influx was blocked by nifedipine indicates that NA is capable of activating Ca^{2+} influx in this tissue through dihydropyridine-sensitive calcium channels.

Four possible explanations can be given to explain the absence of Ca^{2+} influx during stimulation with NA in the absence of procaine: (i) NA-induced increases in intracellular Ca^{2+} may indirectly inactivate Ca^{2+} influx by opening Ca^{2+}-activated K^+ channels. However, if this were the case, then blocking Ca^{2+}-activated K^+ channels should increase ^{45}Ca influx. In the present experiments, TEA, which blocks Ca^{2+}-activated K^+ channels in other smooth muscles (Carl *et al.*, 1993), did not increase NA induced ^{45}Ca influx, but in fact significantly decreased ^{45}Ca influx; (ii) the Ca^{2+} which enters the cell is immediately pumped out by Ca^{2+}-ATPase. This also is unlikely because, even if there were no net change in intracellular Ca^{2+} content, one would expect that an exchange of the ^{45}Ca with the non-radioactive intracellular Ca^{2+} would result in an 'apparent' ^{45}Ca influx; (iii) NA may directly reduce Ca^{2+} channel activity in addition to Ca^{2+} inactivation of Ca^{2+} channels (Imaizumi *et al.*, 1991). This also is unlikely because NA increased ^{45}Ca influx in the presence of procaine. We favour the fourth possibility, which is: (iv) NA-induced increases in intracellular Ca^{2+} from the sarcoplasmic reticulum may directly inactivate the Ca^{2+} influx in a manner similar to the inactivation of potential-dependent calcium currents by intracellular Ca^{2+} (Hagiwara & Byerly, 1981; Tsien, 1983; Chad *et al.*, 1984; Pacaud *et al.*, 1987).

In the presence of procaine, NA was unable to contract the vas deferens despite an increased ^{45}Ca influx and increased ^{45}Ca content of the tissue. Furthermore, procaine completely inhibited the NA-induced ^{45}Ca efflux. This indicates that procaine blocked the NA-induced release of Ca^{2+} from intracellular stores and that this is the primary source of Ca^{2+} for contraction in this tissue. In the presence of procaine, NA did not increase IP_3 generation but did increase ^{45}Ca influx. This increase of ^{45}Ca influx was mediated through α-adrenoceptor stimulation and occurred through dihydropyridine-sensitive calcium channels. The increase of ^{45}Ca influx in the absence of any increase in IP_3 formation is interesting because it has been suggested that in other cells, IP_3 mediates activation of receptor operated Ca^{2+} influx (see review by Putney, 1992). However, it is shown here that procaine inhibited NA-induced IP_3 formation without inhibiting Ca^{2+} influx. These results indicate that, for the rat vas deferens, IP_3 formation was not a required intermediate step in the NA-induced increase of Ca^{2+}-influx.

The present observations that procaine prevented the NA-induced increase in ^{45}Ca efflux (Figure 6) and increased ^{45}Ca content (Figure 5) of the vas deferens, are in accord with the inhibitory effect of procaine on the release of Ca^{2+} from calcium storage sites (Endo, 1977; Itoh *et al.*, 1981; 1982). Furthermore, others have provided evidence in favour of the target for the action of procaine being on the sarcoplasmic reticulum in that procaine has no effect on the pCa-tension relationship in chemically skinned arterial smooth muscle cells (Itoh *et al.*, 1981; 1982). This implies that procaine affects neither the contractile mechanisms directly nor the ability of Ca^{2+} to activate it.

The methodology used in this study is dependent upon retention of Ca^{2+} which has entered the cell. Any Ca^{2+} which is not retained within the cell is not detected. Therefore, it cannot be determined whether NA causes inactivation of Ca^{2+} influx due to raising of cytoplasmic Ca^{2+} or prevents intracellular retention of Ca^{2+} in the sarcoplasmic reticulum. Electrophysiological analysis of the inactivation of the Ca^{2+} channels under different conditions may help to answer these questions.

It is well known that IP_3 will cause Ca^{2+} release from the sarcoplasmic reticulum (Berridge, 1984; Somlyo *et al.*, 1985). The present results are, to our knowledge, the first observation that procaine may inhibit indirectly intracellular Ca^{2+} release by blocking IP_3 formation, as well as directly inhibiting Ca^{2+} release from sarcoplasmic reticulum (Endo, 1977). The mechanism of procaine-induced inhibition of IP_3 formation cannot be determined from these experiments but may be associated with its well known membrane stabilizing activity.

In summary, these results provide further evidence that intracellularly stored Ca^{2+} is the primary mediator of NA-induced contraction in the rat vas deferens. The use of procaine shows that this intracellular Ca^{2+} also inactivates Ca^{2+} influx. The existence of NA-induced Ca^{2+} influx in the presence of procaine while IP_3 formation was blocked indicates that NA-induced Ca^{2+} influx in vas deferens smooth muscle is independent of IP_3 formation.

This work was supported by a grant from the American Heart Association (M.A.K.) and NIH Grants HL 38126 (D.P.W.), HD 26227 (I.L.O.B.).

References

AARONSON, P.I. (1989). Estimation of high K- and noradrenaline-induced ^{45}Ca uptakes in isolated rat aorta: effects of washing in ice-cold solutions. *Pflügers Arch.*, **414**, 579–583.

AHN, H.Y. & KARAKI, H. (1988). Inhibitory effect of procaine on contraction and calcium movement in vascular and intestinal smooth muscles. *Br. J. Pharmacol.*, **94**, 789–796.

APRIGLIANO, O. & HERMSMEYER, K. (1976). In vitro denervation of the portal vein and caudal artery of the rat. *J. Pharmacol. Exp. Ther.*, **198**, 568–577.

BERRIDGE, M.J. (1984). Inositol phosphate and diacylglycerol as second messengers. *Biochem. J.*, **220**, 345–360.

CHAD, J., ECKERT, R. & EWALD, D. (1984). Kinetics of calcium dependent inactivation of calcium current in voltage-clamped neurons of *Aplysia california*. *J. Physiol.*, **347**, 279–300.

CARL, A., FREY, B.W., WARD, S.M., SANDERS, K.M. & KENYON, J.L. (1993). Inhibition of slow wave repolarization and Ca-activated K-channels by quarternary ammonium compounds. *Am. J. Physiol.*, **264**, C625–C631.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.*, **57**, 71–108.

FEDDERSEN, M., VESPERINAS, G., LEWIN, J. & HUIDOBRO-TORO, J.P. (1988). Different calcium sources mobilized by noradrenaline (NA) and adenosine 5'-triphosphate (ATP): a basis for co-transmission in the rat vas deferens. *Eur. J. Pharmacol.*, **153**, 313–314.

HAGIWARA, S. & BYERLY, L. (1981). Calcium channels. *Annu. Rev. Neurosci.*, **4**, 69–125.

HAY, D.W.P. & WADSWORTH, R.M. (1984). Effects of KCl on ^{45}Ca uptake and efflux in the rat vas deferens. *Br. J. Pharmacol.*, **81**, 441–447.

IMAI, S., NAKAZAWA, M., IMAI, H. & NABATA, H. (1984). Effects of procaine on the isolated dog coronary artery. *Arch. Int. Pharmacodyn. Ther.*, **271**, 98–105.

IMAIZUMI, Y., TAKEDA, M., MURAKI, K. & WATANABE, M. (1991). Mechanism of NE-induced reduction of Ca current in single smooth muscle cells from guinea-pig vas deferens. *Am. J. Physiol.*, **260**, C17–C25.

ITOH, K., TAKAKURA, S., SATO, K. & SUTKO, J.L. (1986). Ryanodine inhibits the release of calcium from intracellular stores in guinea-pig aortic smooth muscle. *Circ. Res.*, **58**, 730–734.

ITOH, T., KAJIWARA, M., KITAMURA, K. & KURIYAMA, H. (1982). Roles of stored calcium on the mechanical response evoked in smooth muscle cells of the porcine coronary artery. *J. Physiol.*, **322**, 107–125.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1981). Excitation-contraction coupling in smooth muscle cells of the guinea-pig mesenteric artery. *J. Physiol.*, **321**, 513–535.

JAQUEMOND, V. & ROUGIER, O. (1988). Nifedipine and BAY K inhibit contraction independently from their action on calcium channels. *Biochem. Biophys. Res. Commun.*, **152**, 1002–1007.

KHOYI, M.A., WESTFALL, D.P., BUXTON, I.L.O., AKHTAR-KHAVARI, F., REZAEI, E. SALAICES, M. & SANCHEZ-GARCIA, P. (1988). Norepinephrine and potassium induced calcium translocation in rat vas deferens. *J. Pharmacol. Exp. Ther.*, **246**, 917–923.

KHOYI, M.A., WESTFALL, D.P. & GERTHOFFER, W.T. (1987). Effects of potassium and norepinephrine on calcium influx in guinea-pig vas deferens. *Eur. J. Pharmacol.*, **140**, 55–62.

LOUTZENHISER, R., LEYTON, P., SAIDA, K. & VAN BREEMEN, C. (1985). Calcium compartments and mobilization during contraction of smooth muscle. In *Calcium and Contractility, Smooth Muscle*. ed. Grover, A.K. & Daniel, E.E., pp. 61–92. Clifton, New Jersey: Humana.

MEISHERI, K.D., HWANG, O.K. & VAN BREEMEN, C. (1981). Evidence for two separate Ca^{2+} pathways in smooth muscle plasmalemma. *J. Membrane Biol.*, **59**, 19–25.

PACAUD, P., LOIRAND, G., MIRONNEAU, C. & MIRONNEAU, J. (1987). Opposing effects of noradrenaline on the two classes of voltage-dependent calcium channels of single vascular smooth muscle cells in short-term primary culture. *Pflügers Arch.*, **410**, 557–559.

PUTNEY, J.W. (1992). Inositol phosphates and calcium entry. In *Advances in Second Messenger and Phosphoprotein Research*, Vol. 26, ed. Putney, J.W., pp. 143–160. New York: Raven Press.

RINALDI, G.J., CATTANEO, E.A., MATTIAZZI, A. & CINGOLANI, H.E. (1987). Dissociation between calcium influx blockage and smooth muscle relaxation by nifedipine in spontaneously hypertensive rats. *Circ. Res.*, **60**, 367–374.

SHINOZUKA, K., BJUR, R.A. & WESTFALL, D.P. (1988). Characterization of prejunctional purinoceptors on adrenergic nerves of the rat caudal artery. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 221–227.

SOMLYO, A.V., BAND, M., SOMLYO, A.P. & SCARPA, A. (1985). Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 5231–5235.

TSIEN, R.W. (1983). Calcium channels in excitable cell membranes. *Annu. Rev. Physiol.*, **45**, 341–358.

VESPERINAS, G., FEDDERSEN, M., LEWIN, J. & HUIDOBRO-TORO, J.P. (1989) The use of ryanodine and calcium channel blockers to characterize intra- and extracellular calcium pools mobilized by noradrenaline in the rat vas deferens. *Eur. J. Pharmacol.*, **165**, 309–313.

XUAN, Y., WHORTON, A.R. & WATKINS, W.D. (1989). Inhibition by nicardipine on endothelin-mediated inositol phosphate formation and Ca^{2+} mobilization in smooth muscle cell. *Biochem. Biophys. Res. Commun.*, **160**, 758–764.

ZHANG, L. & BUXTON, I.L.O. (1991). Muscarinic receptors in canine colonic circular smooth muscle. II. Signal transduction pathways. *Mol. Pharmacol.*, **40**, 952–959.

(Received November 23, 1992
Revised July 26, 1993
Accepted August 3, 1993)

Br. J. Pharmacol. (1993), **110**, 1359–1362 © Macmillan Press Ltd, 1993

Inhibition by hydrochlorothiazide of insulin release and calcium influx in mouse pancreatic β-cells

Per-Erik Sandström

Department of Histology and Cell Biology, University of Umeå, S-901 87 Umeå, Sweden

1 The effect of hydrochlorothiazide on insulin release, $^{36}Cl^-$ fluxes and $^{45}Ca^{2+}$ uptake was tested in β-cell-rich mouse pancreatic islets.

2 At high glucose concentrations (10 and 20 mmol l^{-1}), low concentrations of hydrochlorothiazide (0.1–1.0 μmol l^{-1}) reduced insulin release by 22–42%. At lower glucose concentrations (3–8.5 mmol l^{-1}) insulin release was not affected by the drug.

3 Neither short-term influx (3 min) nor net accumulation (60 min) of $^{36}Cl^-$ in the islets was affected by hydrochlorothiazide (0.1–500 μmol l^{-1}).

4 Glucose-stimulated $^{45}Ca^{2+}$ uptake was significantly reduced by hydrochlorothiazide (1–10 μmol l^{-1}).

5 The data suggest that the diabetogenic effect of hydrochlorothiazide, at least in part, can be mediated by direct inhibition of insulin release from the pancreatic β-cells. The inhibition is not mediated by reduced chloride fluxes but may rather be caused by inhibition of calcium uptake.

Keywords: Hydrochlorothiazide; diuretics; β-cells; diabetes mellitus; insulin release; Cl^-; Ca^{2+}

Introduction

Thiazide diuretics may aggravate or even induce diabetes mellitus (Wilkins, 1959; Goldner *et al.*, 1960; Amery *et al.*, 1978) but the underlying mechanisms are largely unknown. Pancreatic as well as extra pancreatic mechanisms have been suggested to explain this diabetogenic action (for review, see Furman, 1981; Brass, 1984).

From studies with human patients it was proposed that thiazide-induced impairment in glucose tolerance is caused by a reduced β-cell response to glucose that is secondary to diuresis-induced hypokalaemia (Helderman *et al.*, 1983). However, it has also been suggested that hypokalaemia may be an aggravation factor rather than a primary factor for development of diuretic-induced disturbances in glucose tolerance (for review, see Furman, 1981).

The few *in vitro* studies on the effect of thiazides on insulin release are contradictory. Malaisse & Malaisee-Lagae (1968) suggested that insulin release is not affected, but it has also been proposed that it can be stimulated by thiazide diuretics (Hermansen *et al.*, 1985). Thus, it is not known whether thiazide-induced impairment of glucose tolerance can be explained by a direct effect on the pancreatic β-cells. Therefore, in the present study, the effect of hydrochlorothiazide on insulin release from isolated β-cell-rich mouse pancreatic islets and the effect on key processes in the stimulus-secretion coupling pathway were investigated.

Methods

Animals and isolation of islets

Eight months old ob/ob mice from the Umeå colony were used as source of pancreatic islets. The hyperplastic islets of these mice contain a high proportion of normal β-cells (>90%, Hellman, 1965) that respond adequately to various stimulators and inhibitors of insulin release (Hahn *et al.*, 1974; Hellman *et al.*, 1974). After overnight food deprivation to normalize the blood sugar (Sandström & Sehlin, 1988a) their pancreatic islets were dissected out without the use of enzymes.

Solutions

A Hepes-buffered Krebs-Ringer solution with the following composition was used as basal medium (mmol l^{-1}): NaCl 130, KCl 4.7, $CaCl_2$ 2.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 20 and D-glucose 3. The medium was supplemented with 1 mg ml^{-1} bovine serum albumin and the pH was adjusted to 7.4 with NaOH.

Hydrochlorothiazide was obtained from Sigma (St. Louis, MO, U.S.A.) and dissolved in basal medium. The pH of the basal medium was not changed by the addition of the drug.

Insulin release

After a preincubation for 30 min in basal medium, groups of 2–3 islets were incubated for 60 min in 300 μl basal medium with or without test substance. The islets were then freeze-dried (−40°C, 0.1 Pascal) and weighed on a quartz-fibre balance. Samples from the incubation medium were assayed for insulin by standard radioimmunoassay (Heding, 1966), using crystalline mouse insulin as standard.

$^{36}Cl^-$ uptake

After preincubation for 30 min in basal medium, groups of 4–5 islets were incubated for 3 or 60 min in control or test medium labelled with $^{36}Cl^-$ (specific radioactivity, 1.9–2.6 GBq mol^{-1}). Then, the islets were washed for 2 min in ice-cold basal medium (0°C) to remove extracellular radioactivity. The islets were freeze-dried and weighed as above and the islet radioactivity was measured by liquid scintillation counting. The method is described in detail elsewhere (Sehlin, 1978).

$^{45}Ca^{2+}$ uptake

The islets were pre-incubated in basal medium as above and then groups of 4–5 islets were incubated for 10 min in control or test medium labelled with $^{45}Ca^{2+}$ (specific activity, 0.3 TBq/mol). Contaminating extracellular radioactivity was removed by washing for 60 min in 2 mmol $l^{-1}LaCl_3$. After freeze-drying and weighing as above, the islet radioactivity was measured by liquid scintillation counting. The method is described in detail elsewhere (Hellman *et al.*, 1976).

Statistics

In the experimental situation, control and test islets were handled in parallel. The differences between control and test groups were analysed by Student's paired *t* test for the number of experiments indicated in the figures and table.

Results

Effect on insulin release

As shown in Figure 1, the effect of hydrochlorothiazide on insulin release was complex. At 10 mmol l^{-1} D-glucose, the release was reduced by 42% by 1 μmol l^{-1} hydrochlorothiazide. At an intermediate concentration (10–100 μmol l^{-1}) no effect was observed, and at a high concentration of hydrochlorothiazide (1 mmol l^{-1}), a potentiation of insulin release was registered. At the higher glucose concentration (20 mmol l^{-1}) a similar pattern was observed. Thus, at 20 mmol l^{-1} D-glucose, a low hydrochlorothiazide concentration (0.1 μmol l^{-1}) reduced insulin release by 22%, whereas at higher concentrations no effect was observed.

At lower glucose concentrations (3–8.5 mmol l^{-1}) hydrochlorothiazide (0.1–1000 μmol l^{-1}) had no significant effect on insulin release (data not shown).

Effect on $^{36}Cl^-$ influx

Inhibition of $^{36}Cl^-$ accumulation in the β-cells is associated with reduced insulin release (Sehlin, 1981; Sandström & Sehlin, 1988b; Sandström, 1990). Since hydrochlorothiazide is an inhibitor of Cl^- transport (Na^+, Cl^- co-transport) in other cell types (for review, see O'Grady *et al.*, 1987) its effect on

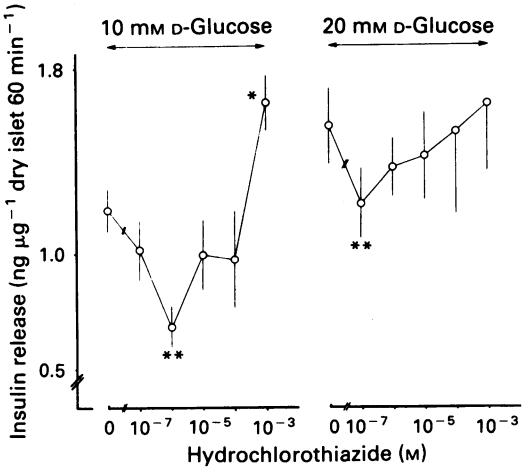


Figure 1 Effect of hydrochlorothiazide on insulin release. After a preliminary incubation for 30 min in basal medium, groups of 2–3 islets were incubated for 60 min in basal medium containing the concentrations of D-glucose and hydrochlorothiazide indicated in the figure. Data are expressed as means ± s.e.mean for 16 experiments. Differences from control were evaluated by Student's *t* test for paired data. $*P<0.05$, $**P<0.025$.

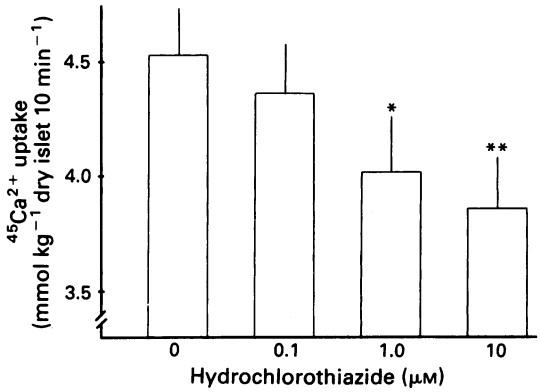


Figure 2 Effect of hydrochlorothiazide on $^{45}Ca^{2+}$ uptake. After a preliminary incubation for 30 min in basal medium, groups of 4–5 islets were incubated for 10 min in basal medium supplemented with $^{45}Ca^{2+}$, 10 mmol l^{-1} glucose and the concentrations of hydrochlorothiazide indicated. Data are expressed as means ± s.e.mean for 20 experiments. Differences from control were evaluated by Student's *t* test for paired data: $*P<0.05$, $**P<0.025$.

$^{36}Cl^-$ influx was also investigated in the β-cells. As shown in Table 1 hydrochlorothiazide had no effect on short-term uptake (3 min) or the accumulation (60 min) of $^{36}Cl^-$ in the islets.

Effect on $^{45}Ca^{2+}$ uptake

Islet uptake of calcium is one of the most distal steps in the stimulus-secretion coupling sequence in the pancreatic β-cells (for review, see Wollheim & Sharp, 1981), and therefore the effect of hydrochlorothiazide on this process was investigated. As shown in Figure 2, the $^{45}Ca^{2+}$ uptake was reduced in a dose-dependent manner by 0.1–10 μmol l^{-1} hydrochlorothiazide.

Discussion

The data in the present work suggest that the adverse effect on carbohydrate metabolism by thiazide diuretics can be mediated by a direct effect on the β-cells. An important observation was that only low concentrations of hydrochlorothiazide inhibited insulin release and that this was observed only when hormone release was stimulated by high (10 and 20 mmol l^{-1}) glucose concentrations. This may help to explain that previous *in vitro* studies with thiazide diuretics showed no effect (Malaisse & Malaisse-Lagae, 1968) or even a stimulation of insulin release (Hermansen *et al.*, 1985). Thus, in the study by Hermansen and co-workers (1985), a

Table 1 Effect of hydrochlorothiazide on $^{36}Cl^-$ influx

	$^{36}Cl^-$ *influx* (mmol kg^{-1} dry islet) *Hydrochlorothiazide* (μmol l^{-1})					
Influx period (min)	*0 (control)*	*0.1*	*1.0*	*10*	*100*	*500*
3 (exptl. series I)	51.65 ± 3.45 (12)	47.32 ± 2.40 (12)	49.62 ± 2.35 (12)	48.89 ± 2.27 (12)		
3 (exptl. series II)	45.05 ± 2.18 (10)			41.02 ± 2.34 (10)	41.061 ± 1.75 (10)	45.59 ± 2.18 (10)
60	82.93 ± 1.78 (14)	80.53 ± 2.50 (14)	84.62 ± 1.40 (12)	80.67 ± 2.63 (14)		

After a preliminary incubation for 30 min in basal medium, groups of 4–5 islets were incubated for 3 or 60 min in basal medium supplemented with $^{36}Cl^-$ and the concentrations of hydrochlorothiazide indicated. Data are expressed as means ± s.e.mean for the number of experiments indicated in parentheses. Differences from control were evaluated by use of Student's *t* test for paired data.

low concentration of thiazide (3 μmol l^{-1}) was tested only at 5.5 mmol l^{-1} D-glucose and no effect was detected. At a higher glucose concentration, where an inhibitory effect of 0.1–1 μmol l^{-1} thiazide was observed in the present study, only high thiazide concentrations (150 μmol l^{-1}) were tested and a stimulation was registered.

In patients treated with hydrochlorothiazide (75 mg/day), the steady state plasma concentration is about 0.4 μmol l^{-1} (Beerman & Groschinsky-Grind, 1978). Therefore, it seems reasonable to believe that the inhibitory effect of low thiazide concentrations on stimulated insulin release observed here could help to explain the adverse action on carbohydrate metabolism seen in clinical practice. Since hydrochlorothiazide never reaches millimolar concentrations in human patients, the stimulatory effect of the high thiazide concentrations on insulin-release is probably not of clinical interest.

The exocytotic release of insulin stimulated by D-glucose is preceded by a number of steps involving depolarization of the plasma membrane by inhibition of K^+ channels, opening of Cl^- channels, and finally the opening of voltage-regulated Ca^{2+} channels, resulting in an increase in $[Ca^{2+}]_i$ (Sehlin, 1978; Wollheim & Sharp, 1981; Pedersen & Findlay, 1987). Thiazide diuretics are sulphonamides, and it is well known that a structurally related compound, diazoxide, hyperpolarizes the β-cells by opening K^+ channels, and that this leads to reduced insulin release (Malaisse & Malaisse-Lagae, 1968; Henquin & Meissner, 1982; Trube *et al.*, 1986). In a recent study it was shown that thiazide diuretics do not affect the activity of K^+ channels in the β-cell plasma membrane (Gillis *et al.*, 1989), and therefore, it seems likely that the inhibitory action of thiazides is not mediated by a hyperpolarization of the β-cell plasma membrane.

It was proposed that the inhibitory effects of loop diuretics (frusemide or bumetanide) on insulin release is due to reduced intracellular chloride levels in the β-cells before stimulation with high glucose concentration (Sandström & Sehlin, 1988b; Sandström, 1990). This reduction is mediated by inhibition of a loop diuretic-sensitive Na^+, K^+, Cl^- co-transport system that functions as a chloride pump in the β-cell plasma membrane (Lindström *et al.*, 1988; Sandström & Sehlin, 1988b; Sandström, 1990; Sandström & Sehlin, 1990). Since thiazide diuretics may reduce chloride transport by inhibition of a Na^+, Cl^- co-transport system (Stokes, 1984), it could be speculated that the inhibitory effect on insulin release is mediated by inhibition of chloride transport. However, in the present study neither the influx nor the accumulation of $^{36}Cl^-$ in the β-cells was affected by hydrochlorothiazide. This shows that the effect of thiazide diuretics on insulin release is not mediated by changed chloride transport and suggests that thiazides and loop diuretics inhibit insulin release by different mechanisms. The lack of effect of hydrochlorothiazide on chloride transport in β-cell-rich islets also shows that a Na^+, Cl^- co-transport system is not present in the β-cell plasma membrane.

The uptake of calcium is one of the distal steps preceding the exocytotic release of insulin (Wollheim & Sharp, 1981). In the present study it was found that the uptake of calcium was significantly reduced by thiazide concentrations that inhibit glucose-induced insulin release. Therefore, it is tempting to speculate that the effect of low concentrations of hydrochlorothiazide on insulin release is, at least in part, mediated by an effect on calcium uptake in the β-cells.

The data in the present study show that thiazide diuretics, in concentrations that are clinically relevant, inhibit glucose-induced insulin release by a direct effect of the pancreatic β-cells. This observation may help to explain the adverse effect on carbohydrate metabolism in human patients. It is suggested that the inhibitory effect of low thiazide concentrations is mediated by an effect on calcium influx in the β-cells.

This work was supported in part by the Swedish Medical Research Council (12x-4756), the Swedish Diabetes Association, Svenska Hoechst AB, the Medical Faculty of Umeå University, and Sahlbergs Fund.

References

AMERY, A., BERTHAUX, P., BULPITT, C., DE SCHAEPDRYVER, A., FAGARD, R., HELLEMANS, J., MUTSERS, A., PERTHAUX, M., DERUYTTERE, C., DOLLERY, C., FORETTE, F., LUND-JOHANSEN, P. & TUOMILEHTO, J. (1978). Glucose intolerance during diuretic therapy. *Lancet*, **i**, 681–683.

BEERMANN, B. & GROSCHINSKY-GRIND, M. (1978). Antihypertensive effect of various doses of hydrochlorothiazide and its relation to the plasma level of the drug. *Eur. J. Clin. Pharmacol.*, **13**, 195–201.

BRASS, E.P. (1984). Effects of antihypertensive drugs on endocrine function. *Drugs*, **27**, 447–458.

FURMAN, B.L. (1981). Impairment of glucose tolerance by diuretics and other drugs. *Pharmacol. Ther.*, **12**, 613–649.

GILLIS, K.D., GEE, W.M., HAMMOUD, A., MCDANIEL, M.L., FALKE, L.C. & MISLER, S. (1989). Effects of sulfonamides on a metabolite-regulated ATP_i sensitive K^+ channel in rat pancreatic β-cells. *Am. J. Physiol.*, **257**, 1119–1127.

GOLDNER, M.G., ZAROWITZ, H. & AKGUN, S. (1960). Hyperglycemia and glucosuria due to thiazide administered in diabetes mellitus. *N. Engl. J. Med.*, **262**, 403–405.

HAHN, H.J., HELLMAN, B., LERNMARK, Å., SEHLIN, & TÄLJEDAL, I.-B. (1974). The pancreatic β-cell recognition of insulin secretagogues: influence of neuroaminidase treatment on the release of insulin and the islet content of insulin, sialic acid, and cyclic adenosine 3':5'-monophosphate. *J. Biol. Chem.*, **249**, 5275–5284.

HEDING, L.A. (1966). A simplified insulin radioimmunoassay. In *Labelled Proteins in Tracer Studies*. ed. Donato, L., Milhaud, G. & Sirchis, L. pp. 345–351. Brussels: Euratom.

HELDERMAN, J.H., ELAHI, D., ANDERSEN, D.K., RAIZES, G.S., TOBIN, J.D., SHOCKEN, D. & ANDRES, R. (1983). Prevention of the glucose intolerance of thiazide diuretics by maintenance of body potassium. *Diabetes*, **32**, 106–111.

HELLMAN, B. (1965). Studies in obese-hyperglycaemic mice. *Ann. N. Y. Acad. Sci.*, **131**, 541–558.

HELLMAN, B., LERNMARK, Å., SEHLIN, J. & TÄLJEDAL, I.-B. (1974). The β-cell recognition of insulin secretagogues: effects of calcium and sodium on glucose metabolism and insulin release. *Biochem. J.*, **138**, 33–45.

HELLMAN, B., SEHLIN, J. & TÄLJEDAL, I.-B. (1976). Effect of glucose on $^{45}Ca^{2+}$ uptake by pancreatic islets as studied with the lanthanum method. *J. Physiol.*, **254**, 639–656.

HENQUIN, J.C. & MEISSNER, H.P. (1982). Opposite effects of tolbutamide and diazoxide on $^{86}Rb^+$ fluxes and membrane potential in pancreatic β-cells. *Biochem. Pharmacol.*, **31**, 1407–1415.

HERMANSEN, K., SCHMITZ, O. & MOGENSEN, C.E. (1985). Effects of a thiazide diuretic (hydroflumethiazide) and a loop diuretic (bumetanide) on the endocrine pancreas: studies in vitro. *Metabolism*, **34**, 784–789.

LINDSTRÖM, P., NORLUND, L., SANDSTRÖM, P.-E. & SEHLIN, J. (1988). Evidence for co-transport of sodium, potassium and chloride in mouse pancreatic islets. *J. Physiol.*, **400**, 223–236.

MALAISSE, W.J. & MALAISSE-LAGAE, F. (1968). Effect of thiazides upon insulin secretion in vitro. *Arch. Int. Pharmacodynam.*, **171**, 235–239.

O'GRADY, S.M., PALFREY, H.C. & FIELD, M. (1987). Characteristics and function of Na-K-Cl cotransport in epithelial tissues. *Am. J. Physiol.*, **253**, 177–192.

PEDERSEN, O.H. & FINDLAY, I. (1987). Electrophysiology of the pancreas. *Physiol. Rev.*, **67**, 1054–1116.

SANDSTRÖM, P.-E. (1990). Bumetanide reduces insulin release by a direct effect on the pancreatic β-cells. *Eur. J. Pharmacol.*, **187**, 377–383.

SANDSTRÖM, P.-E. & SEHLIN, J. (1988a). Furosemide-induced glucose intolerance in mice is associated with reduced insulin secretion. *Eur. J. Pharmacol.*, **147**, 403–409.

SANDSTRÖM, P.-E. & SEHLIN J. (1988b). Furosemide reduces insulin release by inhibition of Cl^- and Ca^{2+} fluxes in β-cells. *Am. J. Physiol.*, **255**, 591–596.

SANDSTRÖM, P.-E. & SEHLIN, J. (1990). Na^+ participates in loop diuretic-sensitive Cl^--cation co-transport in the pancreatic β-cells. *Biochim. Biophys. Acta*, **1023**, 191–196.

SEHLIN, J. (1978). Interrelationship between chloride fluxes in pancreatic islets and insulin release. *Am. J. Physiol.*, **235**, 501–508.

SEHLIN, J. (1981). Are Cl^- mechanisms in mouse pancreatic islets involved in insulin release? *Upsala J. Med. Sci.*, **86**, 177–182.

STOKES, J.B. (1984). Sodium chloride absorbtion by the urinary bladder of the winter flounder. A thiazide-sensitive, electrically neutral transport system. *J. Clin. Invest.*, **74**, 7–16.

TRUBE, G., RORSMAN, P. & OHNO-SHOSAKU, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β-cells. *Pflügers Arch.*, **407**, 493–499.

WILKINS, R.W. (1959). New drugs for the treatment of hypertension. *Ann. Int. Med.*, **50**, 1–10.

WOLLHEIM, C. & SHARP, G.W.G. (1981). Regulation of insulin release by calcium. *Physiol. Rev.*, **61**, 914–973.

(Received May 7, 1993
Revised July 30, 1993
Accepted August 3, 1993)

Br. J. Pharmacol. (1993), **110**, 1363–1368 © Macmillan Press Ltd, 1993

Effects of atrial natriuretic peptide and nitroprusside on isolated pulmonary resistance and conduit arteries from rats with pulmonary hypertension

[1]Janet C. Wanstall, *Justine S. Thompson & *Alyn H. Morice

Department of Physiology and Pharmacology, The University of Queensland, Brisbane, Australia, 4072 and *Department of Medicine and Pharmacology, University of Sheffield, Sheffield, U.K.

1 The relaxant effects of atrial natriuretic peptide (ANP) and nitroprusside were studied on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted preparations of pulmonary resistance vessels (internal diameter 200–500 μm) and main pulmonary arteries taken from rats with pulmonary hypertension induced by monocrotaline (105 mg kg^{-1}, s.c., 4 weeks previously). Control rats received saline.

2 In preparations from monocrotaline-treated rats, the potencies (negative log EC_{50}) of ANP on resistance vessels (8.45) and main pulmonary arteries (8.25) were similar to those obtained in vessels from control rats (8.78 and 8.53 respectively), whereas those of nitroprusside were significantly less than in controls in both resistance vessels (7.13 compared with 7.63 in controls; three fold decrease in potency) and main pulmonary arteries (6.92 compared with 8.17 in controls; 18 fold decrease in potency).

3 On pulmonary resistance vessels from monocrotaline-treated rats, the maximum relaxant responses to ANP and nitroprusside, and also to pinacidil, were increased compared with controls, and reversal of the $PGF_{2\alpha}$-induced contraction by these drugs was greater than 100%. In contrast, on main pulmonary arteries from monocrotaline-treated rats, the maximum relaxations to ANP and nitroprusside were not increased relative to controls, and reversal of $PGF_{2\alpha}$ was not greater than 100%.

4 Since the high potency of ANP on pulmonary resistance and conduit arteries is retained in vessels from rats with pulmonary hypertension, whether induced by monocrotaline (this study) or by chronic hypoxia (previous findings), it is postulated that elevation of plasma levels of ANP by use of drugs that inhibit the breakdown of this endogenous peptide, may be one approach to the pharmacological treatment of pulmonary hypertension.

Keywords: Atrial natriuretic peptide; nitroprusside; pulmonary resistance vessels; pulmonary artery; pulmonary hypertension; monocrotaline; myograph

Introduction

In animals with experimental pulmonary hypertension the responsiveness of pulmonary arteries to vasodilator drugs differs from that seen in normal pulmonary arteries (Altiere *et al.*, 1986; Barer *et al.*, 1989; Wanstall & O'Donnell, 1992). The changes in responsiveness associated with pulmonary hypertension vary depending on the mechanism of action of the vasodilator drug (Wanstall & O'Donnell, 1992). It is possible that they may also depend on the method used to induce pulmonary hypertension, and may differ in resistance and conduit arteries. Each of these possibilities has been considered in the present study in which two potent pulmonary vasodilators, atrial natriuretic peptide (ANP) and nitroprusside, have been examined on pulmonary resistance vessels and main pulmonary arteries taken from rats with monocrotaline-induced pulmonary hypertension.

Both of the vasodilators studied act via the same second messenger, guanosine 3′:5′-cyclic monophosphate (cyclic GMP) but activate different enzymes, viz. particulate and soluble guanylate cyclase, respectively (Rapoport & Murad, 1983; Waldman *et al.*, 1984). ANP was of particular interest because circulating levels of this peptide are increased in pulmonary hypertension, in both animals (McKenzie *et al.*, 1986; Winter *et al.*, 1989) and man (Adnot *et al.*, 1987; Morice *et al.*, 1990), and hence it is possible that this endogenous vasodilator may have a pathophysiological role in counteracting elevations in pulmonary artery pressure.

In *in vivo* experiments (Jin *et al.*, 1988) and in perfused lungs (Jin *et al.*, 1988; Stewart *et al.*, 1991) and isolated resistance vessels (Rogers *et al.*, 1992), the pulmonary vasorelaxant effects of ANP were greater in rats with chronic hypoxic pulmonary hypertension than in control rats. The first aim of the present study was to determine whether responses of pulmonary arteries to ANP were likewise increased when pulmonary hypertension was induced by the toxic plant alkaloid, monocrotaline, instead of chronic hypoxia.

Nitroprusside was studied because the potency of this and other nitrodilators was markedly reduced in isolated main pulmonary artery preparations taken from rats with monocrotaline-induced pulmonary hypertension, when compared with controls (Wanstall & O'Donnell, 1992). The second aim of the study was to determine whether this finding extended to pulmonary resistance vessels.

A preliminary account of some of these data has been presented to a meeting of the British Pharmacological Society (Thompson *et al.*, 1991).

Methods

Pretreatment of animals

Male Wistar rats, aged 6–7 weeks, were treated with a single subcutaneous injection of monocrotaline (105 mg kg^{-1}) or saline (1 ml kg^{-1}; control). Four weeks later the rats were weighed, anaesthetized with pentobarbitone (90 mg kg^{-1}, i.p.) and exsanguinated, via the vena cava, into a heparinized syringe. A blood sample was retained for determination of haematocrit. The main pulmonary artery, the heart and the lungs were then removed.

[1] Author for correspondence.

The heart was dissected, blotted and weighed to determine the ratios: weight of right ventricle:weight of left ventricle plus septum (RV/LV + S) and weight of right ventricle:body weight. Increases in these values were taken as indicating the presence of right ventricular hypertrophy. In previous studies, right ventricular hypertrophy has been shown to be a reliable index of the existence of pulmonary hypertension in rats (Wanstall & O'Donnell, 1990; 1992; Wanstall *et al.*, 1991).

Pulmonary resistance vessel preparations

Pulmonary vascular resistance vessels, internal diameter 200–500 μm, were dissected out from the bronchovascular bundle of the left lung. Ring preparations, 3 mm in length and with the endothelium intact, were mounted on 40 μm stainless steel wires in an automated small vessel myograph (Cambustion, Cambridge, U.K.). The tissue chamber of the myograph contained physiological salt solution (PSS) at 37°C bubbled with 95% O_2:5% CO_2. A passive force equivalent to a transmural pressure of 17.5 mmHg was applied to preparations from saline-treated rats (Rogers *et al.*, 1992). A higher passive force (equivalent to 35 mmHg) was applied to preparations from monocrotaline-treated rats, in order to reflect the higher pulmonary artery pressure, and the associated higher circumferential wall tension, found *in vivo* in rats treated with monocrotaline compared with controls (Wanstall & O'Donnell, 1990; 1992). These passive forces were determined for each individual preparation from passive length-tension curves and the Laplace equation, using the software associated with the automated myograph, and were 3.9 ± 0.27 mN ($n = 13$) and 6.8 ± 0.67 mN ($n = 10$) in preparations from saline- and monocrotaline-treated rats respectively. Active force was recorded isometrically.

Main pulmonary artery preparations

Ring preparations of main pulmonary artery, 3 mm in length and with the endothelium intact, were mounted around two stainless steel wires in a vertical organ bath containing PSS at 37°C. Passive forces were 10 mN and 20 mN in preparations from saline-treated and monocrotaline-treated rats, respectively. These forces, which again reflected the higher pulmonary artery pressure *in vivo* in monocrotaline-treated rats compared with controls, were those determined from passive length-tension curves in previous studies (Wanstall & O'Donnell, 1990). Active force was recorded isometrically using a Statham Universal Transducer (UC3 + UL5) attached to a micrometer (Mitutoyo, Tokyo, Japan).

Experimental protocols

One main pulmonary artery preparation and one or two resistance vessel preparations were obtained from each rat. All preparations were allowed to equilibrate for 1 h before starting the experiment.

On resistance vessel preparations the experimental protocol, selected on the basis of preliminary experiments, was as follows: a cumulative concentration-response (relaxation) curve to ANP (0.02 to 200 nM) or nitroprusside (2 nM to 6 μM) was determined on preparations submaximally contracted with 20 μM $PGF_{2\alpha}$; a contraction was then obtained in response to potassium depolarizing PSS (in which 80 mM NaCl was replaced with 80 mM KCl); a relaxant response to acetylcholine (ACh, 1 μM) was then evoked on preparations contracted with 20 μM $PGF_{2\alpha}$ to confirm the presence of a functional endothelium. Between each of these three components, the tissues were washed for 20–30 min in PSS. The submaximal contractions to 20 μM $PGF_{2\alpha}$ were ($n = 9$): control 2.30 ± 0.23 mN; monocrotaline 2.11 ± 0.50 mN ($P > 0.05$).

On main pulmonary artery preparations, the components of the protocol were the same as on resistance vessels but the sequence was different and was based on that used in previous studies on this preparation (Wanstall & O'Donnell, 1992), viz. a relaxant response to ACh was induced on preparations contracted with 0.1 μM noradrenaline; a contraction was obtained in response to potassium depolarizing PSS; a concentration-response curve was then determined for ANP (0.1 to 100 nM) or nitroprusside (1 nM to 3 μM) on preparations submaximally contracted with 20 μM $PGF_{2\alpha}$. The submaximal contractions to 20 μM $PGF_{2\alpha}$ were ($n = 12$): control 13.3 ± 1.2; monocrotaline 24.3 ± 1.6 mN. Because the size of the contraction was greater ($P < 0.05$) in preparations from monocrotaline-treated rats than controls, some additional preparations from monocrotaline-treated rats were studied in which a smaller contraction was induced by use of a lower concentration of $PGF_{2\alpha}$ viz. 5 μM.

Only one of the vasodilator drugs was examined on any one preparation. When two resistance vessel preparations were obtained from one animal, ANP was examined on one preparation and nitroprusside on the other.

In most of the experiments, on both resistance vessels and main pulmonary arteries, pinacidil (100 μM) was added when the maximal relaxation to ANP or nitroprusside was reached.

Preliminary experiments

The pulmonary artery preparations from control and monocrotaline-treated rats were set up under different passive forces in order to reflect the different pulmonary artery pressures in the two groups of rats (see above). In separate experiments, resistance vessels from control rats were set up at 35 mmHg, i.e. the passive force used throughout the study for preparations from monocrotaline-treated rats, and resistance vessels from monocrotaline-treated rats were set up at 17.5 mmHg, i.e. the passive force used for control preparations. Data for ANP and nitroprusside obtained in these preliminary experiments confirmed that the differences in passive force did not, in themselves, affect the potency or maximum relaxation to the vasodilator drugs on resistance vessels. Previous studies have verified this for main pulmonary artery preparations (Wanstall *et al.*, 1991; 1992; Wanstall & O'Donnell, 1992).

Analysis of data

Contractile responses to $PGF_{2\alpha}$ were expressed as force (mN). Relaxant responses to ANP, nitroprusside or pinacidil were expressed as a percentage of the $PGF_{2\alpha}$-induced contraction (% reversal of $PGF_{2\alpha}$). Thus 100% 'reversal' represents relaxation back to the baseline before the addition of $PGF_{2\alpha}$. Whenever 'reversal' was greater than 100% it indicated that the preparations had inherent tone before the addition of $PGF_{2\alpha}$, and that the relaxant drug had reversed not only the contraction to $PGF_{2\alpha}$ but also at least some of the inherent tone. The potencies of ANP and nitroprusside were expressed

Table 1 Comparison of rats 4 weeks after a single s.c. injection of monocrotaline (MCT, 105 mg kg^{-1}) or saline (Control, 1 ml kg^{-1})

	Control ($n = 15$)	*MCT* ($n = 15$)
Body weight before injection (g)	255 ± 6.9	238 ± 8.1
Weight gain after 4 weeks (g)	166 ± 7.6	34 ± 15.5***
RV/(LV + S)	0.29 ± 0.01	0.65 ± 0.04***
RV/body weight (mg/g)	0.50 ± 0.02	1.29 ± 0.09***
Haematocrit (%)	44 ± 0.6	46 ± 1.2

Values are means ± s.e.mean. n = number of rats.
*** Values significantly different from corresponding value in control rats; $P < 0.001$ (Student's t test).
RV = weight of right ventricle (mg); LV + S = weight of left ventrical + septum (mg).

Table 2 Potency (negative log EC_{50}) of atrial natriuretic peptide (ANP) and nitroprusside on preparations of pulmonary resistance vessel and main pulmonary artery from rats treated with saline (Control) or monocrotaline (MCT)

	Negative log EC_{50}			
	ANP		*Nitroprusside*	
	Control[a]	*MCT*[b]	*Control*[a]	*MCT*[b]
Resistance vessel	8.78 ± 0.25 (5)	8.45 ± 0.14 (5)	7.63 ± 0.11 (4)	7.13 ± 0.17* (4)
Main pulmonary a.	8.53 ± 0.03 (6)	8.25 ± 0.15 (6)	8.17 ± 0.07 (6)	6.92 ± 0.13*** (6)

Preparations were contracted with prostaglandin $F_{2\alpha}$ (20 μM).
Values are means ± s.e.mean. Number of rats in parentheses.
[a]Rats received saline (1 ml kg^{-1}, s.c., 4 weeks previously).
[b]Rats received MCT (105 mg kg^{-1}, s.c., 4 weeks previously).
*Value significantly lower than corresponding value in control rats; $0.05 > P > 0.01$; ***$P < 0.001$ (Student's t test).

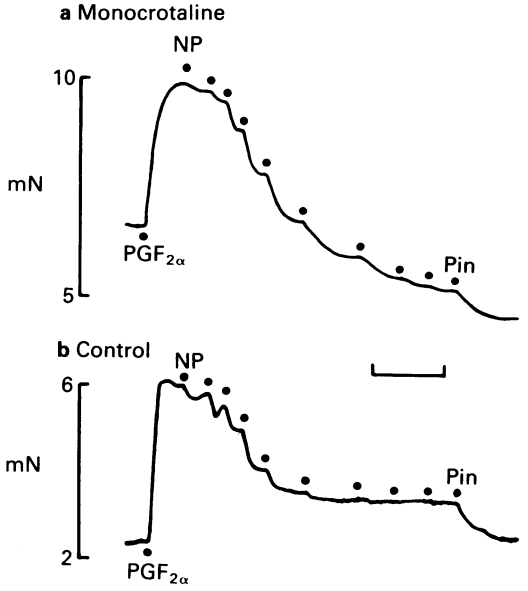


Figure 1 Pulmonary resistance vessel preparations from a monocrotaline-treated rat (a) and a saline-treated rat (b) set up in a single organ bath. Preparations were contracted with 20 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Concentration-response curves to nitroprusside (NP) are shown (NP concentration-range 2 nM to 20 μM; three fold increments). At the end of the curve 100 μM pinacidil (Pin) was added. Time scale = 10 min.

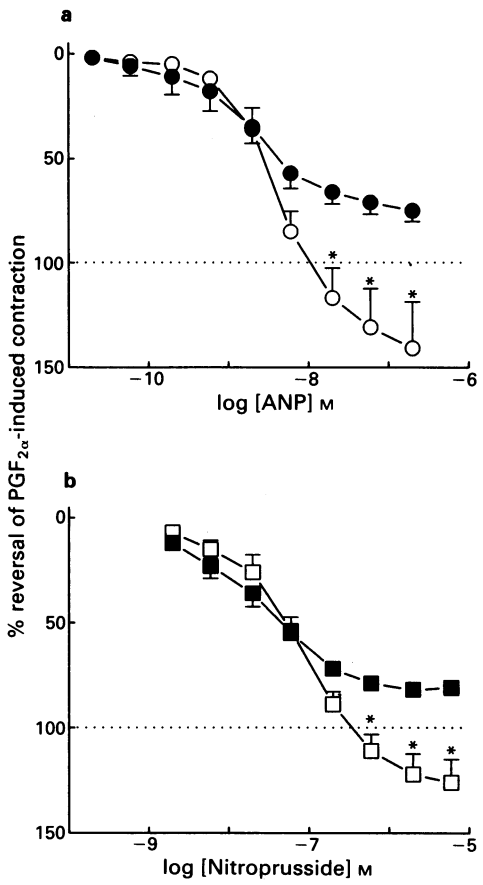


Figure 2 Pulmonary resistance vessels: mean concentration-response (relaxation) curves to (a) atrial natriuretic peptide (ANP) and (b) nitroprusside on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted preparations from rats treated with saline (closed symbols) or monocrotaline (open symbols) 4 weeks before the experiment. Relaxant responses are expressed as percentage reversal of the contraction induced by $PGF_{2\alpha}$ (20 μM). Points are mean values, with s.e.means shown by the vertical lines except when smaller than the symbols. An asterisk indicates that the relaxant response was significantly greater than the corresponding response in the control preparations. $P < 0.05$ (Mann-Whitney U test). For numbers of observations see Table 2.

as the negative log EC_{50}, where EC_{50} is the concentration producing 50% of the maximum response to the particular agent.

Drugs and solutions

The agents used were: acetylcholine chloride (ACh, Sigma), atrial natriuretic peptide (human, ANP, Sigma), monocrotaline (crotaline, MCT, Sigma), (−)-noradrenaline acid tartrate (Sigma), pinacidil (racemic, gift from Leo Pharmaceuticals), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Sigma), sodium nitroprusside (Sigma).

Solutions of monocrotaline (105 mg ml^{-1}) were prepared as the hydrochloride as follows: 500 mg monocrotaline was dissolved in 1.5 ml 1 M HCl, 1 ml of deionized water was added and the pH adjusted to 7.0 with 1 M NaOH. Deionized water was added to make a final volume of 4.76 ml. Solutions of other agents were prepared as follows: ACh (10 mM), $PGF_{2\alpha}$ (10 mM) and sodium nitroprusside (10 mM) in deionized water; ANP (100 μM) in PSS, pinacidil (5 mM) and noradrenaline (10 mM) in 10 mM HCl. Subsequent dilutions were made in PSS.

The composition of the PSS was (mM): NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7, ascorbic acid 1.14 (95% O_2/5% CO_2; pH 7.4).

Statistical analyses

Mean values were calculated from data obtained from preparations from a number (n) of different animals and are

quoted with their standard errors. The significance of differences between mean values was determined by Student's t test (negative log EC_{50} and mN values) or the Mann Whitney U-test (percentage values).

Results

Effects of monocrotaline treatment on rats

Rats treated with monocrotaline gained significantly less weight during the 4 weeks after injection than did control rats treated with saline (Table 1). The ratios RV/LV + S and RV/body weight were significantly greater in monocrotaline-treated rats than in control rats (Table 1), i.e. the rats had right ventricular hypertrophy indicating the presence of pulmonary hypertension. The haematocrit was the same in both groups of rats (Table 1) indicating that the monocrotaline treatment did not cause polycythemia.

Pulmonary resistance vessels and main pulmonary arteries from the monocrotaline-treated rats appeared to have a functional endothelium in that relaxation to ACh was seen in seven out of nine resistance vessel preparations (42 ± 8.2% reversal of $PGF_{2\alpha}$ compared with 66 ± 4.3% in 8/9 control preparations), and in 11 out of 12 main pulmonary artery preparations (18 ± 2.8% reversal of noradrenaline compared with 23 ± 5.5% in 10/12 controls).

Relaxant responses to ANP and nitroprusside on pulmonary artery preparations submaximally contracted with $PGF_{2\alpha}$

Potency The potency (negative log EC_{50}) of ANP on both pulmonary resistance vessels and main pulmonary arteries was unchanged by monocrotaline pretreatment of the rats (Table 2). In contrast, the potency of nitroprusside was significantly lower in preparations from monocrotaline-treated rats, compared with controls (Table 2). The difference in the potency of nitroprusside between the two groups of rats was seen in both resistance vessels (three fold difference) and main pulmonary arteries (18 fold difference) (Table 2). When main pulmonary artery preparations from monocrotaline-treated rats were contracted with 5 μM, instead of 20 μM, $PGF_{2\alpha}$, so that the actual size of the submaximal contraction was closer to that obtained in control rats, the potency of nitroprusside (negative log EC_{50} 6.90 ± 0.15, $n = 3$) was still 18 fold less than in controls.

Maximum relaxation On resistance vessels from control rats, neither ANP nor nitroprusside completely reversed the $PGF_{2\alpha}$-induced contractions (Figures 1 and 2), i.e. maximum relaxations were less than 100% reversal of $PGF_{2\alpha}$ (Table 3). In resistance vessels from monocrotaline-treated rats, maximum relaxations to both ANP and nitroprusside were significantly greater than in control rats and were greater than 100% reversal of $PGF_{2\alpha}$ (Figure 2; Table 3). In resistance vessels from both groups of rats pinacidil (100 μM), added once maximum relaxation to ANP or nitroprusside had been attained, always caused further relaxation (Figure 1). The relaxation to 100 μM pinacidil was significantly greater in preparations from monocrotaline-treated rats than controls (Table 3).

Table 3 Maximum relaxation to atrial natriuretic peptide (ANP), nitroprusside and pinacidil (Pin) in preparations of pulmonary resistance vessel and main pulmonary artery from rats treated with saline (Control) or monocrotaline (MCT)

	Maximum relaxation % reversal of $PGF_{2\alpha}$	
	Control[a]	*MCT*[b]
Resistance vessel		
ANP	75 ± 5.2 (5)	141 ± 22.2* (5)
Nitroprusside	82 ± 3.1 (4)	126 ± 11.1* (4)
Pinacidil[c]	105 ± 4.7 (9)	190 ± 21.1* (9)
Main pulmonary artery		
ANP	70 ± 1.9 (6)	36 ± 7.3† (6)
Nitroprusside	100 ± 1.1 (6)	64 ± 6.1† (6)
Pinacidil[c]	101 ± 1.1 (8)	106 ± 2.9 (7)

Preparations contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 20 μM).
Values are means ± s.e.mean. Numbers of rats in parentheses. Responses are expressed as a percentage of the $PGF_{2\alpha}$-induced contraction (% reversal of $PGF_{2\alpha}$).
[a]Rats received saline (1 ml kg^{-1} s.c., 4 weeks previously).
[b]Rats received MCT (105 mg kg^{-1} s.c., 4 weeks previously).
[c]Response to 100 μM pinacidil added at the end of some of the concentration-response curves to ANP or nitroprusside.
*Value significantly higher than corresponding value in control rats; $P < 0.05$ (Mann-Whitney U test).
†Value significantly lower than corresponding value in control rats; $P < 0.05$ (Mann-Whitney U test).

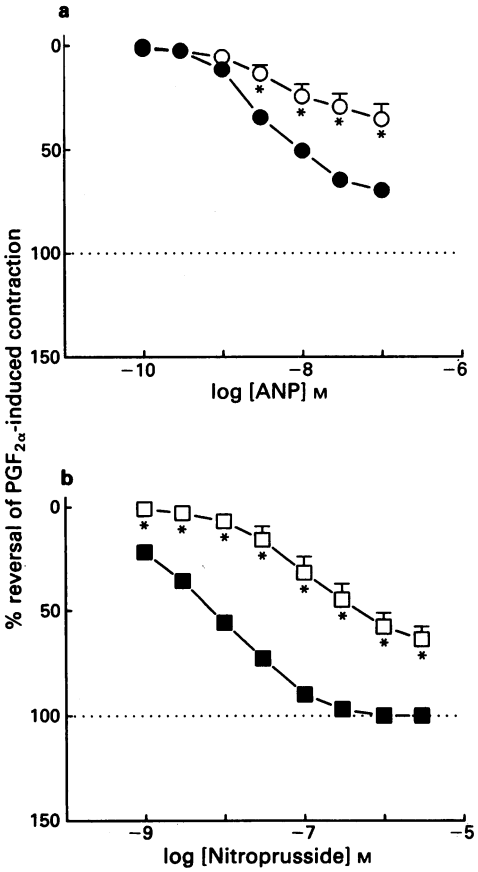


Figure 3 Main pulmonary artery: mean concentration-response (relaxation) curves to (a) atrial natriuretic peptide (ANP) and (b) nitroprusside on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-contracted preparations taken from rats treated with saline (closed symbols) or monocrotaline (open symbols) 4 weeks before the experiment. Relaxant responses are expressed as percentage reversal of the contraction induced by $PGF_{2\alpha}$ (20 μM). Points are mean values, with s.e.means shown by the vertical lines except when smaller than the symbols. An asterisk indicates that the relaxant response was significantly less than the corresponding response in the control preparations ($P < 0.05$, Mann-Whitney U test). For numbers of observations see Table 2.

In main pulmonary arteries from control rats, the contractions to $PGF_{2\alpha}$ were completely reversed by nitroprusside but not by ANP (Figure 3; Table 3). In contrast to the data on resistance vessels (see above), there was no increase in the maximum relaxation to either nitroprusside or ANP in main pulmonary artery preparations from monocrotaline-treated rats. Maximum relaxations were either less than, or similar to, control values, depending on whether the preparations were contracted with 20 μM $PGF_{2\alpha}$ (Figure 3; Table 3) or with 5 μM $PGF_{2\alpha}$ (nitroprusside 86 $\pm$ 6.8% reversal, $n = 3$; ANP 78 and 70% reversal, $n = 2$). Pinacidil (100 μM), added at the end of the curves to either ANP or nitroprusside, caused complete reversal of the contractions in preparations from hypoxic and control rats (Table 3).

Discussion

This study has shown that in rats with monocrotaline-induced pulmonary hypertension, when compared with controls, (a) the potency of ANP on pulmonary resistance vessels and main pulmonary artery was unchanged whilst that of nitroprusside was reduced, and (b) the maximum relaxation to both drugs was increased in resistance vessels but not in the main pulmonary artery. These observations confirm the view that the effect of pulmonary hypertension on the responsiveness of pulmonary arteries to vasodilator drugs varies depending on the mechanism of action of the drug. They also indicate that the changes in pulmonary vascular reactivity in pulmonary hypertension are not identical in resistance vessels and conduit arteries.

The finding that ANP was equally potent in preparations from rats with monocrotaline-induced pulmonary hypertension and control rats confirmed previous findings on pulmonary resistance vessels from rats with hypoxic pulmonary hypertension (Rogers *et al.*, 1992). Hence the existence of pulmonary hypertension of diverse aetiologies does not appear to lead to a reduction in the potency of ANP on pulmonary vascular smooth muscle. Thus, despite the increase in plasma levels of this peptide seen in pulmonary hypertension in animals and man (Mackenzie *et al.*, 1986; Adnot *et al.*, 1987; Winter *et al.*, 1989; Morice *et al.*, 1990), there is no evidence for concomitant desensitization of the vascular smooth muscle to ANP.

The lack of desensitization to ANP contrasted with the finding that the potency of nitroprusside was reduced. A reduction in the potency of nitroprusside has previously been described on main pulmonary arteries contracted with noradrenaline, from rats with both monocrotaline-induced and chronic hypoxic pulmonary hypertension (Wanstall & O'Donnell, 1992). The present findings, on preparations contracted with $PGF_{2\alpha}$, confirm that this effect of pulmonary hypertension on nitroprusside is independent of the spasmogen used, and demonstrate that it is also seen in pulmonary resistance vessels. It was noted that the extent of the reduction in potency was less in resistance vessels (three fold) than in the main pulmonary artery (18 fold).

The mechanism for the loss of potency of nitroprusside is not known. Nitroprusside causes vasorelaxation by activation of soluble guanylate cyclase and consequent elevation of cytosolic cyclic GMP (Ignarro & Kadowitz, 1985). Since no loss in potency was seen for ANP, which also acts through cyclic GMP but by activation of particulate guanylate cyclase (Tremblay & Hamet, 1989), it is unlikely that there is decreased responsiveness of the smooth muscle to the vasorelaxant effect of cyclic GMP. It is more likely that the sensitivity of soluble guanylate cyclase is impaired, as suggested by Wanstall & O'Donnell (1992). This view is supported by data from a recent study in rats with hypoxic pulmonary hypertension (Crawley *et al.*, 1992). These authors showed that the production of cyclic GMP by acetylcholine and by nitroprusside was less in pulmonary arteries from hypoxic rats than in controls, while the activation of cyclic GMP-dependent protein kinase (G-kinase, the enzyme on which cyclic GMP is believed to act to produce vasorelaxation) by exogenous cyclic GMP was no different in pulmonary arteries from hypoxic and control rats. These findings provided evidence that the responsiveness of soluble guanylate cyclase may be reduced in pulmonary arteries from rats with hypoxic pulmonary hypertension, and it is possible that this could also occur in monocrotaline-induced pulmonary hypertension. One hypothesis that has been proposed is that elevated pulmonary artery pressure may lead to increased production of nitric oxide from the endothelium and that this may cause desensitization of soluble guanylate cyclase (Wanstall *et al.*, 1992; Crawley *et al.*, 1992).

Although there was no change in the potency of ANP in the monocrotaline-treated rats, there were changes in the maximal relaxant responses to this peptide, when compared with controls. In resistance vessels maximal relaxation was increased such that reversal of the $PGF_{2\alpha}$ contraction was greater than 100%. This indicated that preparations from monocrotaline-treated rats had inherent tone, and that ANP could reverse not only the contraction to $PGF_{2\alpha}$ but also at least part of the inherent tone. Maximal relaxations to nitroprusside and pinacidil were also increased. Since ANP, nitroprusside and pinacidil each relax vascular smooth muscle by different mechanisms (Tremblay & Hamet, 1989; Ignarro & Kadowitz, 1985; Southerton *et al.*, 1988) this increase in maximum relaxation appears to be non-specific, and may simply reflect the inherent tone in the resistance vessels from monocrotaline-treated rats.

In the main pulmonary artery from monocrotaline-treated rats, in contrast to resistance vessels, there was no increase in maximum relaxation to either ANP or nitroprusside, and reversal of the $PGF_{2\alpha}$-induced contraction was not greater than 100%. On the contrary, maximum relaxation to these two vasodilators was reduced compared with controls. The decreases in maximum relaxation may have occurred because contractions to the selected concentration of $PGF_{2\alpha}$ (20 μM) were larger in main pulmonary artery from monocrotaline-treated rats than in the corresponding control preparations. This possibility is supported by the finding that when a smaller contraction was induced in preparations from the monocrotaline group, by using a lower concentration of $PGF_{2\alpha}$ (5 μM), the decrease in maximum relaxation was no longer seen for ANP and was minimal for nitroprusside. However these data for main pulmonary artery still differed from resistance vessels in that the values for maximum relaxation were neither increased, relative to control values, nor greater than 100%.

In summary, this study has shown that the effect of monocrotaline-induced pulmonary hypertension on the reactivity of rat pulmonary resistance vessels to ANP, viz. an increase in the size of the relaxant responses reflecting an increase in maximum relaxation with no change in potency, is the same as that seen in chronic hypoxic pulmonary hypertension (Rogers *et al.*, 1992). This finding, together with previous findings for pinacidil and nitroprusside on main pulmonary artery (Wanstall & O'Donnell, 1992), suggests that the functional changes in pulmonary vascular smooth muscle in pulmonary hypertension are related to the elevated pulmonary artery pressure and are independent of the method of inducing the pulmonary hypertension. The study has also shown that monocrotaline-induced pulmonary hypertension is associated with a reduction in the potency of nitroprusside in resistance vessels as well as in main pulmonary arteries. However this reduction was smaller in the resistance vessels than in the main pulmonary artery, and was associated with an increase in maximum relaxation not seen in the larger vessels. Hence the functional changes in pulmonary resistance vessels in pulmonary hypertension are not necessarily identical to those seen in conduit arteries.

It has already been postulated that ANP may have a pathophysiological role in pulmonary hypertension (Adnot *et al.*, 1987). The finding that ANP retains its high potency in

resistance and conduit vessels from pulmonary hypertensive rats, irrespective of the cause of the pulmonary hypertension, raises the possibility that this peptide might also have a pharmacological role in the treatment of this disease. ANP itself is unlikely to be of therapeutic benefit because of its rapid degradation by neutral endopeptidases and removal from the circulation by low affinity binding sites (clearance receptors) in the vasculature. However, drugs which inhibit degradation of ANP are currently under development, and preliminary studies with the neutral endopeptidase inhibitors, candoxatrilat (UK 73,967) and SCH 34826 in rats with hypoxic pulmonary hypertension have demonstrated amelioration of pulmonary vascular remodelling (Winter *et al.*, 1991; Stewart *et al.*, 1992). The pharmacological elevation of plasma levels of endogenous ANP in the pulmonary artery may offer some hope for the treatment of patients with pulmonary hypertension.

This research was supported by the National Health and Medical Research Council of Australia. J.C.W. is an NH&MRC Senior Research Fellow. We are grateful to Cambustion, Cambridge, U.K. for the generous loan of an AM10 myograph for use in Australia while this study was in progress. We also thank Agatha Gambino for assistance with some of the experiments, and Leo Pharmaceuticals, Denmark for the gift of pinacidil.

References

ADNOT, S., CHABRIER, P.E., ANDRIVET, P., VIOSSAT, I., PIQUET, J., BRUN-BUISSON, C., GUTKOWSKA, Y. & BRAQUET, P. (1987). Atrial natriuretic peptide concentrations and pulmonary haemodynamics in patients with pulmonary artery hypertension. *Am. Rev. Resp. Dis.*, **136**, 951–956.

ALTIERE, R.J., OLSON, J.W. & GILLESPIE, M.N. (1986). Altered vascular smooth muscle responsiveness in monocrotaline-induced pulmonary hypertension. *J. Pharmacol. Exp. Ther.*, **236**, 390–395.

BARER, G.R., CAI, Y., RUSSELL, P.C. & EMERY, C.J. (1989). Reactivity and site of vasomotion in pulmonary vessels of chronically hypoxic rats; relation to structural changes. *Am. Rev. Resp. Dis.*, **140**, 1483–1485.

CRAWLEY, D.E., ZHAO, L., GIEMBYCZ, M.A., LIU, S., BARNES, P.J., WINTER, R.J.D. & EVANS, T.W. (1992). Chronic hypoxia impairs soluble guanylate cyclase-mediated pulmonary artery relaxation in the rat. *Am. J. Physiol.*, **263**, L325–L332.

IGNARRO, L.J. & KADOWITZ, P.J. (1985). The pharmacological and physiological role of cyclic GMP in vascular smooth muscle. *Annu. Rev. Pharmacol. Toxicol.*, **25**, 171–191.

JIN, H., YANG, R.H., THORNTON, R.M., CHEN, Y.F., JACKSON, R. & OPARIL, S. (1988). Atrial natriuretic peptide lowers pulmonary artery pressure in hypoxia adapted rats. *J. Appl. Physiol.*, **65**, 1729–1735.

MCKENZIE, J.C., TANAKA, I., INAGAMI, T., MISONI, K.S. & KLEIN, R.M. (1986). Alterations in atrial and plasma atrial natriuretic factor (ANF) content during development of hypoxia-induced pulmonary hypertension in the rat. *Proc. Soc. Exp. Biol. Med.*, **181**, 459–463.

MORICE, A.H., PEPKE-ZABA, J., BROWN, M.J., THOMAS, P.S. & HIGENBOTTAM, T.W. (1990). Atrial natriuretic peptide in primary pulmonary hypertension. *Eur. Resp. J.*, **3**, 910–913.

RAPOPORT, R.M. & MURAD, F. (1983). Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role of cyclic GMP. *J. Cyclic Nucleotide Protein Phosphor. Res.*, **9**, 281–296.

ROGERS, T.K., STEWART, A.G. & MORICE, A.H. (1992). Effect of chronic hypoxia on rat pulmonary resistance vessels: vasodilation by atrial natriuretic peptide. *Clin. Sci.*, **83**, 723–729.

SOUTHERTON, J.S., WESTON, J.H., BRAY, K.M., NEWGREEN, D.T. & TAYLOR, S.G. (1988). The potassium channel opening action of pinacidil: studies using biochemical, ion flux and microelectrode techniques. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 310–318.

STEWART, A.G., SHEEDY, W., THOMPSON, J.S. & MORICE, A.H. (1992). Effects of SCH 34826, a neutral endopeptidase inhibitor, on hypoxic pulmonary vascular remodelling. *Pulm. Pharmacol.*, **5**, 111–114.

STEWART, A.G., THOMPSON, J.S., ROGERS, T.K. & MORICE, A.H. (1991). Atrial natriuretic peptide-induced relaxation of preconstricted isolated rat perfused lungs: a comparison in control and hypoxia-adapted animals. *Clin. Sci.*, **81**, 201–208.

THOMPSON, J.S., WANSTALL, J.C. & MORICE, A.H. (1991). The relaxant effects of atrial natriuretic peptide on pulmonary arteries from rats with monocrotaline-induced pulmonary hypertension. *Br. J. Pharmacol.*, **104**, 112P.

TREMBLAY, J. & HAMET, P. (1989). Biochemical mechanisms of atrial natriuretic factor action. *Can. J. Physiol. Pharmacol.*, **67**, 1124–1129.

WALDMAN, S.A., RAPOPORT, R.M. & MURAD, F. (1984). Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. *J. Biol. Chem.*, **259**, 14332–14334.

WANSTALL, J.C., HUGHES, I.E. & O'DONNELL, S.R. (1992). Reduced relaxant potency of nitroprusside on pulmonary artery preparations taken from rats during the development of hypoxic pulmonary hypertension. *Br. J. Pharmacol.*, **107**, 407–413.

WANSTALL, J.C. & O'DONNELL, S.R. (1990). Endothelin and 5-hydroxytryptamine on rat pulmonary artery in pulmonary hypertension. *Eur. J. Pharmacol.*, **176**, 159–168.

WANSTALL, J.C. & O'DONNELL, S.R. (1992). Responses to vasodilator drugs on pulmonary artery preparations from pulmonary hypertensive rats. *Br. J. Pharmacol.*, **105**, 152–158.

WANSTALL, J.C., O'DONNELL, S.R. & KAY, C.S. (1991). Increased relaxation by felodipine on pulmonary artery from rats with monocrotaline-induced pulmonary hypertension does not reflect functional impairment of the endothelium. *Pulm. Pharmacol.*, **4**, 60–66.

WINTER, R.J.D., MELEAGROS, L., PERVEZ, S., JAMAL, H., KRAUSZ, T., POLAK, J.M. & BLOOM, S.R. (1989). Atrial natriuretic peptide levels in plasma and in cardiac tissues after chronic hypoxia in rats. *Clin. Sci.*, **76**, 95–101.

WINTER, R.J.D., ZHAO, L., KRAUTZ, T. & HUGHES, J. (1991). Neutral endopeptidase 24.11 inhibition reduces pulmonary vascular remodelling in rats exposed to chronic hypoxia. *Am. Rev. Resp. Dis.*, **144**, 1342–1346.

(Received June 10, 1992
Revised August 2, 1993
Accepted August 5, 1993)

Br. J. Pharmacol. (1993), **110**, 1369–1374 © Macmillan Press Ltd, 1993

Cardiovascular effects of intrathecally administered bradykinin in the rat: characterization of receptors with antagonists

Paulo Lopes, *Domenico Regoli & [1]Réjean Couture

Department of Physiology, Faculty of Medicine, Université de Montréal, C.P. 6128, Succursale A, Montréal, Québec, Canada H3C 3J7 and *Department of Pharmacology, Faculty of Medicine, Sherbrooke University, Sherbrooke, Québec, Canada J1H 5N4

1 The effects of intrathecal (i.t.) pretreatment with selective B_1 or B_2 kinin receptor antagonists were studied on the cardiovascular response to i.t. injection of bradykinin (BK) in conscious freely moving rats.

2 BK (81 pmol) produced an increase in mean arterial pressure (MAP: 9–13 mmHg) and decrease in heart rate (HR: 20–30 beats min^{-1}) that reached a maximum 2 min after injection.

3 The BK-induced cardiovascular responses were dose-dependently and reversibly reduced by four antagonists with the following rank order of potency: Tyr, D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK = D-Arg[Tyr^3,D-Phe^7,Leu^8]-BK = D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK > D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (Hoe 140). These compounds failed to alter the cardiovascular response to i.t. injection of 8.1 nmol of substance P.

4 Other compounds acting on the B_2 receptor, namely D-Arg[Hyp^3,Gly^6,Leu^8]-BK, D-Arg[Hyp^3,D-Phe^7]-BK, D-Arg[Hyp^2,Thi^{5,8},D-Phe^7]-BK and D-Arg[Hyp^3,Gly^6,D-Phe^7,Leu^8]-BK or on the B_1 receptor, [Leu^8]-desArg^9-BK, did not influence the cardiovascular responses to BK at doses devoid of intrinsic activity on MAP and HR.

5 None of the kinin receptor antagonists caused motor impairment, respiratory arrest or persisting cardiovascular changes.

6 These results confirm that the cardiovascular effects induced by i.t. BK are mediated by the activation of a B_2 receptor in the rat spinal cord. However, the rank order of potency of antagonists does not conform to the classical B_2 functional site characterized in peripheral tissues.

Keywords: Bradykinin; kinin antagonists; kinin receptor subtypes; spinal cord; cardiovascular responses

Introduction

Compelling evidence suggests that bradykinin (BK) is a mediator of pain and inflammation in the periphery (Regoli & Barabé, 1980; Marceau *et al.*, 1983) and a neuromodulator in the central nervous system (Corrêa *et al.*, 1979; Diz, 1985; Lopes & Couture, 1992; Lopes *et al.*, 1992; 1993). The biological activities of BK and of other naturally-occurring kinins (kallidin and T-kinin) are considered to be mediated primarily by two BK receptor types denoted as B_1 and B_2, of which B_1 is induced *de novo* following tissue injury or inflammation (Marceau *et al.*, 1983; deBlois *et al.*, 1989) whereas the B_2 receptor is found to subserve the majority of physiological functions under normal conditions (Regoli, 1984; Taylor *et al.*, 1989; Bathon & Proud, 1991). The B_2 receptor was recently cloned from a rat uterus cDNA library; the deduced amino acid sequence (366 residues) contains seven putative membrane-spanning domains and shows a sequence similarity to members of the G protein-coupled receptor family (McEachern *et al.*, 1991).

Autoradiographic studies (Steranka *et al.*, 1988; Lopes *et al.*, 1993) and binding assays (Fujiwara *et al.*, 1989; Sharif & Whiting, 1991) have revealed the presence of BK binding sites (identified as B_2 receptors) in the guinea-pig spinal cord. Immunoreactive BK has been found to be present in the central nervous system including the spinal cord and the cerebrospinal fluid of different species such as rats, dogs and man (Scicli *et al.*, 1984; Perry & Snyder, 1984; Thomas *et al.*, 1984; Kariya *et al.*, 1985; Hermann *et al.*, 1986). Moreover, the concentrations of BK, T-kinin and kininogen were found to be increased in the rat spinal cord after mechanical injury (Xu *et al.*, 1991). Recently, we have shown that application of BK intrathecally in conscious rats leads to an increase in arterial blood pressure by activating the sympathoadrenal system. The accompanying bradycardia was ascribed to a vagal reflex involving sensory C-fibres (Lopes & Couture, 1992). It was suggested that the cardiovascular effect of BK is mediated by the activation of a B_2 receptor in the spinal cord as des-Arg^9-BK, a highly selective agonist at B_1 receptors, was much less potent than BK, kallidin and T-kinin which were equipotent (Lopes & Couture, 1992). However, the B_2 receptor hypothesis needs to be confirmed by the use of selective antagonists. [Leu^8]-desArg^9-BK is a potent and competitive antagonist of the B_1 receptor but inactive on the B_2 receptor (Regoli & Barabé, 1980). Among the B_2 receptor antagonists described so far, Hoe 140 (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) is considered to be the most potent in several *in vitro* and *in vivo* assays (Hock *et al.*, 1991; Wirth *et al.*, 1991; Lembeck *et al.*, 1991), and is selective for B_2 sites since it is inactive at B_1 receptors (Rhaleb *et al.*, 1991). Nevertheless, several studies using new selective antagonists have highlighted the possible existence of subclasses of B_2 receptors in the periphery (Llona *et al.*, 1987; Rifo *et al.*, 1987; Braas *et al.*, 1988; Steranka *et al.*, 1988; Plevin & Owen, 1988; Taylor *et al.*, 1989; Stewart & Vavrek, 1990; Rhaleb *et al.*, 1991) as well as in the central nervous system (Sharif & Whiting, 1991; Seguin *et al.*, 1992). More recently, two new kinin functional sites denoted as B_{2A} (in the rabbit jugular vein) and B_{2B} (in the guinea-pig ileum) have been described (Regoli *et al.*, 1992).

In the present study, selective antagonists for the B_1 and B_2 receptors were used in an attempt to characterize the kinin receptor subtype which mediates the spinal action of BK on the cardiovascular system of conscious freely moving rats.

[1] Author for correspondence.

Methods

Animal preparation

Male Wistar rats (Charles River, St-Constant, Qué., Canada) weighing 200–250 g were anaesthetized with an intraperitoneal (i.p.) injection of 65 mg kg^{-1} sodium pentobarbitone (Nembutal, M.T.C. Pharmaceuticals, Mississauga, Ont., Canada) and implanted with two polyethylene tubes (Intramedic, Clay Adams, NJ, U.S.A.) as described previously (Hassessian *et al.*, 1988). Briefly, the first catheter (PE-50) filled with physiological saline (0.9% w/v NaCl) containing sodium heparin (100 iu ml^{-1}; Sigma, St. Louis, MO, U.S.A.) was inserted into the medial tail artery. The second catheter (PE-10) was inserted into the spinal subarachnoid space via an incision made in the dura at the atlanto-occipital junction and projected to the 9th thoracic segment (T-9).

Following surgery, the animals were housed individually in a plastic cage for a 24 h recuperation period. The catheters were drawn through the grid cage top and supported upright so that the animals were capable of moving freely in the cage and had free access to food pellets and tap water. Only rats which presented normal behaviour and no apparent motor dysfunction were used in the study. The correct positioning of the i.t. catheter was verified by post-mortem examination at the end of experiments.

Monitoring of cardiovascular parameters

Experiments were performed, 24 h after surgery, in conscious freely moving rats. Water bottles were removed from resident cages at the start of experiments and the cage was covered with an opaque cloth to eliminate visual stimuli during the course of the experiment. Arterial blood pressure was recorded from the intra-arterial catheter connected to a Statham pressure transducer (P231D). Heart rate (HR) was measured with a cardiac tachograph (model 7P4) triggered by the arterial pressure pulses. Both signals were monitored on a Grass polygraph model 79D (Quincy, Mass., U.S.A.).

Experiments started when animals were in a resting state with a stable basal blood pressure and HR. The rats were then challenged with an i.t. injection of 30 µl of artificial cerebrospinal fluid (CSF, aqueous solution of (mM): NaCl 128.6, KCl 2.6, $MgCl_2$ 2.0 and $CaCl_2$ 1.4; pH adjusted to 7.2–7.4). The catheter void volume was 20 µl. Only rats that did not exhibit changes of blood pressure and HR to i.t. injection of CSF for 30 min were used further in the study.

Experimental protocol

After testing CSF, the animals were injected with 81 pmol BK in a volume of 10 µl. The peptide and the CSF flush (20 µl) were given within a total period of 60 s. This dose of BK was selected from a dose-response curve determined in a previous study (Lopes & Couture, 1992). The kinin receptor antagonists were initially administered i.t. in a single dose of 0.7–0.8 nmol (1.0 µg), 2–3 min prior to the second i.t. injection of 81 pmol BK. This period corresponds to the time required for BK to reach its maximal cardiovascular response. BK injections were given 30 min apart and no tachyphylaxis was observed under these experimental conditions (Lopes & Couture, 1992). The response to BK in the presence of an antagonist was compared with that obtained in the absence of antagonist as measured in the same rat. If no blockade was observed, the rats were challenged a second time with the same antagonist at a dose 10 fold higher. On the other hand, if the cardiovascular response to BK was significantly reduced, BK was injected alone 1 h later to determine whether the antagonist blocks in a reversible manner. If the response to BK was entirely reversible, the same antagonist was tested either at higher or lower doses in random order against the same dose of BK (81 pmol). In other rats, the blocking antagonists were tested the same way against 8.1 nmol substance P (SP) to determine their specificity with respect to BK receptors. The potential residual agonistic activities of increasing doses of kinin antagonists were measured for 30 min in separate experiments. Care was taken to detect behavioural or toxic effects by the antagonists.

Maximal changes in MAP and HR have been calculated from the differences between the recordings made 1 min before peptide injection and the maximal values obtained within 3 min after injection.

Peptides

[Leu^8]-desArg^9-BK, D-Arg[Hyp^3,Gly^6, D-Phe^7,Leu^8]-BK, D-Arg[Hyp^2, Thi^{5,8}, D-Phe^7]-BK, D-Arg[Hyp^3,Thi^5, D-Tic^7,Oic^8]-BK (Hoe 140), D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK, D-Arg[Tyr^3,D-Phe^7,

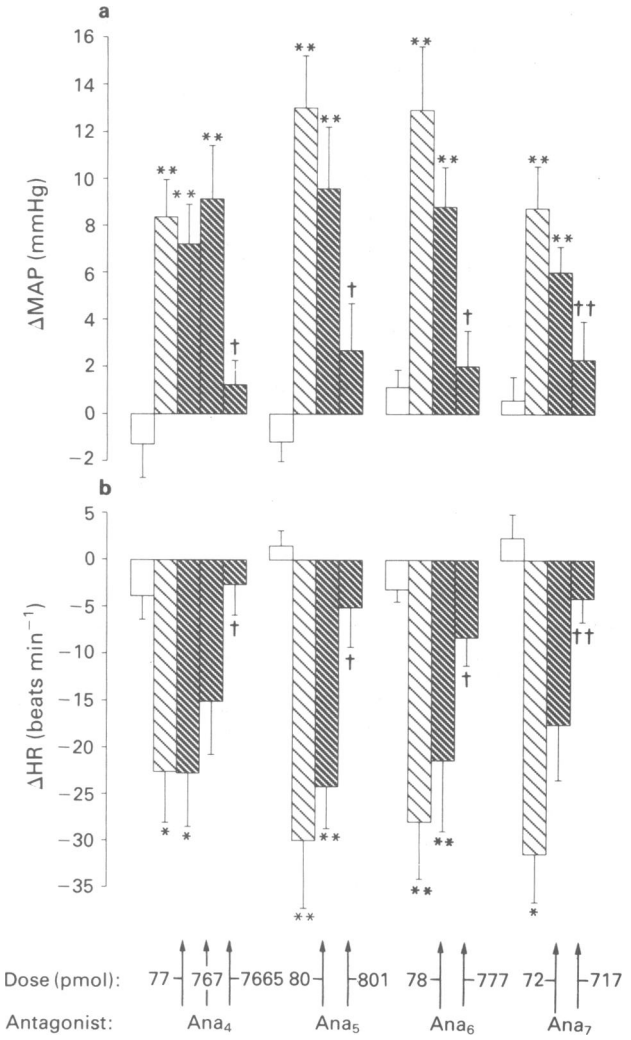


Figure 1 Effects of various bradykinin (BK) antagonists on the maximal changes in mean arterial pressure (MAP; a) and heart rate (HR; b) induced by the i.t. injection of 81 pmol BK. BK was injected before (▨) and 2–3 min after (▩) the intrathecal injection of D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (Ana_4), D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK (Ana_5), D-Arg[Tyr^3,D-Phe^7,Leu^8]-BK (Ana_6) or Tyr,D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK (Ana_7) at the indicated doses. Each point represents the mean ± s.e.mean results of 8–10 rats in each group. The statistical significance of the differences between CSF (□) and BK was calculated with the ANOVA and the Dunnett test as indicated by $*P<0.05$; $**P<0.01$. The statistical significance of the differences between values before and after treatment with the antagonist was calculated with Student's t test for paired samples: $\dagger P<0.05$; $\dagger\dagger P<0.01$. Respective baseline MAP (mmHg) and HR (beats min^{-1}) were 86.1 ± 2.8 and 340 ± 22 for Ana_4, 91.4 ± 2.9 and 345 ± 12 for Ana_5, 90.5 ± 2.3 and 349 ± 16 for Ana_6, 89.5 ± 4.0 and 373 ± 25 for Ana_7.

Leu[8]-BK, Tyr,D-Arg[Hyp[3],D-Phe[7],Leu[8]]-BK, D-Arg[Hyp[3],D-Phe[7]]-BK, and D-Arg[Hyp[3],Gly[6],Leu[8]]-BK were used in the present study. The selectivity and antagonist activity of these compounds have been published elsewhere (Rhaleb *et al.*, 1991; Regoli *et al.*, 1992). SP, BK and [Leu[8]]-desArg[9]-BK were purchased from Hükabel Scientific Ltd. (Montréal, Québec, Canada). Hoe 140 was made available from Hoechst AG (Frankfurt, Germany). All other peptides were synthesized by the solid-phase method and purified by high-performance liquid chromatography (Drapeau & Regoli, 1988). Their structure was assessed by fast atomic bombardment (FAB) mass spectrometry. All peptides were dissolved directly in CSF and stock solutions (1 mg ml^{-1}) were divided into 100 µl aliquots and stored at − 20°C until used. Daily dilutions were made in CSF.

Statistical analysis of data

The results are expressed as means ± s.e.mean. Statistical significance of differences were evaluated with Student's *t* test for paired samples. A one-way analysis of variance (ANOVA) with a *post-hoc* Dunnett test was used for multiple comparisons with one control group. Only probability values (*P*) less than 0.05 were considered to be statistically significant.

Results

Cardiovascular response to bradykinin administered intrathecally

The i.t. administration of 81 pmol BK, at the T-9 spinal cord level to freely moving rats, caused transient increases in mean arterial blood pressure (MAP) of 9–13 mmHg and decreases in heart rate (HR) of 20–30 beats min^{-1} that reached a maximum 2 min after injection (Figure 1). At this dose of BK, the pressor effect returned to baseline values within 10 min while the bradycardia lasted for about 15–20 min. Furthermore, no tachyphylaxis was observed when 3–4 successive injections of 81 pmol BK were given 30 min apart. The i.t. injection of a similar volume of CSF was without appreciable effect on MAP and HR (Figure 1). Baseline MAP and HR were not significantly different among groups (Figure 1 and Table 1).

Effects of BK receptor antagonists on the cardiovascular response to BK

The receptor responsible for the cardiovascular response induced by the i.t. injection of BK was characterized with a series of antagonists selective either for the B_2 or the B_1 kinin receptor (Table 1 and Figure 1). When administered intrathecally 2–3 min prior to 81 pmol BK, four analogues out of nine blocked in a dose-dependent manner the cardiovascular response to BK with the following rank order of potency: Tyr,D-Arg[Hyp[3],D-Phe[7],Leu[8]]-BK = D-Arg[Tyr[3],D-Phe[7],Leu[8]]-BK = D-Arg[Hyp[3],D-Phe[7],Leu[8]]-BK > D-Arg[Hyp[3], Thi[5],D-Tic[7], Oic[8]]-BK (Hoe 140). The first three compounds blocked significantly the MAP and HR responses to BK at a molar ratio antagonist/agonist of 10:1, whereas the last compound blocked only at a dose 100 fold (7.6 nmol) higher than BK (Figure 1). The antagonism was no longer present when BK was re-injected alone 1 h later (data not shown). Hoe 140 (767 pmol) was also tested for longer pretreatment periods (10, 20 and 30 min) to determine whether its apparent weakness was dependent on the equilibration time with the B_2 receptor before BK was injected. Such a possibility is however unlikely as this dose of Hoe 140 was inactive against BK whatever the time-exposure ($n = 8$, data not shown). D-Arg[Hyp[3],Gly[6],D-Phe[7],Leu[8]]-BK and D-Arg[Hyp[2],Thi[5,8],D-Phe[7]]-BK had no significant effect on the cardiovascular response elicited by 81 pmol BK when administered at doses 10 and 100 times higher than the agonist (Table 1). The antagonistic activities of these compounds could not be measured at doses higher than 40 nmol as they produced direct effects on MAP (Table 1). D-Arg[Hyp[3],D–Phe[7]]-BK and D-Arg[Hyp[3],Gly[6],Leu[8]]-BK were unable to block BK at doses

Table 1 Effects of kinin receptor antagonists on mean arterial pressure (MAP) and on the MAP response to 81 pmol bradykinin

Antagonist	n	*Dose* (nmol)	*Basal MAP* (mmHg)	*Direct effect on MAP* (mmHg)	*Changes in MAP to BK* (mmHg)	
					Before antagonist	*After antagonist*
[Leu[8]]-desArg[9]-BK	8	CSF	91.8 ± 5.6	1.7 ± 0.8		
		1.0		2.4 ± 0.9	7.1 ± 1.3	8.3 ± 0.8
		10.3		1.9 ± 1.1		7.2 ± 1.5
	4	51.3		10.0 ± 2.0**		
		102.7		9.2 ± 2.6**		
D-Arg[Hyp[3],Gly[6],D-Phe[7],Leu[8]]-BK	8	CSF	96.1 ± 8.0	0.6 ± 0.6		
		0.8		3.3 ± 1.5	12.9 ± 2.6	16.3 ± 2.3
		8.2		3.3 ± 1.7		9.6 ± 2.1
	4	41.0		39.5 ± 6.4**		
		82.1		38.5 ± 9.7**		
D-Arg[Hyp[2],Thi[5,8],D-Phe[7]]-BK	10	CSF	93.8 ± 5.1	0.0 ± 1.2		
		0.8		3.2 ± 1.5	11.9 ± 2.5	11.1 ± 4.6
		8.4		1.5 ± 0.8		7.5 ± 1.7
		41.9		6.7 ± 1.0**		
		83.7		11.0 ± 2.2**		
D-Arg[Hyp[3],D-Phe[7]]-BK	8	CSF	88.0 ± 4.4	0.4 ± 1.2		
		0.8		4.1 ± 1.3	15.0 ± 3.9	12.5 ± 2.3
		8.3		4.5 ± 1.3		12.9 ± 1.4
		41.7		10.2 ± 1.2**		7.5 ± 1.0
D-Arg[Hyp[3],Gly[6],Leu[8]]-BK	8	CSF	91.5 ± 3.7	0.6 ± 0.9		
		0.9		2.4 ± 0.7	11.3 ± 3.3	8.6 ± 1.7
		8.6		5.6 ± 1.1		10.4 ± 1.2
		42.8		10.3 ± 1.9**		4.1 ± 1.1†

Values represent the mean ± s.e.mean of (*n*) rats. Statistical differences between values before and after the i.t. injection of the antagonist is indicated by †$P < 0.05$. Statistical comparison to CSF values is indicated by *$P < 0.05$ and **$P < 0.01$.

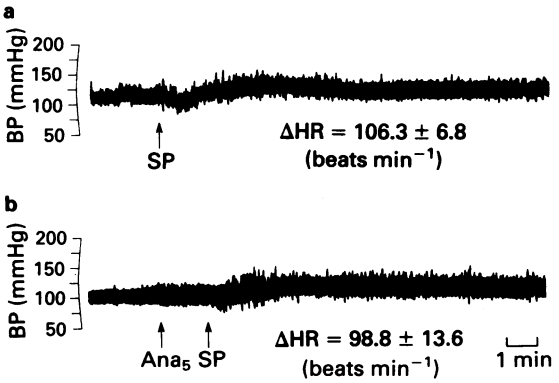


Figure 2 Typical tracings showing the arterial blood pressure (BP) response to 8.1 nmol substance P (SP) before (a) and after (b) 0.8 nmol D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK) (Ana_5) injected intrathecally in one rat. ΔHR represents the maximal changes in heart rate measured 5 min after SP administration in 6 rats.

up to 100 times that of the agonist. At a fairly high dose (42 nmol), only D-Arg[Hyp^3,Gly^6,Leu^8]-BK reduced significantly the BK-induced increase in MAP although the analogue displayed an intrinsic effect on MAP (Table 1). The B_1 antagonist, [Leu^8]-desArg^9-BK, at doses ranging from 1 nmol to 10.3 nmol, failed to antagonize the cardiovascular response to 81 pmol BK. At higher doses, this analogue also displayed a direct pressor effect.

Intrathecal effect of B_2 receptor antagonists against substance P

The i.t. injection of 8.1 nmol subtance P produced an increase of MAP and HR as reported earlier (Hassésian *et al.*, 1988). This cardiovascular response to SP was not significantly different from that obtained in animals which received a prior i.t. injection of Tyr,D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK, D-Arg[Tyr^3,D-Phe^7,Leu^8]-BK, D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK or D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK at doses of 0.7–0.8 nmol. An example is given in Figure 2 with the analogue D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK.

Intrinsic activity of BK antagonists

The intrinsic activity of each antagonist administered at increasing doses was assessed on MAP in separate groups of rats. All analogues failed to produce any significant effect on MAP when administered alone at doses lower than 8–10 nmol. However, with doses 4–5 times higher, most of the antagonists showed an increase in MAP similar to that observed with 81 pmol BK. D-Arg[Hyp^3,Gly^6,D-Phe^7,Leu^8]-BK (41.0 nmol) and Tyr,D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK (35.8 nmol) produced the greatest effect on MAP with maximal increases of 39.5 ± 6.4 mmHg (Table 1) and 41.2 ± 12.0 mmHg, respectively (data not shown). Note that the pressor effects evoked by these two analogues were 2–3 times greater than that evoked by 81 pmol BK and could not be reached with a supramaximal dose of BK (81 nmol) (Lopes & Couture, 1992). All other antagonists, at doses ranging from 40–100 nmol, caused a small (about 10 mmHg) but significant change in MAP (Table 1). At the doses tested, neither BK nor BK analogues produced motor impairment, respiratory arrest or persisting cardiovascular changes.

Discussion

In a previous study, we have shown that BK, kallidin and T-kinin are equipotent in eliciting increases in MAP and decreases in HR when injected intrathecally at the ninth thoracic segment of the conscious rat (Lopes & Couture, 1992). In contrast, des-Arg^9-BK, a selective agonist at the B_1 receptor, was 1000 fold less potent. On the basis of this finding, it was concluded that the cardiovascular effect induced by BK applied to the spinal cord is mediated by B_2 receptors. Likewise, the pressor response to BK administered intracerebroventricularly in rats was attributed to activation of B_2 receptors (Lindsey *et al.*, 1989). In the present study, the hypothesis of the existence of B_2 receptors in the spinal cord of the rat has been confirmed by the use of antagonists. While the B_1 receptor antagonist [Leu^8]-des-Arg^9-BK had no effect, four B_2 receptor antagonists blocked in a dose-dependent and reversible manner the cardiovascular response to BK: D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK = D-Arg[Tyr^3,D-Phe^7, Leu^8]-BK = Tyr,D-Arg[Hyp^3,D-Phe^7, Leu^8]-BK > D-Arg[Hyp^3, Thi^5,D-Tic^5,Oic^8]-BK (Hoe 140) when pre-injected intrathecally at molar antagonist/agonist ratio equal or superior to 10. However, this rank order of potency for B_2 receptor antagonists does not conform to that found in peripheral tissues, where Hoe 140 has been shown to be the most active compound (Hock *et al.*, 1991; Rhaleb *et al.*, 1991; Regoli *et al.*, 1992). Moreover, several compounds which have been shown to be antagonists in peripheral tissues, namely D-Arg[Hyp^3,Gly^6,Leu^8]-BK, D-Arg[Hyp^3,D-Phe^7]-BK, D-Arg[Hyp^2, Thi^{5,8},D-Phe^7]-BK and D-Arg[Hyp^3,Gly^6,D-Phe^7,Leu^8]-BK were unable to reduce the effects of BK, while other compounds (D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK, D-Arg[Tyr^3,D-Phe^7,Leu^8]-BK and Tyr,D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK) pre-injected at the same molar doses (generally 810 pmol) were found to reduce significantly the cardiovascular effects of BK. The first group of compounds which were found to be inactive have also been shown to act as partial agonists in peripheral organs (Regoli *et al.*, 1992). The major discrepancy between the central and peripheral effects of kinin receptor antagonists is the weakness of Hoe 140 which cannot be attributed to differences in metabolic degradation since Hoe 140 has been shown to be more resistant to peptidases than the other antagonists (Wirth *et al.*, 1991; Lembeck *et al.*, 1991). Although other factors such as the rate of diffusion into nervous structures cannot be completely ruled out, it is possible that the functional site involved in the spinal cardiovascular effect of BK is somewhat different from that found in the vascular endothelium or in various smooth muscles. The BK receptor of the rat spinal cord may be similar to the high-affinity site (denoted B_{2C}) described in the guinea-pig brain and lung membranes by Seguin *et al.* (1992) at which Hoe 140 displays low affinity.

Most analogues of BK (B_1 or B_2 receptor antagonists) exhibited intrinsic activities at doses 50 fold greater than that of BK (0.81 nmol) required to elicit the maximal effect (Lopes & Couture, 1992). It is unknown, whether these cardiovascular changes (increase of BP and decrease of HR) are attributable to direct actions on B_2 receptors (agonist effect) or to non specific effects in the spinal cord. Nevertheless, none of the analogues tested caused distressed behaviour, hindlimb paralysis or death of the animals. Moreover, the four analogues which blocked the BK-induced cardiovascular effects were inactive against the blood pressure and heart rate increases induced by the i.t. injection of SP, supporting the interpretation that they act specifically on B_2 receptors in the spinal cord. On the other hand, the i.t. injection of an antagonist of SP (± CP-96,345) did not modify the effects of BK (Pham & Couture, 1993), while blocking those of SP. This indicates that BK is not acting via the release of SP. This possibility had to be excluded since *in vitro* quantitative receptor autoradiographic studies have shown that [^{125}I-Tyr^8]-BK receptor binding sites are localized predominantly in superficial layers of the dorsal horn in the rat and guinea-pig spinal cord, where numerous endings of thin sensory myelinated (Aδ) and non-myelinated (C) fibres containing SP are found (Lopes *et al.*, 1992; 1993). Although the selective destruction of these fibres with the neurotoxin, capsaicin, reduced significantly the BK radiolabelled sites in

the rat dorsal horn (Lopes *et al.*, 1992), this treatment had no effect on the pressor response to BK (Lopes & Couture, 1992) thus ruling out the possible involvement of other sensory neuropeptides such as calcitonin gene-related peptide, galanin, somatostatin and cholecystokinin as mediators of BK-induced cardiovascular responses. In fact, these peptides are depleted from sensory C-fibres together with SP by capsaicin (Holzer, 1988; Maggi, 1991).

A rather low, but significant density of specific [^{125}I-Tyr^8]-BK binding sites was also detected in the intermediolateral horn where cell bodies and dendrites of preganglionic sympathetic fibres are located (Lopes *et al.*, 1992; 1993). These specific BK sites may be involved in mediating the spinal action of kinins on the cardiovascular system. Moreover, the possibility that BK activates preganglionic sympathetic fibres through interneurones and other transmitters present in this tissue cannot be excluded. However, compelling evidence rules out peripheral and supraspinal sites of action for i.t. BK and related analogues (Lopes & Couture, 1992).

In the rat and guinea-pig spinal cord, we found that Hoe 140 was the most potent displacer of [^{125}I-Tyr^8]-BK specific binding followed by D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK, Tyr,D-Arg[Hyp^3,D-Phe^7,Leu^8]-BK, D-Arg[Tyr^3,D-Phe^7,Leu^8]-BK (Lopes *et al.*, 1992; 1993). This rank order of potency, and in particular the high activity of Hoe 140, contrasts with the results obtained in the present study. These findings suggest that the functional B_2 site involved in the cardiovascular effects of BK is different from the major (abundant) site labelled by [^{125}I-Tyr^8]-BK and occupied by Hoe 140. It thus appears that the sites located in superficial layers of the dorsal horn as revealed by autoradiographic studies, are not involved in the spinal cardiovascular action of BK.

In summary, the i.t. injection of BK in the conscious rat produces a transient increase in MAP and decrease in HR which appear to be mediated by the activation of a B_2 receptor in the spinal cord. This hypothesis is supported by the finding that the cardiovascular response to BK is blocked in a dose-dependent, reversible and specific manner by the intrathecal administration of antagonists selective for the B_2 but not for the B_1 receptor. The rank order of potency of the antagonists suggests, however, that the B_2 receptor involved in the cardiovascular action of BK belongs to a functional site which, pharmacologically, does not conform to the B_2 receptors that are found in a large variety of peripheral tissues.

This study was supported by a Grant-in-aid (MT-8925) from the Medical Research Council of Canada (MRCC) and the Quebec Heart Foundation. R.C. is a senior scholar of the Fonds de la recherche en santé du Quebec. D.R. is a career investigator of the MRCC and P.L. holds studentships from the Heart and Stroke Foundation of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR). The secretarial assistance of Ms Christiane Laurier and the graphic work of Mr Claude Gauthier are gratefully acknowledged.

References

BATHON, J.M. & PROUD, D. (1991). Bradykinin antagonists. *Annu. Rev. Pharmacol. Toxicol.*, **31**, 129–162.

BRAAS, K.M., MANNING, D.C., PERRY, D.C. & SNYDER, S.H. (1988). Bradykinin analogues: differential agonist and antagonist activities suggesting multiple receptors. *Br. J. Pharmacol.*, **94**, 3–5.

CORRÉA, F.M.A., INNIS, R.B., UHL, G.R. & SNYDER, S.H. (1979). Bradykinin-like immunoreactive neuronal systems localized histochemically in rat brain. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 1489–1493.

DE BLOIS, D., BOUTHILLIER, J. & MARCEAU, F. (1989). Pharmacological modulation of the up-regulated responses to des-Arg^9-bradykinin in vivo and in vitro. *Immunopharmacology*, **17**, 187–198.

DIZ, D.I. (1985). Bradykinin and related peptides in central control of the cardiovascular system. *Peptides*, **6** (Suppl. 2), 57–64.

DRAPEAU, G. & REGOLI, D. (1988). Synthesis of bradykinin analogs. In: Immunochemical techniques. Part n. Chemotaxis and Inflammation. *Methods Enzymol.*, **163**, 263–272.

FUJIWARA, Y., MANTIONE, C.R., VAVREK, R.J., STEWART, J.M. & YAMAMURA, H.I., (1989). Characterization of [^{3}H]bradykinin binding sites in guinea pig central nervous system: possible existence of B_2 subtypes. *Life Sci.*, **44**, 1645–1653.

HASSÉSSIAN, H., DRAPEAU, G. & COUTURE, R. (1988). Spinal action of neurokinins producing cardiovascular responses in the conscious freely moving rat: evidence for a NK-1 receptor mechanism. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 649–654.

HERMANN, K., SCHAECHTELIN, G. & MARIN-GREZ, M. (1986). Kinins in cerebrospinal fluid: reduced concentration in spontaneously hypertensive rats. *Experientia*, **42**, 1238–1239.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, ST., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–773.

HOLZER, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience*, **24**, 739–768.

KARIYA, K., YAMAUCHI, A. & SASAKI, T. (1985). Regional distribution and characterization of kinin in the CNS of the rat. *J. Neurochem*, **44**, 1892–1897.

LEMBECK, F., GRIESBACHER, T., ECKHARDT, M., HENKE, S., BREIPOHL, G. & KNOLLE, J. (1991). New, long-acting, potent bradykinin antagonists. *Br. J. Pharmacol.*, **102**, 297–304.

LINDSEY, C.J., NAKAIE, C.R. & MARTINS, D.T.O. (1989). Central nervous system kinin receptors and the hypertensive response mediated by bradykinin. *Br. J. Pharmacol.*, **97**, 763–768.

LLONA, I., VAVREK, R., STEWART, J. & HUIDOBRO-TORO, J.P. (1987). Identification of pre- and postsynaptic bradykinin receptor sites in the vas deferens: evidence for different structural prerequisites. *J. Pharmacol. Exp. Ther.*, **241**, 608–614.

LOPES, P. & COUTURE, R. (1992). Cardiovascular responses elicited by intrathecal kinins in the conscious rat. *Eur. J. Pharmacol.*, **210**, 137–147.

LOPES, P., KAR, S., TOUSIGNANT, C., REGOLI, D., QUIRION, R. & COUTURE, R. (1993). Autoradiographic localization of [^{125}I-Tyr^8]-bradykinin receptor binding sites in the guinea pig spinal cord. *Synapse*, **15**, 48–57.

LOPES, P., REGOLI, D., THAKUR, M. & COUTURE, R. (1992). Use of selective antagonists to substantiate the existence of a B_2-kinin receptor subtype in spinal cardiovascular control. *Soc. Neurosci.*, Abst. 1984.

MAGGI, C.A. (1991). The pharmacology of the efferent function of sensory nerves. *J. Auton. Pharmacol.*, **11**, 173–208.

MARCEAU, F., LUSSIER, A., REGOLI, D. & GIROUD, J.P. (1983). Pharmacology of kinins: their relevance to tissue injury and inflammation. *Gen. Pharmacol.*, **14**, 209–229.

MCEACHERN, A.E., SHELTON, E.R., BHAKTA, S, OBERNOLTE, R., BACH, C., ZUPPAN, P., FUJISAKI, J., ALDRICH, R.W. & JARNAGIN, K. (1991). Expression cloning of a rat B_2 bradykinin receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 7724–7728.

PERRY, D.C. & SNYDER, S.H. (1984). Identification of bradykinin in mammalian brain. *J. Neurochem.*, **43**, 1072–1080.

PHAM, T.M. & COUTURE, R. (1993). Inhibitory action of (±)CP-96,345 on the cardiovascular responses to intrathecal substance P and neuropeptide K in the conscious freely moving rat. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, 34–41.

PLEVIN, R. & OWEN, P.J. (1988). Multiple B_2 kinin receptors in mammalian tissues. *Trends Pharmacol. Sci.*, **9**, 387–389.

REGOLI, D. (1984). Neurohumoral regulation of precapillary vessels: the kallikrein-kinin system. *J. Cardiovasc. Pharmacol.*, **6**, S401–S412.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

REGOLI, D., JUKIC, D., TOUSIGNANT, C. & RHALEB, N.-E. (1992). Kinin receptor classification. *Agents Actions*, **38**, 475–486.

RHALEB, N.-E., TÉLÉMAQUE, S., ROUISSI, N., DION, S., JUKIC, D., DRAPEAU, G. & REGOLI, D. (1991). Structure-activity studies of bradykinin and related peptides; B_2-receptor antagonists. *Hypertension*, **17**, 107–115.

RIFO, J., POURRAT, M., VAVREK, R.J., STEWART, J.M. & HUIDOBRO-TORO, J.P. (1987). Bradykinin-receptor antagonists used to characterize the heterogeneity of bradykinin-induced responses in the rat vas deferens. *Eur. J. Pharmacol.*, **142**, 305–312.

SCICLI, A.G., FORBES, G., NOLLY, H., DUJOVNY, M. & CARRETERO, O.A. (1984). Kallikrein-kinins in the central nervous system. *Clin. Exp. Hypert.*, **A6**, 1731–1738.

SEGUIN, L., WIDDOWSON, P.S. & GIESEN-CROUSE, E. (1992). Existence of three subtypes of bradykinin B_2 receptors in the guinea pig. *J. Neurochem.*, **59**, 2125–2133.

SHARIF, N.A. & WHITING, R.L. (1991). Identification of B_2-bradykinin receptors in guinea pig brain regions, spinal cord and peripheral tissues. *Neurochem. Int.*, **18**, 89–96.

STERANKA, L.R., MANNING, D.C., DEHAAS, C.J., FERKANY, J.W., BOROSKY, S.A., CONNOR, J.R., VAVREK, R.J., STEWART, J.M., SNYDER, S.H. (1988). Bradykinin as a pain mediator: receptors are localized to sensory neurons, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3245–3249.

STEWART, J.M. & VAVREK, R.J. (1990). Kinin antagonists: design and activities. *J. Cardiovasc. Pharmacol.*, **15**, S69–S74.

TAYLOR, J.E., DEFEUDIS, F.V. & MOREAU, J.P. (1989). Bradykinin antagonists: therapeutic perspectives. Current Trends Review. *Drug Devel. Res.*, **16**, 1–11.

THOMAS, G.R., THIBODEAUX, H., MARGOLIUS, H.S. & PRIVITERA, P.J. (1984). Cerebrospinal fluid kinins and cardiovascular function effects of cerebroventricular melittin. *Hypertension*, **6**, 46–50.

WIRTH, K., HOCK, F.J., ALBUS, U., LINZ, W., ALPERMANN, H.G., ANAGNOSTOPOULOS, H., HENKE, S.T., BREIPOHL, G., KÖNIG, W., KNOLLE, J. & SCHÖLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vivo* studies. *Br. J. Pharmacol.*, **102**, 774–777.

XU, J., HSU, C.Y., JUNKER, H., CHAO, S., HOGAN, E.L. & CHAO, J. (1991). Kininogen and kinin in experimental spinal cord injury. *J. Neurochem.*, **57**, 975–980.

(Received March 19, 1993
Revised June 18, 1993
Accepted August 9, 1993)

Br. J. Pharmacol. (1993), **110**, 1375–1380 © Macmillan Press Ltd, 1993

Zeneca ZD7114 acts as an antagonist at β_3-adrenoceptors in rat isolated ileum

[1]James W. Growcott, *Brian Holloway, Michelle Green & *Campbell Wilson

VIM and *Cardiovascular Research Departments, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG

1 The relaxant effects of Zeneca ZD7114, BRL37344 (putative β_3-adrenoceptor agonists) and various phenylethylamine-based agonists were studied in isolated ileum of the rat where tone was increased with carbachol (0.5 μM). Agonist-induced relaxation was measured under equilibrium conditions with α-, β_1- and β_2-adrenoceptors inhibited.

2 Relaxant responses were obtained to isoprenaline, noradrenaline, and BRL37344, although, the efficacy of this latter agent was significantly lower than that of isoprenaline. Salbutamol caused weak relaxation (<20%) at high concentrations (10 μM) and ZD7114 was without significant relaxant effect even at high concentrations (10 μM).

3 Relaxant responses to isoprenaline and BRL37344 were weakly antagonized by high concentrations of (±)-propranolol (10 and 100 μM) yielding pK_B values of 5.7 with isoprenaline as the agonist and 5.5 with BRL37344 as the agonist.

4 The non-selective β-adrenoceptor antagonist, (±)-alprenolol (1–100 μM) caused competitive antagonism of the relaxant responses to isoprenaline (pA_2 value = 6.5). A similar pK_B value was obtained when BRL37344 was used as the agonist (6.4).

5 Relaxant effects of isoprenaline and BRL37344 were also antagonized by ZD7114 (1–100 μM) yielding pA_2 and pK_B values of 6.3 and 6.7 respectively.

6 The low potencies of (±)-propranolol and (±)-alprenolol as antagonists of the relaxant responses to isoprenaline and BRL37344 indicate that both the agonists and antagonists employed in the current study may interact with β_3-adrenoceptors in the rat isolated ileum. Contrary to the previous findings in guinea-pig ileum, where BRL37344 and ZD7114 were full agonists, in the current study, BRL37344 was a partial agonist and ZD7114 an antagonist at the β_3-adrenoceptor in rat ileum.

Keywords: Rat isolated ileum; β_3-adrenoceptor; Zeneca ZD7114; BRL37344

Introduction

Atypical or β_3-adrenoceptors have been identified in rat adipocytes (Arch *et al.*, 1984) and a variety of gastrointestinal smooth muscle preparations including guinea-pig and rat ileum (Bond & Clarke, 1987; 1988; van der Vliet *et al.*, 1990). Similar receptors have been implicated in the neuronal activation of the myenteric plexus of the guinea-pig (Taneja & Clarke, 1991). These sites can be differentiated from the classical β-adrenoceptors by the use of propranolol which exhibits low affinity for β_3-adrenoceptors. Moreover, agents such as BRL37344 and ZD7114 have been shown to have a high degree of selectivity for the β_3-adrenoceptor (Arch *et al.*, 1984; Holloway *et al.*, 1991a,b). These latter agents cause an inhibition of histamine-raised tone in guinea-pig ileum and are similar in potency and efficacy to the classical β-adrenoceptor agonist, isoprenaline (Growcott *et al.*, 1993). However, other workers have noted that BRL37344 may not always produce a full agonist response when compared to isoprenaline. For example, in human adipocytes, BRL37344 was found to be significantly less efficacious than isoprenaline in causing lipolysis whereas, in rat adipocytes, each agent possessed similar efficacy (Hollenga *et al.*, 1990). Further, in rat jejunum, where effects on resting tone were measured, BRL37344 produced a significantly lower maximum response when compared to isoprenaline (van der Vliet *et al.*, 1990). Although the authors gave no explanation for these differences in efficacy, similar observations have been previously noted with other β-adrenoceptor agonists such as, prenalterol (Kenakin & Beek, 1980). These authors addressed the issue by demonstrating that the intrinsic activity of prenalterol was determined, in part, by the receptor reserve of the tissue under study, i.e. in guinea-pig trachea, prenalterol was a full agonist, whereas in guinea-pig extensor digitorum, the agent possessed no agonist activity whatsoever and could act as an antagonist. Hence, receptor reserve could determine the final effect of a putative agonist – where reserve was high, full agonism would be observed, where the reserve was low, partial agonism or antagonism would be observed.

We have extended this argument to the current study in an attempt to establish the identity and function of the adrenoceptor present on the smooth muscle of rat isolated ileum. Using ZD7114, BRL37344 and a range of other agents, we have attempted to determine whether this site is different from that previously characterized in guinea-pig isolated ileum (Bond & Clarke, 1987; 1988; Growcott *et al.*, 1993).

A preliminary account of this work was presented at the meeting of the British Pharmacological Society, London, September, 1992.

Methods

Tissue preparation

Alderley Park rats (200–300 g) of either sex were killed by a blow to the head and cervical dislocation. The terminal 10 cm of ileum was removed and placed in Krebs solution. Segments (2–3 cm in length) were cut and the lumen washed through with Krebs solution to remove any traces of food. The segments were set up in 10 ml organ baths under a resting tension of 0.5 g in Krebs solution, maintained at 37°C and gassed with 95% O_2:5% CO_2. The composition (mM) of the Krebs solution was as follows: NaCl 118.9, $NaHCO_3$ 25.0, KCl 4.9, KH_2PO_4 1.2, glucose 11.1, $CaCl_2$ 2.5 and ascorbic acid 0.11. CGP20712A ((±)-1-[2-(3-carbamoyl-4-hydr-

[1] Author for correspondence.

oxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluromethyl-2-imidazolyl)-phenoxy]-2-propanol), ICI 118551 (erythro-(±)-1-(7-methyindan-4-yloxy)-3-isopropylaminobutan-2-ol) and prazosin (all 0.1 μM) were present throughout the experiments to inhibit β_1-, β_2- and α_1-adrenoceptors respectively. Tissues were allowed to equilibrate for at least 30 min before any experimental procedures were begun.

Agonist activity

The relaxant effects of agonists were determined by measuring the inhibition of carbachol (0.5 μM)-induced tone by addition of the agonists. This concentration of carbachol was found to produce between 50–70% of the maximal achievable tone (data not shown). Adrenoceptor agonists were added cumulatively until a maximal relaxant effect was observed. Responses to agonists were then repeated 60 min later to check for any changes in tissue sensitivity.

Activity was expressed as an IC_{50} value, i.e. the concentration of agonist which produced a relaxant response that was 50% of the maximal effect.

Antagonist activity

Antagonist effects on the relaxant actions of the adrenoceptor agonists were assessed by using paired tissues, i.e. one tissue where the concentration-response curve to the agonist was constructed in the absence of antagonist and the other tissue where the same procedure was carried out in the presence of the antagonist. This procedure helped to assess the contribution of other factors such as tachyphylaxis. The equilibration time for the antagonists was 30 min.

Antagonist activity was determined by comparing the IC_{50} value obtained from the agonist-response curve in the absence of antagonist to that obtained in the presence of antagonist and calculated as a concentration-ratio. In some cases, a plot of log (agonist concentration-ratio −1) versus log [antagonist] was constructed. If the slope of the regression line was not significantly different from unity, then the antagonism was considered to be competitive (Arunlakshana & Schild, 1959). Where single concentrations of antagonist were used or where the antagonist effect was shown to be non-competitive, pK_B values were derived from the equation: pK_B = log(antagonist concentration ratio −1) −log[antagonist], according to the method of Furchgott (1972).

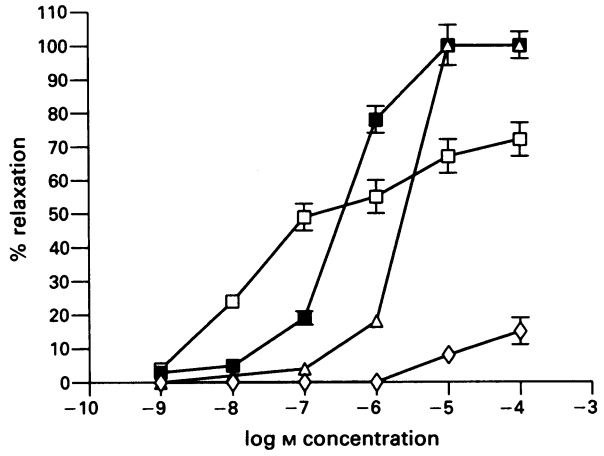


Figure 1 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves for isoprenaline (■, $n = 81$), BRL37344 (□, $n = 50$), noradrenaline (Δ, $n = 4$) and salbutamol (◇, $n = 4$) in the presence of CGP20712A, ICI 118551 and prazosin (all 0.1 μM). Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size. ZD7114 was without significant relaxant activity up to 10 μM.

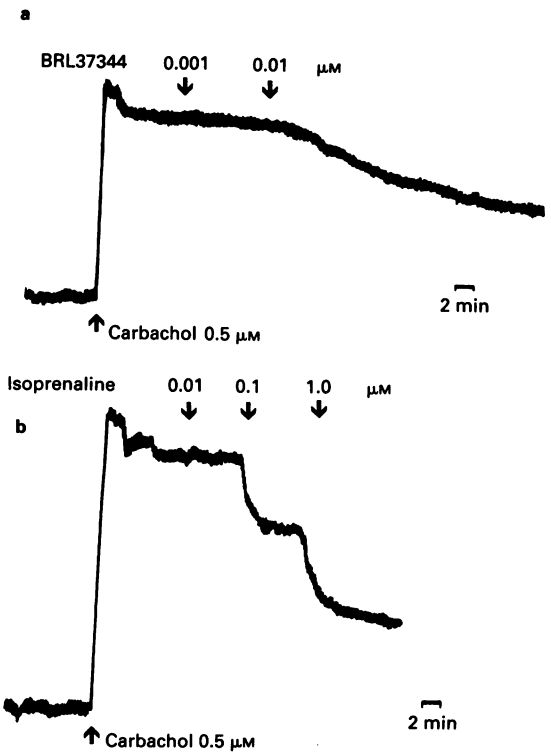


Figure 2 Typical traces showing the effects of (a) BRL37344 and (b) isoprenaline on carbachol (0.5 μM)-induced tone in the isolated ileum of the rat. Prazosin, CGP20712A and ICI 118551 (all 0.1 μM) were present throughout. Arrows indicate where agonists were added. Tone measurements were recorded isotonically.

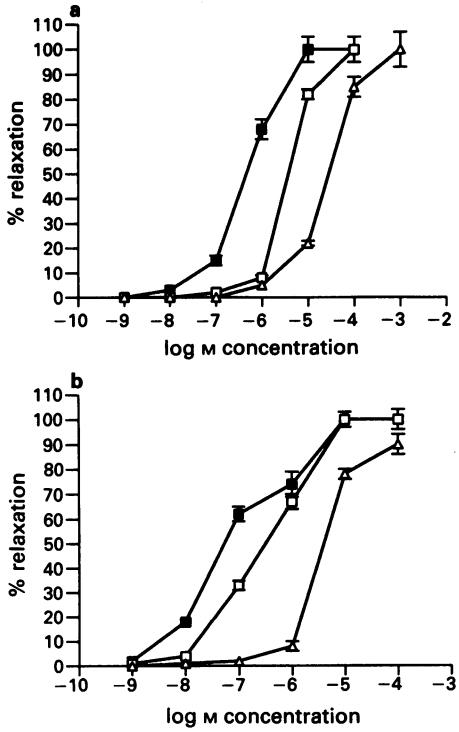


Figure 3 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves to (a) isoprenaline and (b) BRL37344 in the absence (■, $n = 8$ and 9 respectively) and presence of propranolol 10 μM (□, $n = 4$) and 100 μM (Δ, $n = 4$) respectively. CGP 20712A, ICI 118551 and prazosin (all 0.1 μM) were present throughout. Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size.

Statistics

Results throughout are expressed as geometric mean values together with their respective 95% confidence limits. The numbers of rats used, n, given in parentheses. Slopes of the regression lines are given as 95% confidence limits. Statistical significance was determined using Student's t test where $P<0.05$ was considered to be significant.

Drugs used

The following drugs were used in the current study: (−)-isoprenaline hydrochloride (Sigma), (−)-noradrenaline bitartarate (Sigma), salbutamol hemisulphate (Sigma), carbachol chloride (Sigma) (±)-propranolol (Zeneca), (±)-alprenolol (Zeneca), CGP 20712A (CIBA-Geigy), ICI 118551 (Zeneca), ZD7114 ((S)-4-[2-hydroxy-3-phenoxy-propylamino-ethoxy]-N-(2-methoxyethyl)-phenoxyacetamide) and BRL37344 (sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylaminopropyl]phenoxy-acetate sesquijudrate) (both synthesized by B Rao, Research Department, Zeneca Pharmaceuticals, Alderley, Park Macclesfield). (±)-Alprenolol, ZD7114, BRL37344 were initially dissolved in dimethylsulphoxide (DMSO) and subsequently diluted in distilled water. All other agents were dissolved in distilled water.

Results

Agonist activity

Isoprenaline, noradrenaline, and BRL37344 all caused graded relaxation of the tissues where tone had been raised with carbachol. Salbutamol produced a relaxation only at concentrations above 10 μM (i.e. between 10 and 20% of the isoprenaline maximum response) and ZD7114 was without any significant relaxant effect up to 10 μM. BRL37344 (EC_{50} 0.05 μM [0.004–0.9 μM], $n = 50$), was nine times more potent than isoprenaline (IC_{50} 0.45 μM [0.18–1.3 μM], $n = 81$) and 47 times more potent than noradrenaline (IC_{50} 2.3 μM [2.0–2.8 μM], $n = 4$). However, the maximum response obtained with BRL37344 (i.e. 71.4 ± 4.5%) was significantly ($P<0.05$) lower than that achieved with isoprenaline. Data are illustrated in Figure 1. The response profiles obtained with BRL 37344 and isoprenaline were quite different. BRL37344 produced a response that was slow in onset and continued to develop slowly, taking around 25 min to reach a plateau (Figure 2a). Isoprenaline, on the other hand produced a relatively rapid reduction in tone, responses reaching a plateau in about 5 min (Figure 2b).

It was noted that tachyphylaxis was present with the adrenoceptor agonists when concentration-response curves were repeated in the same tissues at hourly intervals (data not shown). Hence for antagonist studies (next section), paired tissue segments were used, allowing for one concentration curve per tissue.

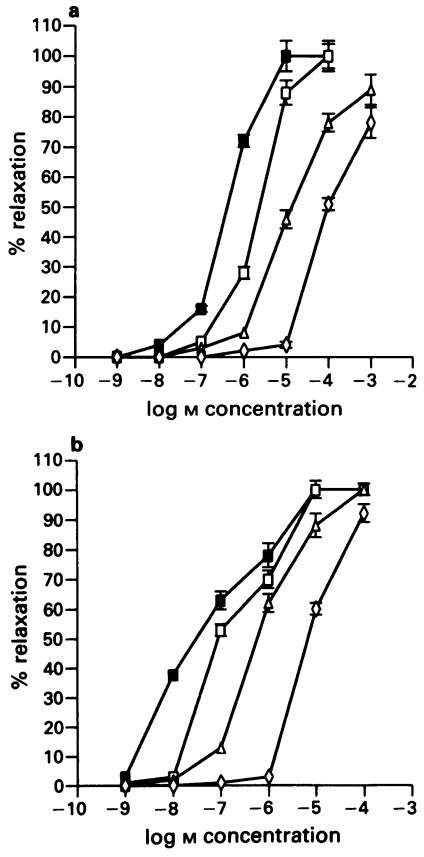


Figure 4 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves to (a) isoprenaline and (b) BRL37344 in the absence (■, $n = 12$) and presence of alprenolol 1 μM (□, $n = 4$), 10 μM (△, $n = 4$) and 100 μM (◇, $n = 4$). CGP20712A, ICI 118551 and prazosin (all 0.1 μM) were present throughout. Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size.

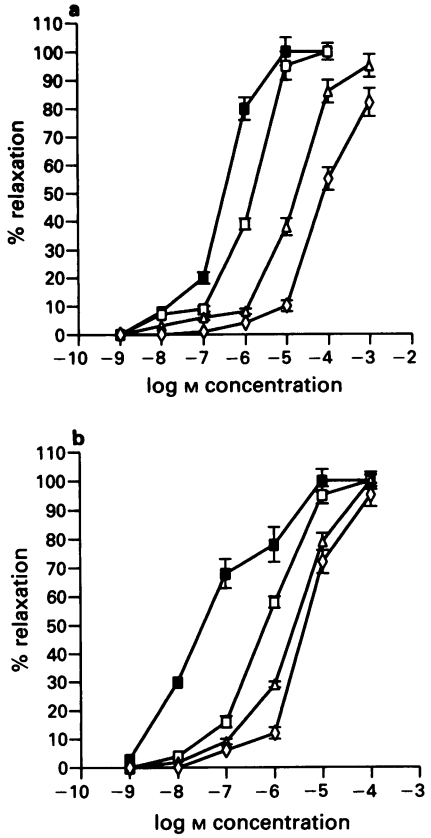


Figure 5 Carbachol (0.5 μM)-contracted rat isolated ileum. Relaxation-response curves to (a) isoprenaline and (b) BRL37344 in the absence (■, $n = 12$) and presence of ZD7114 1 μM (□, $n = 4$),10 μM (△, $n = 4$) and 100 μM (◇, $n = 4$). CGP20712A, ICI 118551 and prazosin (all 0.1 μM) were present throughout. Each point represents the mean percentage relaxation and the number of rats is given as n. Vertical bars indicate s.e.mean only where this exceeds the symbol size.

Antagonist activity

(±)-Propranolol (10 and 100 μM) weakly antagonized the relaxant reponses to both isoprenaline and BRL37344 (Figure 3a and b). pK_B values derived from these concentrations

of propranolol were 5.7 [5.3–6.4, $n=8$] and 5.5 [4.5–5.9, $n=9$] versus isoprenaline and BRL37344 respectively. These were not significantly different from each other. (±)-Alprenolol (1–100 μM) competitively antagonized the relaxant responses to isoprenaline (Figure 4a). The Arunlakshana-Schild plot of the data revealed a pA_2 of 6.5 [5.9–6.9, $n=12$, Figure 6a], the slope of the regression line not being significantly different from unity (0.9 [0.8–1.0]). When BRL 37344 was used as the agonist, its relaxant responses were also antagonized by (±)-alprenolol (1–100 μM, Figure 4b). However, the antagonist effect did not appear to be competitive as the slope of the Arunlakshana-Schild plot was significantly different from unity (0.5 [0.1–0.8], $P<0.05$). The pK_B value derived from these concentrations was 6.4 [5.8– 6.9, $n=12$], which was not significantly different from that obtained with (±)-alprenolol versus isoprenaline.

As ZD7114 was without significant agonist activity, it was tested as a potential antagonist of the relaxant responses to isoprenaline and BRL37344. When isoprenaline was used as the agonist, ZD7114 (1–100 μM) caused competitive antagonism of the relaxant responses (Figure 5a). An Arunlakshana-Schild plot of the data revealed a pA_2 value of 6.3 [5.9–6.8, $n=12$, Figure 6b), the slope of the regression line not being significantly different from unity (1.1 [0.8–1.2]). However, when BRL37344 was used as the agonist, the antagonist effect of ZD7114 (1–100 μM) did not appear to be competitive (Figure 5b) as the slope of the Arunlakshana-Schild plot was significantly different from unity (0.6 [0.5–0.6], $P<0.05$). The pK_B value derived from these concentrations of ZD7114 was 6.7 [5.9–7.4, $n=12$]. This value was not significantly different from that obtained with ZD7114 when isoprenaline was used as the agonist.

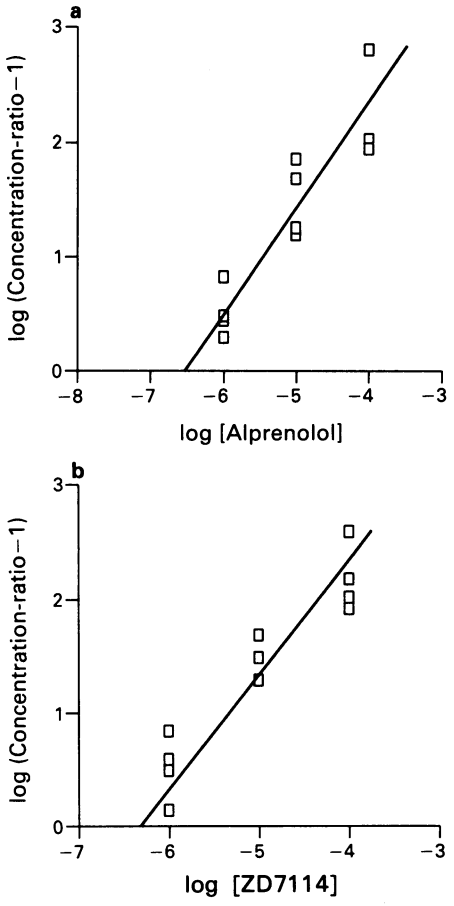


Figure 6 Arunlakshana-Schild plots of the data from (a) antagonism of isoprenaline by alprenolol, 1–100 μM (concentration-responses shown in Figure 4a), and (b) antagonism of isoprenaline by ZD7114, 1–100 μM (concentration-responses shown in Figure 5a).

Discussion

We have previously demonstrated that ZD7114 and BRL 37344 act at atypical or β_3-adrenoceptors in the guinea-pig (Growcott *et al.*, 1993). In this preparation, both agents possessed similar efficacy to isoprenaline, although both agents were less potent. In the current study, BRL37344 was the most potent of the agonists tested, although its efficacy was significantly lower than that of isoprenaline. ZD7114, on the other hand, was without any significant agonist activity. The reduced efficacy of BRL37344 compared to isoprenaline has been noted by other workers who have used the compound to evaluate potential β_3-adrenoceptor agonist activity in, for example, rat jejunum (van der Vliet *et al.*, 1990), human white adipocytes (Hollenga *et al.*, 1990) and colon (McLaughlin *et al.*, 1991). It was also interesting to note the difference in agonist profiles between isoprenaline and BRL 37344. Isoprenaline produced a fast relaxation whereas the response to BRL37344 was slow in onset and duration, the concentration responses covering some five log units. Although no direct reference to the speed of action of BRL 37344 was made by McLaughlin & MacDonald (1990), these workers did comment on the ability of this agent to produce profound tachyphylaxis in rat colon. Indeed, a close analogue of BRL37344, (BRL35135), has also been reported to produce tachyphylaxis in guinea-pig gastric fundus (Coleman *et al.*, 1987). The latter workers pointed out that neither isoprenaline nor salbutamol produced tachyphylaxis. The tendency for the BRL37344 compound to produce tachyphylaxis could possibly be explained by its slow onset and duration of action. Indeed, it has been suggested (Arch, 1989) that the bulky N-substituent of the BRL compounds could bind to accessory sites (possibly unrelated to the β-adrenoceptor). This might, in turn, mean that the rate at which these compounds access the β_3-adrenoceptor would be much slower than the classical ligands such as isoprenaline and noradrenaline. It was also noted that, with BRL37344 in the current studies, the concentration-responses appeared to be of a biphasic nature, suggesting that BRL37344 was interacting with more than one site. It seems unlikely that BRL 37344 would have interacted with more than one population of adrenoceptor as high concentrations of selective antagonists were employed in order to prevent this. Perhaps the postulated interaction with the accessory binding site (Arch, 1989) can again account for this or the compound may interact with some other mechanism which remains unknown. The answer to this question remains to be resolved.

The low efficacy of ZD7114 has also been noted in other parts of the rat gastrointestinal tract, i.e. distal colon (MacDonald & Lamont, 1992) and in human colon (Growcott, unpublished observations). Although speculation has been raised as to the homogeneity of atypical adrenoceptors (Bianchetti & Manara, 1990), it would seem inappropriate to apply that argument in the current study. It could be argued that differences in agonist efficacy result from differences in receptor coupling, i.e. in tissues where BRL37344 and ZD 7114 act as full agonists, receptor-coupling mechanisms are highly efficient, whereas in tissues where the efficacy of these agents was low, the efficiency of the receptor-coupling mechanisms was also low. If this were the case then it would be predicted that with an agonist possessing high intrinsic efficacy, such as isoprenaline, in a tissue with an efficient receptor coupling mechanism, e.g. guinea-pig ileum, this agent would be significantly more potent than in a tissue where receptor coupling efficiency was low (rat tissue, for example). Partial agonists would appear to lose efficacy as coupling efficiency declined (Kenakin, 1984). However, this argument cannot be the sole explanation for the observations made in the current study. Isoprenaline possessed full intrinsic efficacy in both guinea-pig (Growcott *et al.*, 1993) and rat ileum (present study). Indeed, there was no significant difference between the potency obtained with this agent on guinea-pig tissue when compared to that obtained on rat

tissue (IC_{50} values 0.50 and 0.45 μM respectively). However, both BRL37344 and ZD7114, compounds that possessed equal intrinsic activities when compared to isoprenaline in guinea-pig ileum, exhibited lower or no measurable intrinsic activities in rat ileum. Indeed, the latter compound exhibited antagonist activity in the rat ileum. An alternative explanation for these differences could be that guinea-pig and rat tissues contain differing levels of β_3-adrenoceptor reserves: in guinea-pig this is high, whereas in the rat, these levels are low. Hence, the efficacy of an agent at the β_3-adrenoceptor would be, in part, dependent upon the level of β_3-adrenoceptor reserve. Indeed, it appears that in the rat ileum, the β_3-adrenoceptor reserve is sufficiently low to allow ZD7114 to act as a competitive β_3-adrenoceptor antagonist (current study and MacDonald & Lamont, 1992) and for BRL37344 to act as a partial agonist. Indeed, in the absence of a suitable alkylating agent or irreversible antagonist to demonstrate receptor reserve and, hence alter the stimulus-response characteristics of tissues, it has been reported that functional antagonism of agonist responses can produce effects on the concentration-responses that resemble the effects observed with alkylating agents (Kenakin, 1984). Thus, functional antagonism can be achieved by addition of an agent, which through an effect at some other receptor, opposes the action of the primary stimulus. Using this technique in guinea-pig ileum, we have been able to demonstrate that ZD7114 can act as an antagonist of the responses to isoprenaline (Growcott *et al.*, 1992).

It is highly probable that the adrenoceptors involved in the responses to the agents used in the current study were of the atypical or β_3-subtype. The affinities of propranolol and alprenolol, as antagonists of the responses to both isoprenaline and BRL37344, in the current study, were comparable to those reported by other workers, i.e. pA_2 values of <7.0. These are significantly lower than would be expected for effects of these agents at classical β-adrenoceptors (i.e. pA_2 values in excess of 8.0). However, it is interesting to note that some workers find that responses to either isoprenaline and/or BRL37344 are completely insensitive to blockade with propranolol (Blue *et al.*, 1990), whereas others find that the actions of these two compounds can be blocked (McLaughlin & MacDonald, 1990). The affinity of propranolol in these latter studies, was however, lower than that obtained at classical adrenoceptors. In fact, the range of affinities expressed for propranolol at the atypical β-adrenoceptor is shared by another non-selective antagonist, alprenolol. The data obtained with this agent in the current study (i.e. pA_2 value of 6.5 versus isoprenaline) was significantly lower than would be expected for effects at classical β-adrenoceptors (i.e. pA_2 typically 8.6–9.0). This is in good agreement with other workers, e.g. Blue *et al.* (1990) using guinea-pig ileum (pA_2 value of 6.5 versus isoprenaline) but different from that obtained by van der Vliet *et al.* (1990) using rat jejunum (pA_2 value of 7.5 versus isoprenaline) and Bianchetti & Manara (1990) using rat colon (pA_2 value of 7.6 versus isoprenaline). A possible explanation for these apparent 'discrepancies' could be that in the studies where 'high' affinities have been obtained for propranolol or alprenolol a heterogeneous population of β-adrenoceptors exists and the affinities of these antagonists is an overestimate, including their effects at β_1-, β_2- and β_3-adrenoceptors. Interestingly, the pK_B value obtained for the antagonist effect of ZD7114 versus isoprenaline in the current study (6.33) was lower than that obtained for the same agent in the study of MacDonald & Lamont (1992), i.e. 7.3. In this latter study, it is not clear whether selective antagonists were present throughout the study to eliminate any possible contribution from classical adrenoceptors. Hence a direct comparison is not possible.

In the current study, the antagonist effects of propranolol, alprenolol and ZD7114 versus BRL37344 did not appear to be of a competitive nature. A possible explanation for this effect could be that because the responses elicited by BRL 37344 were composed of effects of the agent at more than one site (see above). This would lead to a non-linear Schild plot with an 'aggregate' slope less than unity (Kenakin, 1984). A further possibility could be that at the higher concentrations of BRL37344 'breakthrough' of the antagonists cocktail occurred and the resultant effect was due to an interaction with more than one type of adrenoceptor.

Whether the data generated in the animal tissues can be directly related to a human situation is, at present, difficult to answer. It would appear from comparison of the genomic sequences that there are only minor differences between the β_3-adrenoceptor in man, mouse and rat (Granneman *et al.*, 1992). Even so, there appear to be significant differences in the functional activity of recombinant rat and human β_3-adrenoceptors, when expressed in Chinese hamster ovary cells (Liggett, 1992). These differences could account, at least in part, for the results obtained in functional studies using isolated tissues which have implied marked discrepancies between human and animal β_3-adrenoceptors. However, differences in receptor density may also account for some of the inter-species differences seen in studies with isolated tissues. Only when agonists with high efficacy at the β_3-adrenoceptor are developed will some of these arguments be resolved.

References

ARCH, J.R.S. (1989). The brown adipocyte β-adrenoceptor. *Proc. Nutr. Soc.*, **48**, 215–233.

ARCH, J.R.S., AINSWORTH, A.T., CAWTHORNE, M.A., PIERCEY, V., SENNITT, M.V., THODY, V.E., WILSON, C. & WILSON, S. (1984). Atypical β-adrenoceptor on brown adipocytes as a target for anti-obesity drugs. *Nature*, **309**, 163–165.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol Chemother.*, **14**, 48–57.

BIANCHETTI, A. & MANARA, L. (1990). *In vitro* inhibition of intestinal motility by phenylethanolamines: evidence of atypical β-adrenoceptors in rat colon. *Br. J. Pharmacol.*, **100**, 831–839.

BLUE, D.R., BOND, R.A., ADHAM, N., DELMONDO, R., MICHEL, A.D., EGLEN, R., WHITING, R.L. & CLARKE, D.E. (1990). Interaction of dihydroalprenolol and cyanopindolol with atypical β-adrenoceptors in guinea-pig ileum. *J. Pharmacol. Exp. Ther.*, **252**, 1034–1042.

BOND, R.A. & CLARKE, D.E. (1987). A response to isoprenaline unrelated to α- or β-adrenoceptor agonism. *Br. J. Pharmacol.*, **91**, 683–686.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95**, 723–724.

COLEMAN, R.A., DENYER, L.H. & SHELDRICK, K.E. (1987). β-adrenoceptors in guinea-pig gastric fundus – are they the same as the 'typical' β-adrenoceptors in rat adipocytes? *Br. J. Pharmacol.*, **89**, 463P.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology*, Vol. 33, pp. 283–335. Berling: Springer Verlag.

GRANNEMAN, J.G., LAHNERS, K.N. & RAO, D.D. (1992). Rodent and human β_3-adrenoceptor genes contain an intron within the protein-coding block. *Mol. Pharmacol.*, **42**, 964–970.

GROWCOTT, J.W., GREEN, M. & HOLLOWAY, B. (1992). Atypical β-adrenoceptor antagonist profile of ICI D7114 in guinea-pig and rat isolated ileum. *Br. J. Pharmacol.*, **107**, 372P.

GROWCOTT, J.W., WILSON, C., HOLLOWAY, B. & MAINWARING, S. (1993). Evaluation of ICI D7114-a putative stimulant of brown adipocytes – on histamine pre-contracted guinea-pig ileum. *Br. J. Pharmacol.*, **109**, 1212–1218.

HOLLENGA, C., HAAAS, M., DEINUM, J.T. & ZAAGSMA, J. (1990). Discrepancies in lipolytic activities induced by β-adrenoceptor agonists in human and rat adipocytes. *Horm. Metab. Res.*, **22**, 17–21.

HOLLOWAY, B.R., BRISCOE, M., GROWCOTT, J.W., WILSON, C. & STOCK, M.J. (1991a). *In vitro* and *in vivo* selectivity of ICI D7114 for brown fat and thermogenesis. In *Adrenoceptors: Structures, Mechanisms, Function, Advances in Pharmacological Sciences*, pp. 313–314. Basel: Birkhauser Verlag.

HOLLOWAY, B.R., HOWE, R., RAO, B.S., STRIBLING, D., MAYERS, R.M., BRISCOE, M. & JACKSON, J. (1991b). ICI D7114 a novel selective β-adrenoceptor agonist selectively stimulates brown fat and increases whole body oxygen consumption. *Br. J. Pharmacol.*, **103**, 97–104.

KENAKIN, T.P. (1984). The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

KENAKIN, T.P. & BEEK, D. (1980). Is prenalterol (H33/80) really a selective β_1-adrenoceptor agonist? Tissue selectivity resulting from differences in stimulus-response relationships. *J. Pharmacol. Exp. Ther.*, **213**, 406–413.

LIGGETT, S.B. (1992). Functional properties of the rat and human β_3-adrenergic receptors: Differential agonist activation of recombinant receptors in Chinese hamster ovary cells. *Mol. Pharmacol.*, **42**, 634–637.

MACDONALD, A. & LAMONT, M. (1992). Relative contributions of β-adrenoceptor subtypes to isoprenaline-induced relaxation of rat distal colon. *Br. J. Pharmacol.*, **107**, 24P.

MCLAUGHLIN, D.P., FULTON, J.D., MACDONALD, E., SCOTT, P.J.W. & MACDONALD, A. (1991). β-Adrenoceptors mediating relaxation in human colon *in vitro*. *Br. J. Pharmacol.*, **104**, 152P.

MCLAUGHLIN, D.P. & MACDONALD, A. (1990). Evidence for the existence of atypical β-adrenoceptors (β_3-adrenoceptors) in the rat distal colon *in vitro*. *Br. J. Pharmacol.*, **101**, 569–574.

TANEJA, D.T. & CLARKE, D.E. (1991). Evidence for a noradrenergic innervation to 'atypical' β-adrenoceptors (or putative β_3-adrenoceptors) in the ileum of the guinea-pig. *J. Pharmacol. Exp. Ther.*, **260**, 192–200.

VAN DER VLIET, A., RADEMAKER, B. & BAST, A. (1990). A β-adrenoceptor with atypical characteristics is involved in the relaxation of the rat small intestine. *J. Pharmacol. Exp. Ther.*, **255**, 218–226.

(Received May 4, 1993
Revised August 5, 1993)
Accepted August 11, 1993)

Br. J. Pharmacol. (1993), **110**, 1381–1386 © Macmillan Press Ltd, 1993

Inhibition by rapamycin of leukocyte migration and bronchial hyperreactivity induced by injection of Sephadex beads to guinea-pigs

[1]Janetti Nogueira de Francischi, Dolores M. Conroy, Karim Maghni & [2]Pierre Sirois

Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, P.Q., Canada J1H 5N4

1 The effect of rapamycin (0.001 to 5 mg kg^{-1}) on the increased leukocyte counts in bronchoalveolar lavage (BAL) fluid and hyperreactivity of isolated bronchial strips to histamine and acetylcholine (ACh) was studied following the intravenous injection of Sephadex beads to guinea-pigs.

2 The intramuscular (i.m.) injection of rapamycin (0.012 to 5 mg kg^{-1}) dose-dependently inhibited the increase in leukocyte counts in BAL fluid. Rapamycin (5 mg kg^{-1}) reduced the numbers of eosinophils neutrophils, macrophages and lymphocytes in BAL fluid by 64, 55, 19 and 50% respectively. In addition, rapamycin (0.012 to 5 mg kg^{-1}) significantly inhibited the Sephadex-induced hyperreactivity of bronchial tissue to both histamine and ACh.

3 At a dose of 0.001 mg kg^{-1}, rapamycin did not significantly reduce leukocyte infiltration or bronchial hyperreactivity.

4 Cyclosporin (5 mg kg^{-1}) significantly reduced both lymphocyte and eosinophil numbers in BAL fluid of Sephadex-injected guinea-pigs whereas dexamethasone (1 mg kg^{-1}) significantly reduced lymphocyte numbers. Neither drug affected the bronchial hyperreactivity to histamine and ACh.

5 It is concluded that the new immunosuppressive drug, rapamycin, is a potent inhibitor of leukocyte migration and bronchial hyperreactivity observed following the intravenous injection of Sephadex beads to guinea-pigs. Rapamycin also appears to be more effective than cyclosporin or dexamethasone in reducing leukocyte counts and bronchial hyperreactivity in this model.

6 Our results suggest that inflammatory mechanisms which are inhibited by rapamycin may be important in the induction of Sephadex-induced hyperreactivity.

Keywords: Rapamycin; cyclosporin; dexamethasone; leukocytes; guinea-pig lung; Sephadex beads; bronchial hyperreactivity

Introduction

Rapamycin, a macrolide antibiotic produced by *Streptomyces hygroscopicus* (Vezina *et al.*, 1975) is a very potent immunosuppressive drug as it was shown to inhibit the development of experimental allergic encephalomyelitis and adjuvant arthritis, the production of IgE antibodies (Martel *et al.*, 1977) and tissue allograft rejection (Calne *et al.*, 1989; Stepkowski *et al.*, 1991).

Rapamycin, like cyclosporin A (Sigal *et al.*, 1991) and FK 506 (Kay *et al.*, 1991), a structural analogue of rapamycin, can inhibit the *in vitro* proliferation of T-lymphocytes induced by various mitogenic agents. However, the profile of the suppressive mechanism of action shown by rapamycin on lymphocyte proliferation assays differed from that observed with FK 506 and cyclosporin A in that only the former could also inhibit T-lymphocyte proliferation induced by interleukin (IL)-2 (Kay *et al.*, 1991; Sigal *et al.*, 1991). Rapamycin suppresses a wider spectrum of T- and B-cell activation pathways than FK 506 or cyclosporin (Chang *et al.*, 1991).

There is now increasing evidence that T-lymphocytes play an important role in the pathogenesis of human asthma (reviewed by Rochester & Rankin, 1991). Lymphocytes and eosinophils have been detected in bronchial biopsies of stable atopic asthmatic patients (Azzawi *et al.*, 1990). T-cell derived cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3 and IL-5 have been shown to modulate IgE production by B lymphocytes and mediate the migration, activation/survival of eosinophils and other leukocytes (Hamblin, 1991; Corrigan & Kay, 1992). In addition, a significant increase in lymphocyte number in bronchoalveolar lavage (BAL) fluid from ovalbumin-sensitized rats has been shown to correlate with the increase in bronchial hyperresponsiveness (Elwood *et al.*, 1991). Sanjar *et al.* (1990) showed a hyperresponsiveness to methacholine and a significant increase in lymphocyte number in BAL of ovalbumin-sensitized guinea-pigs. In addition, an increased number of activated T-lymphocytes in both peripheral blood and BAL fluid of asthmatic patients has been reported (Walker *et al.*, 1991). Both cyclosporin and FK 506, which suppress the activation of T-lymphocytes, have been shown to inhibit antigen-induced bronchial hyperresponsiveness in the guinea-pig (Fukuda *et al.*, 1991).

In the present investigation, we have studied the effect of rapamycin on leukocyte infiltration in guinea-pig lung following the injection of Sephadex beads. This technique has been used previously in our laboratory to induce a pulmonary eosinophilia in the guinea-pig (Maghni *et al.*, 1991). In addition, we have examined the effect of rapamycin on the reactivity of isolated bronchial strips to acetylcholine and histamine from normal and Sephadex-treated animals. A preliminary account of part of this work was presented to the British Pharmacological Society (Francischi *et al.*, 1992).

Methods

Male and female Dunkin Hartley guinea-pigs (300–400 g) were used throughout this study.

[1] Present address: Department of Pharmacology, Biological Science Institute, Federal University of Minas Gerais, CEP 31270-010, Brazil.

[2] Author for correspondence.

Induction of leukocyte infiltration in guinea-pig lungs by Sephadex beads

Leukocyte infiltration into guinea-pig lungs was induced by an intravenous injection (i.v.) of Sephadex beads as described by Maghni *et al.* (1991). Briefly, 0.13 ml of a suspension of Sephadex G50 (superfine) beads (55 mg ml^{-1}) in sterile saline was injected into the ear vein of conscious guinea-pigs. The injections were performed immediately following dilution of the beads. In all the experiments, the animals were killed by cervical dislocation 24 h after Sephadex injection and exsanguinated by cutting the inferior vena cava.

Total and differential cell counts in bronchoalveolar lavage (BAL) fluid

Immediately after exsanguinating the animals, the trachea was cannulated with a catheter joined by a three-way stopcock attached to two 50 ml syringes. Fifty-five ml of phosphate-buffered saline (PBS, pH 7.4, 37°C) was injected into the lungs in 5 ml aliquots and aspirated after gentle massage of the thorax. The total amount of fluid recovered from the lavages was similar in both naïve and Sephadex-injected animals. The cell suspensions were submitted to a first centrifugation (400 *g*, 10 min). The red blood cells present in the cell pellet were lysed by hypotonic shock and after correction of osmolarity, the cells were centrifuged (400 *g*, 10 min). The final cell pellet was resuspended in 20 ml PBS for total cell counts in a Neubauer chamber. Cell viability was simultaneously assessed by the Trypan blue exclusion technique. Differential cell counts were done from duplicate cytospin smears of the original total cell suspensions using Wright's stain. The results are presented as mean cell number ± s.e. mean. The number of cells was assessed without the experimenter being aware of the treatment of the animals.

Preparation of bronchial strips from naïve and Sephadex-injected animals

After opening the thoracic cavity, the lungs were removed. Internal bronchi from each lobe (0.5–2.0 mm internal diameter) were isolated, cleaned of surrounding parenchymal tissue and cut into spiral strips. The strips were attached to Grass isometric transducers coupled to a Grass polygraph under a 2 g initial tension. The tissues were superfused with Krebs-Henseleit solution (mM composition: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.18 and glucose 11.1) at 37°C, pH 7.0 in a cascade system. Dose-response curves to histamine (10^{-10} to 10^{-6} M) and acetylcholine (ACh; 10^{-10} to 10^{-5} M) were obtained following an equilibration period of 60 min. All agonists were administered as bolus injections.

Administration of immunosuppressive drugs

A stock solution of rapamycin (25 mg ml^{-1}) was prepared in 0.2 ml of ethanol and 0.8 ml of Cremophor el. Dilutions of rapamycin were further prepared from stock solution as required. Rapamycin was administered by the intramuscular (i.m.) route as described by Calne *et al.* (1989), 2 h before injection of Sephadex beads. Control animals were injected with a vehicle solution (0.2 ml ethanol:0.8 ml Cremophor el) in the same schedule as that used for rapamycin. Each animal received 0.1 ml of drug or vehicle per 100 g of body weight. Cyclosporin A and dexamethasone, diluted in sterile physiological saline immediately before use, were injected by the i.m. route in a separate group of animals, 2 and 1.5 h respectively before injection of Sephadex beads.

Materials

The following compounds were used: Sephadex beads G50 (superfine: 10–40 μm, Pharmacia); rapamycin was kindly supplied by Wyeth-Ayerst Research, Princeton, NJ, U.S.A.; cyclosporin A (i.v. solution, Sandimmune, Canada); dexamethasone (Hexadrol Phosphate Injection, Organon Tekniks, Canada); histamine dihydrochloride, acetylcholine chloride, Cremophor el, Wright's stain and Trypan blue dye were purchased from Sigma, St Louis, MO, U.S.A.

Statistics

Data from BAL studies were analysed by Student's *t* test for single comparisons, and by Dunnett's *t* test (Dunnett, 1964) for multiple comparisons (unpaired, one-tailed). Data for dose-response curves to histamine and ACh from control and Sephadex-treated guinea-pigs were analysed by analysis of variance. In all cases a *P* value of less than 0.05 was considered statistically significant ($P < 0.05$).

Results

Increase in leukocyte number in BAL fluid collected from Sephadex-injected guinea-pigs

A greater than three fold increase (from 21.99 to 83.28 × 10^6 cells) in total cell number recovered in BAL fluid was observed 24 h following the i.v. injection of Sephadex beads to guinea-pigs. Cytospin preparations of BAL fluid cells after Wright's staining showed that eosinophil, neutrophil, macrophage and lymphocyte numbers were all increased following Sephadex injection (Table 1), with a pronounced eosinophil (ten fold increase), neutrophil (235 fold increase) and lymphocyte (20 fold increase) infiltration. The polymorphonuclear cells (eosinophils and neutrophils) comprised 57%, and mononuclear cells (macrophages plus lymphocytes) represented 43% of total cells in BAL fluid from Sephadex-injected animals compared to 16% polymorphonuclear and 84% mononuclear cells in BAL fluid from naïve guinea-pigs. The cells which were considered as macrophages were a mixture of monocyte (no cytoplasmic vacuoli), fully vacuolised macrophages and alveolar macrophages.

Table 1 Leukocyte number in guinea-pig bronchoalveolar lavage fluid (BAL) 24 h after injection of Sephadex beads

Groups	*Leukocyte number in BAL* (cells × 10^6) *Differential*			
	Eosino	*Neutro*	*Macro*	*Lympho*
Naïve ($n = 5$)	3.45 ± 0.62	0.08 ± 0.05	18.26 ± 1.34	0.20 ± 0.08
Sephadex ($n = 6$)	33.60 ± 4.84[b]	18.86 ± 2.93[b]	31.92 ± 5.08[a]	3.91 ± 0.76[b]

Results are expressed as mean number of leukocytes ± s.e.mean.
Eosino = eosinophils, Neutro = neutrophils, Macro = macrophages and Lympho = lymphocytes.
[a]$P < 0.05$; [b]$P < 0.01$ (Student's *t* test for unpaired data).

Increase in bronchial reactivity to histamine and acetylcholine induced by injection of Sephadex beads into guinea-pigs

An increase in reactivity of isolated bronchial tissue to histamine and ACh was observed 24 h following i.v. injection of Sephadex beads into guinea-pigs (Figure 1a and b). The bronchial strips from control and Sephadex-injected guinea-pigs responded over the same dose-range to both agonists. However, the maximum response of the bronchial strips from Sephadex-injected animals was significantly greater than that observed for control tissues. This is reflected in an increase in the gradient of the log dose-response curves to histamine and ACh for bronchial strips from Sephadex-treated animals compared to naïve animals and is referred to here as 'bronchial hyperreactivity' (Figures 1a and b). The maximum contraction of the bronchial strips to histamine and ACh was increased from 0.223 ± 0.039 and 0.174 ± 0.03 g to 0.533 ± 0.048 and 0.388 ± 0.031 g ($P<0.01$, $n=10$) respectively following the injection of Sephadex beads.

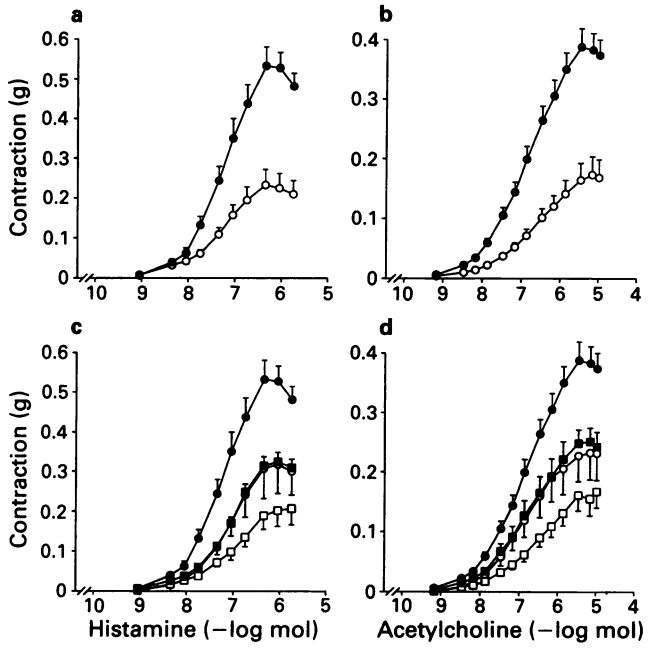


Figure 1 Dose-response curves of bronchial strips from vehicle (○) and Sephadex-injected (●) guinea-pigs to histamine (a) and acetylcholine (ACh) (b). Effect of rapamycin 0.012 (○), 0.12 (□) and 5 mg kg^{-1} (■) on the Sephadex-induced bronchial hyperreactivity to histamine (c) and ACh (d), $n=6-12$. Rapamycin diluted in cremophor el and ethanol (vehicle) was administered 2 h before the injection of Sephadex beads. Bronchial strips were prepared 24 h following the injection of the beads. Results are presented as mean contraction (g) ± s.e.mean.

Effect of rapamycin on leukocyte counts in BAL fluid and bronchial hyperreactivity of Sephadex-injected animals

Rapamycin (0.001–5 mg kg^{-1}) administered 2 h before the injection of Sephadex beads dose-dependently reduced the total leukocyte number present in BAL fluid. At a dose of 5 mg kg^{-1}, rapamycin significantly reduced the total leukocyte count from 83.79 ± 7.52 to $43.91 \pm 4.41 \times 10^6$ cells ($P<0.01$). This decrease in total leukocyte numbers induced by rapamycin (5 mg kg^{-1}) was related to a significant reduction of the number of eosinophils, neutrophils, macrophages and lymphocytes present in BAL fluid (Table 2). A significant inhibition of eosinophil numbers was also observed with 0.12 mg kg^{-1} rapamycin ($P<0.01$). At the lowest dose of rapamycin (0.001 mg kg^{-1}) there was no significant inhibition of the total or differential cell numbers.

Increasing the dose of rapamycin to 25 mg kg^{-1} did not further reduce the leukocyte counts in BAL fluid (data not shown). In addition, i.m. injection of rapamycin (5 mg kg^{-1}) into naïve animals did not affect the total and differential cell counts recovered in BAL fluid when compared to animals treated with vehicle (results not shown). Furthermore, administration of the vehicle, 2 h before the Sephadex injection, did not cause a significant change in leukocyte profile found in BAL fluid (Table 2) when compared to results from animals which were injected with the Sephadex beads (Table 1).

Rapamycin (0.012 to 5 mg kg^{-1}) significantly reduced the hyperreactivity of isolated bronchial strips to histamine and ACh observed 24 h following the injection of Sephadex beads (Figure 1c and d). At a dose of 5 mg kg^{-1}, the maximal contraction of the tissue to histamine was significantly reduced from 0.533 ± 0.048 to 0.324 ± 0.024 g and that induced by ACh was reduced from 0.388 ± 0.031 to 0.257 ± 0.023 g ($P<0.01$, $n=10-12$). This represented a 70% reduction of the maximal contraction of bronchial strips to both agonists. A similar significant inhibition of the maximum contractile response of isolated bronchi to histamine and ACh was also observed with 0.012 and 0.12 mg kg^{-1} rapamycin (Figure 1c and d). Increasing the dose of rapamycin to 25 mg kg^{-1} did not further suppress the bronchial hyperreactivity (results not shown). However, the lowest dose of rapamycin (0.001 mg kg^{-1}) did not inhibit the hyperreactivity of bronchial smooth muscle induced by the injection of Sephadex beads. Rapamycin (5 mg kg^{-1}) did not attenuate the maximal contraction of tissues isolated from naïve guinea-pigs (data not shown).

Effects of cyclosporin and dexamethasone on leukocyte counts in BAL fluid and bronchial hypereactivity of Sephadex-injected guinea-pigs

Cyclosporin (5 mg kg^{-1}) significantly reduced both eosinophil and lymphocyte numbers in BAL fluid from Sephadex-

Table 2 Inhibition by rapamycin of leukocyte counts in BAL from guinea-pigs injected with Sephadex beads

	Leukocyte number in BAL (cells × 10^6)			
		Differential		
Rapamycin (mg kg^{-1})	*Eosino*	*Neutro*	*Macro*	*Lympho*
0 ($n=10$)	32.08 ± 2.04	16.06 ± 2.04	28.47 ± 0.43	3.75 ± 0.43
0.001 ($n=5$)	23.91 ± 4.73	14.95 ± 3.16	29.86 ± 3.88	2.78 ± 0.43
0.012 ($n=6$)	22.67 ± 3.45	7.30 ± 2.69[a]	23.91 ± 2.53	4.62 ± 1.16
0.12 ($n=5$)	13.26 ± 5.93[b]	8.94 ± 3.46	29.19 ± 3.82	2.40 ± 0.81
5.0 ($n=7$)	11.65 ± 0.82[b]	7.24 ± 1.40[b]	23.13 ± 1.90[a]	1.87 ± 0.29[a]

Rapamycin (i.m.) injection was administered 2 h before i.v. injection of Sephadex beads. BAL was performed 24 h after Sephadex injection. Control animals received i.m. vehicle (Cremophor el + ethanol) injection 2 h before the beads.
Eosino = eosinophils, Neutro = neutrophils, Macro = macrophages and Lympho = lymphocytes.
[a]$P<0.05$; [b]$P<0.01$ (Dunnett's t test for unpaired data).

injected animals (control; $P<0.05$, Figure 2), but did not significantly affect neutrophil and macrophage numbers. In addition, cyclosporin did not reduce the hyperreactivity of isolated bronchial strips from Sephadex-injected guinea-pigs to histamine or ACh (Table 3). Dexamethasone (1 mg kg^{-1}) significantly reduced only the number of lymphocytes in BAL fluid (Figure 2), but not the hyperreactivity of bronchial tissues from Sephadex-injected animals (Table 3).

Discussion

In the present study, we have examined the effect of rapamycin, a new immunosuppressive drug (reviewed by Morris, 1992), in a model of guinea-pig airway inflammation induced by an intravenous injection of Sephadex beads. A marked lung infiltration of leukocytes accompanied by an increase in reactivity of bronchial tissues were two features of this model. The influx of eosinophils (ten fold increase), neutrophils (235 fold increase) and lymphocytes (20 fold increase) into the airways was observed 24 h after the administration of the beads. This technique was previously used in our laboratory to increase the number of eosinophils in BAL fluid (Maghni et al., 1991). In addition, i.v. administration of Sephadex beads in rats also causes eosinophilia but with a longer time-course than in guinea-pigs, as observed by Spicer et al. (1990). In the present study we have shown that in addition to an increase in eosinophils, there was also an increase in neutrophils, macrophages and lymphocytes recovered in BAL fluid from Sephadex-injected guinea-pigs.

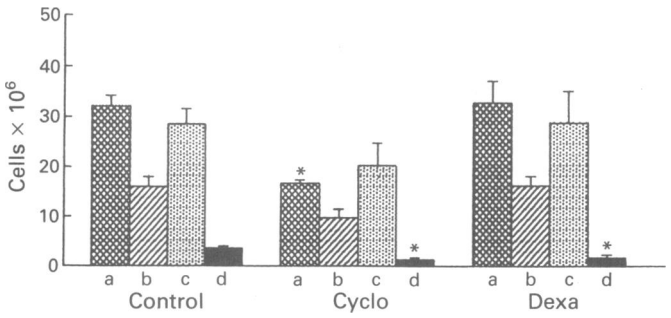


Figure 2 Effect of cyclosporin and dexamethasone on leukocyte number in BAL fluid from guinea-pigs injected with Sephadex beads. Dexamethasone (Dexa, 1 mg kg^{-1}, $n=4$) and cyclosporin (Cyclo, 5 mg kg^{-1}, $n=4$) were injected by i.m. route 1.5 and 2 h, respectively, before injection of Sephadex beads. Control animals received an i.m. injection of vehicle (cremophor el + ethanol). BAL was performed 24 h following injection of Sephadex. (a) = eosinophils, (b) = neutrophils, (c) = macrophages, (d) = lymphocytes. *$P<0.05$, Dunnett's t test for unpaired data.

Table 3 Lack of effect of cyclosporin and dexamethasone on the hyperreactivity of bronchial strips from Sephadex-injected guinea-pigs

	Bronchial responsiveness (area under curve)	
	Histamine	*ACh*
Control ($n=6$)	2.48 ± 0.35	2.45 ± 0.27
Cyclo ($n=4$)	2.01 ± 0.30	2.30 ± 0.38
Dexa ($n=4$)	3.00 ± 0.35	2.79 ± 0.36

Area under the curves was obtained from graphic integral of log dose-response curves to histamine and ACh. Dexamethasone (Dexa, 1 mg kg^{-1}) and cyclosporin (Cyclo, 5 mg kg^{-1}) were administered by intramuscular route 1.5 and 2 h, respectively, before the injection of Sephadex beads. The tissues were isolated 24 h after the injection of Sephadex.

The pronounced increase in neutrophil numbers after the injection of Sephadex beads is probably related to the fact that these cells were only rarely found in the lavage fluid of naïve animals. The macrophage number was also significantly increased 24 h following the injection of the beads, but not to the same extent as that observed for the other leukocytes.

Concomitant with this marked cell influx, an increase in the reactivity of isolated bronchial strips to histamine and ACh was also observed 24 h after the injection of the beads. This hyperreactivity of the isolated bronchial strips to both spasmogens is characterized by an increase in the gradient of the log dose-response curves to both agonist rather than a shift to the left of the log dose-response curve. Histological examination of lung samples from Sephadex-treated animals showed that the inflammatory cells were prominent in and around the epithelial layer of the lower bronchus and throughout the lung parenchyma, while the upper bronchus showed little evidence of cell infiltration. In addition, bronchial epithelial cell damage was not observed following Sephadex bead injections (Maghni et al., 1992). Using the model described above, we have assessed the effect of rapamycin on the bronchopulmonary inflammation and on the increased bronchial hyperreactivity induced by Sephadex beads. Rapamycin (0.012–5 mg kg^{-1}) significantly reduced the Sephadex-induced bronchial hyperreactivity to histamine and ACh. Rapamycin (5 mg kg^{-1}) reduced by approximately 70% the maximal contraction of the bronchi to histamine and ACh. At this dose of rapamycin there was a significant inhibition of eosinophils, neutrophils, macrophages and lymphocytes in BAL fluid of Sephadex-injected guinea-pigs. Despite a significant inhibition of bronchial hyperreactivity with 0.012 and 0.12 mg kg^{-1} rapamycin, this could only be related to a significant decrease in neutrophil and eosinophil counts respectively. The lowest dose of rapamycin (0.001 mg kg^{-1}) neither inhibited leukocyte infiltration nor the bronchial hyperreactivity observed following the injection of Sephadex beads. It must be noted, however, that it was not necessary to inhibit fully the leukocyte migration in order to achieve almost total inhibition of bronchial hyperreativity by rapamycin.

Our results also show that specific inhibition of T-lymphocyte activity by cyclosporin A, although associated with a significant suppression of eosinophil and lymphocyte influx did not inhibit bronchial hyperreactivity. This suggests that the inhibitory effect of cyclosporin A on eosinophil and lymphocyte infiltration is not adequate to suppress bronchial hyperreactivity in this model. In addition, dexamethasone which significantly inhibited the increase of lymphocytes in BAL fluid in Sephadex-treated guinea-pigs failed to reduce bronchial responsiveness. Elwood et al. (1992) recently reported that cyclosporin A failed to attenuate allergen-induced airway hyperresponsiveness in sensitized rats despite a significant inhibition of eosinophil and lymphocyte influx. However, in the same study dexamethasone inhibited the cell infiltration and the increase in airway responsiveness after allergen exposure.

A lack of inhibition by dexamethasone on bronchial reactivity despite a decrease in eosinophils and neutrophils in BAL fluid was also observed by Sanjar et al. (1990) using a long term treatment. In another study from our laboratory, a high dose of dexamethasone (5 mg kg^{-1}, i.p. for 7 days) induced a reduction in leukocyte number with only a significant inhibition of lymphocyte counts in BAL fluid. However, this dose did not significantly attenuate bronchial hyperreactivity induced by Sephadex beads (unpublished results). It may be argued that the analysis of BAL fluid which consists of surface washings from bronchioles and alveoli does not necessarily reflect events within the respiratory tissue itself. On the other hand, Snella et al. (1987) have shown a good correlation between bronchial tissue eosinophilia and eosinophils collected in BAL fluid but whether the same is true for other leukocytes is currently unknown. In addition it may not simply be the number of inflammatory cells re-

cruited to the lungs, but the activation state of these cells that may be relevant to the development of bronchial hyperreactivity in our model. It is not known if the Sephadex-induced increase in leukocyte count in BAL fluid is accompanied by an activation of these cells. However, preliminary results from our laboratory have shown that two populations of eosinophils, normodense and hypodense, could be isolated from BAL of Sephadex-injected guinea-pigs. The presence of hypodense eosinophils in this model suggests that these cells are activated. Elevated numbers of hypodense eosinophils have been detected in blood from asthmatics (Fukuda *et al.*, 1985). Furthermore, the number of activated eosinophils was reported to correlate with the degree of airway reactivity in asthmatics (Azzawi *et al.*, 1985). In addition, there are now many reports showing the presence of activated T-lymphocytes in bronchial biopsies (Azzawi *et al.*, 1990) as well as in blood and BAL from asthmatic patients (Walker *et al.*, 1991). T-cell derived cytokines such as GM-CSF, IL-3 and IL-5 have pronounced effects on neutrophils and eosinophils, including chemotaxis, prolongation of survival and activation (Corrigan & Kay, 1992). Human recombinant GM-CSF and IL-3 have been shown to induce eosinophilia in the guinea-pig (Kings *et al.*, 1990) and intra-tracheal injection of murine recombinant (mr) IL-5 induced a significant cellular infiltration into guinea-pig lungs (Iwama *et al.*, 1992). It is interesting to note that there was a significant increase in lymphocytes counts in BAL fluid from Sephadex-injected guinea-pigs although the role of these cells in the model is currently unknown.

From the results presented here, rapamycin appears to be more effective than cyclosporin A or dexamethasone in inhibiting cell infiltration to the lung and bronchial hyperreactivity. Our data suggest that inflammatory mechanisms not inhibited by cyclosporin or dexamethasone but suppressed by rapamycin may be important in the induction of Sephadex-induced bronchial hyperreactivity.

In conclusion, the present study shows that the intravenous injection of Sephadex beads to guinea-pigs induces a marked leukocyte infiltration in the lungs and a hyperreactivity of bronchial tissue to both histamine and ACh. Rapamycin significantly inhibited the Sephadex-induced bronchopulmonary inflammation and significantly attentuated the bronchial hyperreactivity to both spasmogens. In contrast, dexamethasone and cyclosporin failed to attenuate the bronchial hyperresponsiveness in spite of reducing leukocyte counts in BAL fluid, thus suggesting different mechanisms of immunosuppressor activity for these three drugs. It is conceivable that inflammatory mechanisms not inhibited by dexamethasone or cyclosporin but reduced by rapamycin may be important in the induction of Sephadex-induced airway hyperreactivity. A direct effect of rapamycin on bronchial smooth muscle can be ruled out since this immunosuppressive drug (100 nM–1.0 μM) had no effect on the contractile responses of bronchial tissues isolated from either naïve or Sephadex-injected guinea-pigs (data not shown). The mechanism of the inhibitory effects of rapamycin on Sephadex-induced hyperreactivity and airway cell migration requires further study. Rapamycin may prove to be a useful drug for the treatment of asthma where airway hyperreactivity and cell infiltration are characteristic features.

We wish to thank the Conselho Nacional de Pesquisa, Brasil (grant n. 201.087/90.1) (CNPq), the Medical Research Council of Canada (M.R.C.) for support and Miss Solange Cloutier for excellent technical assistance. D.M.C., K.M. and P.S. have fellowship, studentship and scientist awards from the M.R.C., respectively.

References

AZZAWI, M., BRADLEY, B., JEFFERY, P.K., FREW, A.J., WARDLAW, A.J., KNOWLES, G., ASSOUFI, B., COLLINS, J.V., DURHAM, S. & KAY, A.B. (1990). Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am. Rev. Resp. Dis.*, **142**, 1407–1413.

CALNE, R.Y., LIM, S., SAMAAN, A., COLLIER, D.S., POLLARD, S.G., WHITE, D.J. & THIRU, S. (1989). Rapamycin for immunosuppression in organ allografting. *Lancet*, **ii**, 227.

CHANG, J.Y., SEHGAL, S.N. & BANSBACH, C.C. (1991). FK 506 and rapamycin: novel pharmacological probes of the immune response. *Trends Pharmacol. Sci.*, **12**, 218–223.

CORRIGAN, C.J. & KAY, A.B. (1992). Asthma. Role of T-lymphocytes and lymphokines. *Br. Med. Bull.*, **48**, 72–84.

DUNNETT, C.W. (1964). New tables for multiple comparisons with a control. *Biometrics*, **20**, 482–491.

ELWOOD, W., LOTVALL, J.O., BARNES, P.J. & CHUNG, K.F. (1991). Characterization of allergen-induced bronchial hyperresponsiveness and airway inflammation in actively sensitized Brown-Norway rats. *J. Allergy Clin. Immunol.*, **88**, 951–960.

ELWOOD, W., LOTVALL, J.O., BARNES, P.J. & CHUNG, K.F. (1992). Effect of dexamethasone and cyclosporin A on allergen-induced airway hyperresponsiveness and inflammatory cell responses in sensitized Brown-Norway rats. *Am. Rev. Resp. Dis.*, **145**, 1289–1294.

FRANCISCHI, J.N., CONROY, D.M., MAGHNI, K. & SIROIS, P. (1992). Inhibition of Sephadex-induced leukocyte infiltration into guinea-pig lung by rapamycin. *Br. J. Pharmacol.*, **107**, 51P.

FUKUDA, T., AKUTSU, I., MOTOJIMA, S. & MAKINO, S. (1991). Inhibition of antigen-induced late asthmatic response and bronchial hyperresponsiveness by cyclosporin and FK 506. *Int. Arch. Allergy, Appl. Immunol.*, **94**, 259–261.

FUKUDA, T., DUNNETTE, S.L., REED, C.E., ACKERMAN, J., PETERS, M.S. & GLEICH, G.J. (1985). Increased numbers of hypodense eosinophils in the blood of patients with bronchial asthma. *Am. Rev. Resp. Dis.*, **132**, 981–985.

HAMBLIN, A.S. (1991). The role of cytokines in asthma. *Ann. N.Y. Acad. Sci.*, **629**, 250–261.

IWAMA, T., NAGAI, H., SUDA, H., TSURUOKA, N. & KODA, A. (1992). Effect of murine recombinant interleukin-5 on the cell population in guinea-pig airways. *Br. J. Pharmacol.*, **105**, 19–22.

KAY, J.E., KROMWEL, L., DOE, S.E.A. & DENYER, M. (1991). Inhibition of T and B lymphocyte proliferation by rapamycin. *Immunology*, **72**, 544–549.

KINGS, M.A., CHAPMAN, I., KRISTERSSON, A., SANJAR, S. & MORLEY, J. (1990). Human recombinant lymphokines and cytokines induce pulmonary eosinophilia in the guinea pig which is inhibited by ketotifen and AH 21-132. *Int. Arch. Allergy Appl. Immunol.*, **91**, 354–361.

MAGHNI, K., DE BRUM-FERNANDES, A.J., FOLDES-FILEP, E., GAUDRY, M., BORGEAT, P. & SIROIS, P. (1991). Leukotriene B_4 receptors on guinea-pig alveolar eosinophils. *J. Pharmacol. Exp. Ther.*, **258**, 784–789.

MAGHNI, K., LANOUE, C., CLOUTIER, S., CRISTOL, J.P., CADIEUX, A. & SIROIS, P. (1992). Sephadex bead-induced bronchial hyperreactivity and airway eosinophilia. *Pharmacologist*, **34**, 358.

MARTEL, R.R., KLICIUS, J. & GALET, S. (1977). Inhibition of the immune response by rapamycin, a new antifungal antibiotic. *Can. J. Physiol. Pharmacol.*, **55**, 48–51.

MORRIS, R.E. (1992). Rapamycins; antifungal, antitumor, antiproliferative, and immunosuppressive macrolides. *Transplant. Rev.*, **6**, 39–87.

ROCHESTER, C.L. & RANKIN, J.A. (1991). Is asthma T-cell mediated? *Am. Rev. Resp. Dis.*, **144**, 1005–1007.

SANJAR, S., AOKI, S., KRISTERSSON, A., SMITH, D. & MORLEY, J. (1990). Antigen challenge induces pulmonary airway eosinophil accumulation and airway hyperreactivity in sensitized guinea-pigs: the effect of anti-asthma drugs. *Br. J. Pharmacol.*, **99**, 679–686.

SIGAL, N.H., LIN, C.S. & SIEKIERKA, J.J. (1991). Inhibition of human T-cell activation by FK 506, rapamycin and cyclosporine A. *Transplant. Proc.*, **23**, 1–5.

SNELLA, M.-C., VENAILLE, T., HOLT, P. & RYLANDER, R. (1987). Interstitial and free lung cells in acute inflammation in the guinea-pig. *Agents Actions*, **22**, 261–269.

SPICER, B.A., BAKER, R.C., HATT, P.A., LAYCOCK, S.M. & SMITH, H. (1990). The effects of drugs on Sephadex-induced eosinophilia and lung hyperresponsiveness in the rat. *Br. J. Pharmacol.*, **101**, 821–828.

STEPKOWSKI, S.M., CHEN, H., DALOZE, P. & KAHAN, B.D. (1991). Rapamycin, a potent immunosuppressive drug for vascularized heart, kidney, and small bowel transplantation in the rat. *Transplantation*, **51**, 22–26.

VEZINA, C., KUDELSKI, A. & SEHGAL, S.N. (1975). Rapamycin (AY-22989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot. (Tokyo)*, **28**, 721–726.

WALKER, C., KAEGI, M.K., BRAUN, P. & BLASER, K. (1991). Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J. Allergy Clin. Immunol.*, **88**, 935–942.

(Received July 7, 1993
Accepted August 11, 1993)

Br. J. Pharmacol. (1993), **110**, 1387–1394 © Macmillan Press Ltd, 1993

Cardiotonic actions of selective phosphodiesterase inhibitors in rat isolated ventricular cardiomyocytes

Elizabeth J. Kelso, [1]Barbara J. McDermott & Bernard Silke

Department of Therapeutics and Pharmacology, The Queen's University of Belfast, Whitla Medical Building, 97 Lisburn Road, Belfast BT7 9BL

1 The contractile effects of the novel cardiotonic agent HN-10200 (2-[3-methoxy-5-methylsulphinyl-2-thienyl]-1H-imidazo-[4,5-c]-pyridine hydrochloride), were examined and comparisons made with the responses obtained to a structurally similar compound, sulmazole, and to a number of other compounds which are known to inhibit phosphodiesterase (PDE) isoenzymes with differing selectivities; namely, enoximone (PDE III inhibitor), Ro 20-1724 (PDE IV inhibitor) and 3-isobutyl-1-methylxanthine (non-selective PDE inhibitor).

2 Contractile function, as measured by mechanical shortening, and biochemical systems involving cyclic AMP were investigated in ventricular cardiomyocytes isolated from adult Sprague-Dawley rats (200–250 g).

3 HN-10200 exerted a concentration-dependent (10^{-8} M–10^{-4} M) positive contractile effect, which was independent of α- or β-adrenoceptor, or histamine receptor stimulation.

4 The efficacies of the contractile responses to the PDE inhibitors were of the order: HN-10200 > IBMX > sulmazole > enoximone and maximum stimulations, which were obtained at concentrations of 10^{-4} M, were 54 ± 4%, 41 ± 7%, 38 ± 7% and 26 ± 5% (mean ± s.e.) greater than basal levels, respectively ($n = 6$); the basal value of contractile amplitude (dL), in the absence of PDE inhibitors was 7.39 ± 0.18% (mean ± s.e.). Ro 20-1724 did not have any effect on contractile activity.

5 Due to low basal levels of cyclic nucleotides in isolated cells, accumulation of cyclic AMP due to the presence of the PDE inhibitors was detected only when the levels of cyclic nucleotide were enhanced with forskolin (10 μM).

6 The PDE inhibitors increased levels of cyclic AMP only at concentrations > 10^{-4} M. HN-10200 and sulmazole had similar concentration-dependent profiles for the accumulation of cyclic AMP; their potencies were lower than that of IBMX (concentrations of forskolin required to increase cyclic AMP by 4 pmol mg^{-1} protein, in the presence of maximum concentrations of the PDE inhibitors, were 13 ± 3 μM, 14 ± 3 μM and 3 ± 0.6 μM [mean ± s.e.], respectively).

7 These results indicate that a similar mechanism, probably through a weak inhibition of the cyclic AMP-specific PDE isoenzymes, is responsible for the increase in levels of cyclic AMP by HN-10200 and sulmazole. However, cyclic AMP is only partially responsible for the positive contractile effect of HN-10200 and, similarly, sulmazole and IBMX. The lack of apparent increase in levels of cyclic AMP by enoximone, highlights its degree of selectivity for the PDE III isoenzyme, such that the PDE IV isoform is still present in sufficient quantity to degrade cyclic AMP within the cell. On the other hand, the potent action of Ro 20-1724 on accumulation of cyclic AMP, in addition to the lack of effect on contractile function, is in agreement with the selectivity of this compound for the PDE IV isoenzyme and compartmentalization of cyclic AMP in rat isolated ventricular cardiomyocytes.

Keywords: Ventricular cardiomyocytes; cyclic AMP; contractility; selective phosphodiesterase inhibitors; IBMX; forskolin

Introduction

Extensive efforts to find alternatives to the conventional therapy of congestive heart failure have resulted in the development of several new cardiotonic agents, which combine positive inotropic effects with vasodilator activity to achieve maximum improvement in cardiac performance (Weishaar *et al.*, 1985; Hartmann *et al.*, 1986; Thompson, 1991). Whilst the mechanisms of action of these agents have not been fully elucidated, these compounds have been shown to derive their inotropic and vasodilator actions, at least in part, from the selective inhibition of phosphodiesterase (PDE) isoenzymes, PDE III and PDE IV, which are specific for adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Colucci *et al.*, 1986; Prigent *et al.*, 1988; Schmitz *et al.*, 1989; Silver *et al.*, 1989; Beavo & Reifsnyder, 1990; Nicholson *et al.*, 1991). The effectiveness of these drugs at increasing intracellular levels of cyclic AMP varies considerably and evidence indicates that a potentially more useful drug would have a lesser effect mediated through cyclic AMP (Hohl & Li, 1991).

[1] Author for correspondence.

It has been reported that sulmazole inhibits the PDE III isoenzyme extracted from the hearts of various mammalian species, including rat (Parsons *et al.*, 1988; Verrijk *et al.*, 1989). However, the degree of selectivity on isoenzymes of PDE, and the efficacy of this drug, are unclear. Moreover, the extent of such inhibition to the inotropic action of the compound is particularly controversial (Endoh *et al.*, 1985; Ruegg, 1986; Williams & Holmberg, 1990). It has been demonstrated that the inotropic action of sulmazole involves a direct action involving Ca^{2+} sensitization of the myofilaments (Solaro & Ruegg, 1982; Kitada *et al.*, 1989; Ruegg & Morano, 1989). Inotropic activity mediated through this mechanism is possibly advantageous, since an increase in cyclic AMP may not only lead to positive inotropic but also positive chronotropic and possibly arrhythmogenic effects (Scholz & Meyer, 1986).

HN-10200 (2-[3-methoxy-5-methylsulphinyl-2-thienyl]-1H-[imidazo-[4,5-c]-pyridine hydrochloride) is a novel compound which is structurally similar to sulmazole and has been found to possess vasodilator and positive inotropic activities. In anaesthetized and conscious dogs, HN-10200, whether

administered intravenously or orally, produced a positive inotropic effect with little change in heart rate and a dose-dependent decrease in total peripheral resistance, diastolic blood pressure and left ventricular end-diastolic pressure (Stroßnig & Pruss, 1991). Also, HN-10200 reversed heart failure induced by either propranolol or pentobarbitone. The only *in vitro* study carried out using HN-10200 (Kuhberger *et al.*, 1991) has shown that the compound exerts a positive inotropic effect in a number of species including rat, guinea-pig, pig and man. This action was not attributable to mechanisms involving H_1-, β-, or α-receptors, release of endogenous noradrenaline, ouabain-like agonist actions, or antagonism of adenosine receptors or muscarinic receptors. Positive actions of HN-10200 reported by Kuhberger *et al.* (1991) include a weak Ca^{2+}-sensitizing effect on ventricular skinned muscle fibres of pig, and a weak inhibition of PDEs in failing human heart tissue. However, the latter was not accompanied by a significant increase in accumulation of cyclic AMP or contraction. It was not clear from this study as to what effect the compound had on the individual isoenzymes of PDE.

In this study it was of interest to investigate to what extent cyclic AMP was involved in the contractile response of the new cardiotonic agent, HN-10200. An integral part of this study included a comparison of the actions of HN-10200 with the structurally similar compound, sulmazole, and to other compounds with differing selectivities for PDE isoenzymes (Figure 1). While these compounds are known to have positive inotropic activity (Blinks & Endoh, 1984; Ruegg, 1986; Kuhberger *et al.*, 1991), the extent by which this action is attributed to mechanisms involving cyclic nucleotides is unclear. Ro 20-1724 was chosen as a compound with reported selectivity for the PDE IV isoenzyme (Reeves *et al.*, 1987; Muller *et al.*, 1990), and it has been clearly demonstrated that IBMX is a non-selective PDE inhibitor (Beavo, 1988; Nicholson *et al.*, 1991). The isolated adult ventricular cardiomyocyte is a useful model in which to investigate the mechanisms of inotropic agents in heart muscle due to the fact that mechanical and biochemical effects can be studied in a homogeneous population of cells which is uncomplicated by the interactions of neural and mechanical influences which are intrinsic factors of whole tissue preparations.

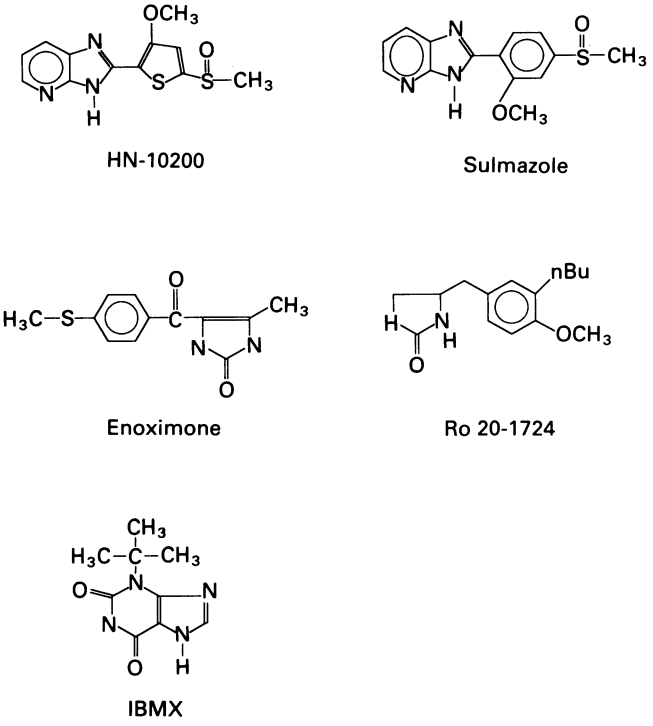


Figure 1 Molecular structures of phosphodiesterase inhibitors.

Methods

Cell preparation

Ca^{2+}-tolerant ventricular cardiomyocytes were obtained from the hearts of adult male Sprague-Dawley rats (200–250 g) by a modification of a protocol previously described (Piper *et al.*, 1982). Hearts were retrogradely perfused and equilibrated with a modified Krebs Ringer solution (KRB), containing (mM): NaCl 110, KCl 2.6, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2 and glucose 11 (pH 7.4), which had just previously been gassed with 95% O_2/5% CO_2 at 37°C. After the initial period of washout, collagenase was added to the perfusate at a concentration of 0.04% (w/v), which was recirculated through the heart in the presence of low levels of $CaCl_2$ (25 μM) for 20–25 min (Powell & Twist, 1976). Following removal of the atria, the ventricles were dissected and chopped into cubes of 0.7 mm^3 with a mechanical tissue chopper (McIlwain Chopper, Mickle Laboratory Engineering Co. Ltd., Surrey). The minced tissue was placed in the collagenase perfusate which had been supplemented with 0.1% (w/v) BSA, and the mixture triturated using a 10 ml serological pipette for approximately 5 min. The disaggregated cell suspension was filtered through a nylon mesh gauze of pore size, 200 μm, to remove undigested material and then centrifuged at 25 *g* for 60 s. The sediment was washed twice in modified KRB solutions containing 250 μM and 500 μM $CaCl_2$, respectively. Finally, the cells were layered onto a solution of 4% (w/v) BSA containing 1 mM $CaCl_2$, and left to settle by gravity at 37°C. After approximately 5 min, the supernatant was aspirated and the resulting cell material resuspended at a density of 1–2 mg protein ml^{-1} in modified Tyrode solution containing (mM): NaCl 125, KH_2PO_4 1.2, KCl 2.6, $MgSO_4$ 1.2, $CaCl_2$ 1, glucose 10 and HEPES 10 at pH 7.4. Suspensions of cardiomyocytes were >70% viable as estimated by their elongated rod-shaped morphology.

Modulation of adenylate cyclase

Cells were incubated for 1 h at 37°C and in an atmosphere of 95% O_2/5% CO_2 prior to their use, to allow cells to recover from any biochemical trauma incurred during the isolation procedure. Effects of pharmacological treatment of the isolated cells on the accumulation of cyclic AMP were studied by a method previously described (Millar *et al.*, 1988). All experiments were performed in the presence of adenosine deaminase at 5 units ml^{-1}, used to eliminate endogeneous adenosine. Cells were incubated at 37°C for 15 min with the various PDE inhibitors. In PDE inhibitor comparison studies, forskolin (10 μM) was used to potentiate the levels of cyclic AMP. Experiments were terminated by centrifugation of the suspensions for 1 min at 12,000 *g* through a 1:1 mixture of silicon oils into 6% (w/v) perchloric acid. After neutralization with a Tris-HCl (0.05 M) buffer solution in which K_2CO_3 (0.5 M) had been dissolved, potassium perchlorate was removed by centrifugation for 2 min at 12,000 *g*. Cyclic AMP was determined by a protein binding assay (Tovey *et al.*, 1974), using a [^{3}H]-cyclic AMP assay system. Protein concentrations were determined by a modified Lowry procedure (Lowry *et al.*, 1951; Ohnishi & Barr, 1978) using BSA as a standard. Concentrations of cyclic AMP are expressed as picomoles per milligram of protein (pmol mg^{-1} protein).

Attachment of cells

Cells were suspended at a density of 2×10^5 cells ml^{-1} in modified Tyrode solution. For optimal cell attachment, prior treatment of the glass coverslips (9 mm^2) was necessary. The

coverslips were placed in water at 100°C for 15 min, transferred to 100% (v/v) alcohol and then washed individually in fresh alcohol before being flamed dry prior to use. The coverslips were placed singly in wells of tissue culture plates (24 well plates, Falcon type 3047) under sterile conditions. Laminin in modified Tyrode solution (15 $\mu g\ ml^{-1}$), was added to each dish (250 μl/well). The laminin solution was removed after 45 min and the coverslips were washed with modified Tyrode solution. Aliquots of cell suspension were then placed on top of laminin-precoated glass coverslips (250 μl/well), and incubated for 2 h in an atmosphere of 95% O_2/5% CO_2 at 37°C. The coverslips with attached viable cells were washed twice in modified Tyrode solution (500 μl/well) and then transferred, individually, to 35 mm plastic petri dishes (type 1-50318, Nunc). The cells were allowed to settle for 20 min in fresh modified Tyrode solution (2 ml per dish) supplemented with KCl (1.5 mM), $CaCl_2$ (1 mM) and adenosine deaminase (5 units ml^{-1}), before the start of each experiment.

Measurement of contractility

In response to electrical stimulation, unloaded contractions of isolated cardiomyocytes were studied by use of a video microscopic technique previously described by Piper *et al.* (1989). A culture dish containing a coverslip with adherent cells was placed on the moveable stage, which was thermostatically controlled (37°C) by means of an electronic two-channel temperature control unit (TRZ 3700, Carl Zeiss, Germany). Two silver chloride electrodes, positioned in parallel across the petri-dish, were immersed to a depth of 5 mm into the medium, and electrical biphasic stimuli composed of two opposite rectangular 60 V stimuli, with a pulse width of 0.5 ms duration, were applied at a frequency of 0.5 Hz. Mechanical activity (shortening) of the cells was continually monitored by means of the video camera and recorder (U-recorder U-matic VO-9600P, Sony, Japan). Cells were selected for contractile measurements on the basis of their viability, histological appearance and response to electrical field stimulation. Although 98% of adherent cells were rod-shaped, a number of other criteria were defined for selection: (1) only uniformly shaped cells with clear sarcomere striations and absence of membrane blebs or granulations were selected for measurement, (2) cells, although adherent at various surface sites, were required to elicit significant shortening at both ends when stimulated, (3) cells were selected with clearly defined borders, that is, not touching or overlapping with another cell, and (4) cells which did not show spontaneous contractions when unstimulated. The images of the cells were recorded on video cassette at a final magnification of × 280 and were subsequently analysed by means of replay of the cell activity in slow motion. Mechanical activity was determined from measurements of the maximum diastolic length of the cell occurring 0.5 s before the contraction (DL), and the fully contracted systolic length (SL).

Data analysis

Data are presented as mean values ± standard error (s.e.) of 5 to 8 heart cell preparations. Levels of cyclic AMP were calculated by the Multicalc immunoassay data management programme (Pharmacia, Finland). Concentration-response data were fitted using a quadratic equation (SPSS-PC) and the potencies of the PDE inhibitors determined by calculating the concentration of forskolin required to increase the level of cyclic AMP by 4 pmol mg^{-1} protein. The contractile response (dL) was expressed as a percentage, (DL − SL) × 100/DL where DL is the diastolic length and SL is the systolic length of the cells. At each concentration of drug monitored, 30 to 35 cells were measured and mean values ± standard deviations were determined. Data are presented as mean values ± standard error (s.e.) of 5–8 heart cell preparations. Statistical analysis of the data was performed by multivariate analysis with repeated measures and multiple comparison test (Student-Neuman-Keuls). Values are given as mean ± s.e.mean in the text.

Materials

Drugs were obtained from the following sources: HN-10200 (2-[3-methoxy-5-methylsulphinyl-2-thienyl]-1H-imidazo-[4,5-c]-pyridine hydrochloride) was a gift from Hafslund Nycomed, Linz, Austria; sulmazole (2-[2-methoxy-4-methylsulphinyl)-phenyl]-1H-imidazo-[4,5-b]-pyridine) was a gift from Boehringer Ingelheim Ltd., Berkshire; Ro 20-1724 (4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone) was purchased from Roche Products (Hertfordshire); 3-isobutyl-1-methylxanthine (IBMX), forskolin, adenosine deaminase and bovine serum albumin (BSA) fraction V (fatty acid free) were obtained from Sigma Chemical Co. (Poole, Dorset). Solutions of IBMX, sulmazole, and Ro 20-1724 (0.2 M) were prepared by dissolving the compounds in dimethyl sulphoxide (DMSO) and further dilutions made in modified Tyrode solution. Care was taken to prevent precipitation of compounds by adding solutions of DMSO to a large volume (1:1 000) of modified Tyrode solution and immediately agitating the mixture using a Vortex (Leicestershire). HN-10200 was soluble directly in modified Tyrode solution.

Other materials included collagenase (Type I) purchased from Serva (Heidelberg, Germany), laminin from Gibco Ltd. (U.K.) and silicon oils (AR 20 and AR 200) from Wacker (Munich, Germany). Kits for the determination of cyclic AMP, by protein binding assay, were obtained from Amersham International (Buckinghamshire). Optiphase 'Hisafe' liquid scintillation fluid was purchased from LKB Scintillation Products (Leicestershire). All other chemicals were of Analar analytical grade (U.K.). Twice distilled water which had been de-ionized through a Millipore-Q system (Millipore, Harrow) was used in all experiments.

Results

Effects of PDE inhibitors on the accumulation of cyclic AMP

Sulmazole, HN-10200, enoximone, Ro 20-1724 and IBMX affected levels of cyclic AMP within isolated cardiomyocytes to varying degrees (Figure 2). The non-selective PDE inhibitor, IBMX (3×10^{-4} M) produced a larger response (10.85 ± 1.65 pmol cyclic AMP mg^{-1} protein) than both sulmazole, and the structurally related compound, HN-10200, (3.31 ± 0.19 and 3.65 ± 0.41 pmol cyclic AMP mg^{-1} protein, respectively) but at equimolar concentrations was comparable in efficacy to that of the PDE IV inhibitor, Ro 20-1724 (13.42 ± 4.70 pmol mg^{-1} protein). Sulmazole and HN-10200 increased levels of cyclic AMP to a similar degree, while enoximone, at an equimolar concentration, did not have any effect on the stimulated levels of cyclic AMP. The concentrations of cyclic AMP under control conditions with forskolin, and with the addition of enoximone, were 1.42 ± 0.19 and 1.58 ± 0.28 pmol mg^{-1} protein, respectively (Figure 2). Enoximone, sulmazole and HN-10200 increased levels of cyclic AMP, in the presence of forskolin (10 μM) by 1.1, 2.6 and 2.4 fold, respectively, compared to the much larger increases in levels of cyclic AMP of 7.7 and 9.5 fold in the presence of IBMX and Ro 20-1724, respectively.

Concentration-dependent effects of HN-10200, sulmazole and IBMX were demonstrated over a range of concentrations of forskolin (10^{-7} M -10^{-4} M). Forskolin alone produced an increase in levels of cyclic AMP at concentrations greater than 10^{-6} M, and likewise the potentiating effects of HN-10200, sulmazole and IBMX were found only under such conditions of high stimulation. Figure 3a shows that significant elevation ($P<0.05$) of levels of cyclic AMP by

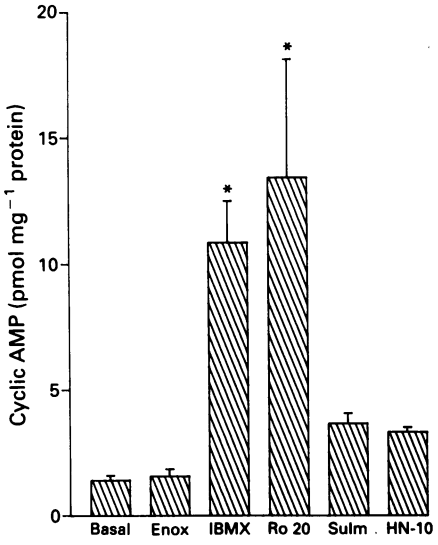


Figure 2 Effect of PDE inhibitors (3×10^{-4} M) on the accumulation of cyclic AMP in rat ventricular cardiomyocytes which were stimulated with forskolin (10 μM). Data are presented as the mean ± s.e. for 6 heart cell preparations. *Denotes, in the presence of forskolin, a significant difference ($P < 0.05$) of treatment with respect to the response to forskolin alone (10 μM). Enox = enoximone; Sulm = sulmazole; IBMX = 3-isobutyl-1-methylxanthine; HN-10 = HN-10200; Ro 20 = Ro20-1724.

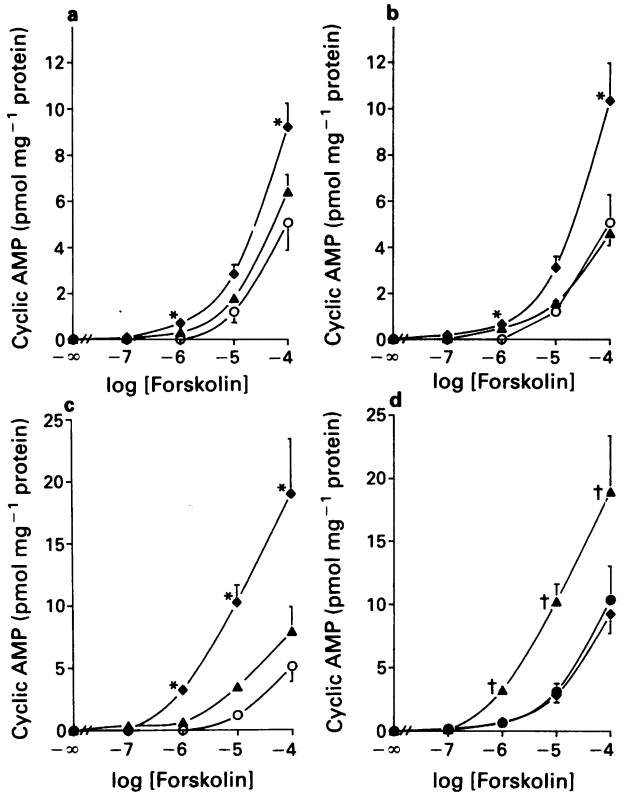


Figure 3 Effects of (a) HN-10200, (b) sulmazole and (c) 3-isobutyl-1-methylxanthine (IBMX) on the accumulation of cyclic AMP in rat ventricular cardiomyocytes, stimulated with increasing concentrations of forskolin. Responses were carried out in the absence (○) and presence of PDE inhibitors at concentrations of 10^{-4} M (▲) and 10^{-3} M (◆). (d) Demonstrates a comparison of the effects of IBMX (▲), HN-10200 (●) and sulmazole (◆) at a concentration of 10^{-3} M. Absolute values in the absence of forskolin are 0.36 ± 0.28 (mean ± s.e.) pmol mg^{-1} protein. Data are presented as the mean ± s.e. of 6 heart cell preparations. *Denotes a significant difference ($P < 0.05$) with respect to the appropriate basal value; †denotes a significant difference ($P < 0.05$) between the PDE inhibitors, sulmazole, HN-10200 and IBMX, at each concentration of forskolin.

HN-10200 was evident only at a maximal concentration (10^{-3} M) of the PDE inhibitor and at high levels of forskolin ($> 10^{-6}$ M). An analogous concentration-response relationship was obtained for the novel compound, sulmazole (Figure 3b). Both compounds demonstrated comparable efficacies, which were 9.22 ± 1.03 and 10.35 ± 1.63 pmol cyclic AMP mg^{-1} protein at maximal concentrations of the PDE inhibitors (10^{-3} M) (HN-10200 and sulmazole, respectively). Similarly, IBMX produced a significant shift in the concentration-response curve of forskolin only at a concentration of 10^{-3} M (Figure 3c). IBMX had a greater efficacy (18.9 ± 4.45 pmol mg^{-1} protein) at the maximal concentration than either sulmazole or HN-10200. The greater potency and efficacy of IBMX by comparison to these characteristics of the selective PDE inhibitors, HN-10200 and sulmazole, is demonstrated in Figure 3d.

Table 1 shows the potencies of the various compounds expressed as the concentrations of forskolin which increased cyclic AMP by 4 pmol mg^{-1} protein in the presence of each PDE inhibitor. HN-10200 and sulmazole were equally potent, and at maximal concentrations (10^{-3} M) required approximately three fold less concentration of forskolin to increase the accumulation of cyclic AMP to the same level as under basal conditions. IBMX (10^{-3} M), on the other hand, required a 13 fold less concentration of forskolin to increase the level of cyclic AMP accumulation to the same degree, and 3.5 fold less stimulation when a lower concentration of IBMX was used (10^{-4} M). Comparable data obtained for HN-10200 and sulmazole (10^{-4} M) were 1.63 and 1.34 fold changes, respectively.

Effects of PDE inhibitors on contractile activity

Electrically-paced (0.5 Hz) ventricular cardiac cells which were exposed to increasing concentrations of PDE inhibitors (10^{-8} M – 10^{-4} M) increased the contractile activity of the cells in the presence of forskolin (10 μM) (Figure 4). The contractile amplitude (dL) in the absence of PDE inhibitors was 7.39 ± 0.18% (basal value). The efficacies of the contractile responses were of the order: HN-10200 > IBMX > sulmazole > enoximone, and maximum stimulations which were attained at a concentration of 10^{-4} M were 54 ± 4%, 41 ± 7%, 38 ± 7% and 26 ± 4% greater than the basal levels, respectively. Ro 20-1724 did not increase the contractile amplitude of the cells (Figure 4). The effects of equimolar concentrations (10^{-5} M) of sulmazole and Ro 20-1724 were examined in the presence of forskolin (10 μM). The combined effect of Ro 20-1724 and sulmazole on ventricular cell shortening (10.64 ± 0.38%) was not greater than sulmazole alone (10.55 ± 0.42%). Concentration-dependent increases in contractile activity in the presence of either HN-10200, sulmazole or IBMX were observed only at high concentrations (> 10 μM) in the absence of stimulation with forskolin.

Table 1 Potencies of phosphodiesterase (PDE) inhibitors to increase cellular accumulation of cyclic AMP in the presence of forskolin

Drug	*Concentration*	*[Forskolin]** × 10^{-5} M
(Basal)	–	4.12 ± 0.47
Sulmazole	10^{-4} M	3.08 ± 0.80
	10^{-3} M	1.32 ± 0.34
HN-10200	10^{-4} M	2.83 ± 0.51
	10^{-3} M	1.46 ± 0.33
IBMX	10^{-4} M	1.18 ± 0.25
	10^{-3} M	0.32 ± 0.06

*Denotes the concentration of forskolin required to increase cyclic AMP levels by 4 pmol mg^{-1} protein. Data are given as mean values ± s.e. of 6 heart cell preparations.

Comparison of contractile function and accumulation of cyclic AMP

Figure 5 shows the effects of HN-10200, sulmazole, enoximone and IBMX on both contractile function and accumulation of cyclic AMP. HN-10200, sulmazole and IBMX increased levels of cyclic AMP only at concentrations at which a maximal response in contractile function occurred (10^{-4} M). Ro 20-1724, while producing a greater effect on accumulation of cyclic AMP than HN-10200, sulmazole or IBMX did not have any effect on contractile activity.

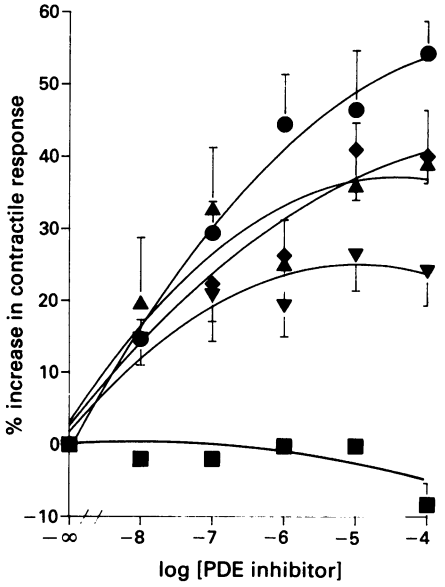


Figure 4 Contractile response of ventricular cardiomyocytes subjected to increasing concentrations of the PDE inhibitors, HN-10200 (●), sulmazole (▲), enoximone (▼), 3-isobutyl-1-methylxanthine (IBMX) (◆) and Ro 20-1724 (■) in the presence of forskolin (10 μM). Data are presented as the mean ± s.e. of 6 heart cell preparations. Contractile response, expressed as a percentage, is the maximum shortening relative to the prestimulus cell length (%dL).

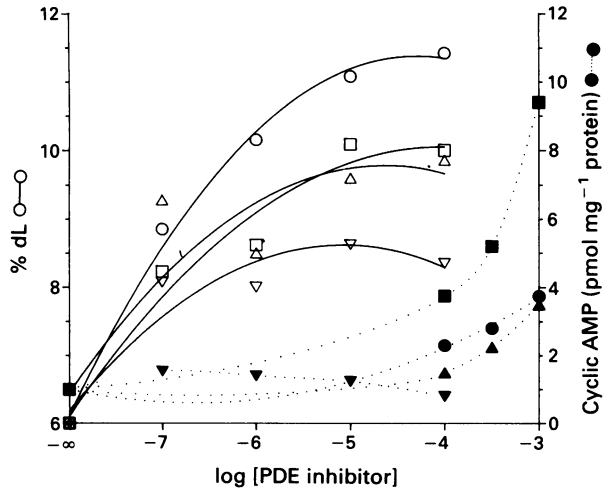


Figure 5 Relationship between cellular accumulation of cyclic AMP (solid symbols) and contractile response (open symbols) for increasing concentrations of HN-10200 (○ and ●), sulmazole (△ and ▲), enoximone (▽ and ▼) and 3-isobutyl-1-methylxanthine (□ and ■). Data are presented as the mean ± s.e. of 6 heart cell preparations.

Effects of receptor antagonists on positive contractile response of HN-10200

Pretreatment of ventricular cardiomyocytes with the non-selective β-adrenoceptor antagonist, propranolol (10^{-6} M), did not cause a significant influence on the positive concentration-dependent effect of HN-10200 on cellular contraction. Similarly, there was no effect of pretreatment of the cells with the non-selective α-adrenoceptor antagonist, phentolamine (10^{-6} M), the histamine H_1 receptor antagonist, pyrilamine (10^{-6} M) or the histamine H_2 receptor antagonist, cimetidine (10^{-5} M). These concentrations of antagonist, in the appropriate cases, were sufficient to block contractile responses induced by isoprenaline (10^{-7} M), phenylephrine (10^{-6} M) or histamine (10^{-7} M).

Discussion

The use of isolated ventricular cardiomyocytes is particularly suited to biochemical studies involving determination of intracellular cyclic AMP, where the physical and chemical environment can be specifically controlled, and also in studies of contractile measurement in which the integrity of the cells can be continually monitored. The viability and cellular dimensions of the cells used in this investigation were in accordance with those reported by other investigators (Powell *et al.*, 1984; Horackova & Mappleback, 1988). Microscopic examination prior to each experiment established the reproducibility between population of cells; that is, cells which were longitudinal and uniform, with clearly defined borders and regular sarcomere striations. In this study it was of particular interest to assess the biochemical actions of HN-10200 and sulmazole in relation to inhibition of the PDE III and IV isoenzymes. Both these isoenzymes are present in isolated ventricular cardiomyocytes, analogous to those in whole ventricle (Bode *et al.*, 1990).

Basal levels of cyclic AMP are low in isolated heart muscle cells (Endoh *et al.*, 1980; Nicholson *et al.*, 1991; Kelso *et al.*, 1992). Hence forskolin, which acts directly on the catalytic subunit of adenylate cyclase to activate the enzyme (Lindner & Metzger, 1983; Rodger & Shahid, 1984; Bristow *et al.*, 1984; Evans, 1986) was a useful tool with which to magnify the effects of the selective PDE inhibitors investigated. The efficacy of forskolin in increasing levels of cyclic AMP was lower in isolated cardiomyocytes than in the intact myocardium (Rodger & Shahid, 1984; Buschmans *et al.*, 1985) possibly due to the absence of other cell types. Levels of cyclic AMP, in the presence of either Ro 20-1724 or IBMX, are similar to those reported by Millar *et al.* (1988) in isolated ventricular cardiomyocytes.

Comparison of the effects of the novel compound, HN-10200, with that of sulmazole has shown close similarities with regard to accumulation of cyclic AMP in isolated ventricular cardiomyocytes. It is known that sulmazole produces an increase in levels of cyclic AMP in isolated tissue through inhibition of the cyclic AMP-specific PDE III isoenzyme (Verrijk *et al.*, 1989). In this study, sulmazole increased levels of cyclic AMP to a significantly lower extent than that of the non-selective PDE inhibitor, IBMX, or indeed that of Ro 20-1724, but was comparable to the increase achieved by HN-10200, suggesting that the latter inhibits similarly the PDE III isoenzyme. However, it is possible that sulmazole also has a weak action on the PDE IV isoenzyme at high concentrations (10^{-3} M) since enoximone, a potent and selective PDE III inhibitor, did not produce any apparent increase in levels of cyclic AMP.

Interestingly, enoximone did not augment the forskolin-stimulated effect on the accumulation of cyclic AMP. These results are in marked contrast to those of other investigators (Ahn *et al.*, 1986; Endoh & Blinks, 1987; Hsieh *et al.*, 1987) who indicated a significant elevation in levels of cyclic AMP,

in accordance with evidence showing that this compound is a highly selective inhibitor of the PDE III isoform. The effect of enoximone on rat isolated cardiomyocytes in this study was an unusual contrast to the positive action of all the other PDE inhibitors examined.

Ahn *et al.* (1986) reported a similar peculiarity using sulmazole, which did not increase levels of cyclic AMP, either in the absence or presence of forskolin, in guinea-pig ventricular slices. On the other hand, they showed that enoximone increased levels of cyclic AMP, in the presence and absence of stimulation by forskolin, and with a greater potency than IBMX (Ahn *et al.*, 1986). Reasons for the failure of sulmazole to promote the accumulation of cyclic AMP in this model were not well defined; it was suggested only that the lack of effect might result from poor penetration of the drug into the cell (Endoh *et al.*, 1985; Ahn *et al.*, 1986; George *et al.*, 1991) but further investigation by Ahn *et al.* (1986) in ventricular and atrial homogenates showed that sulmazole increased levels of cyclic AMP to a small degree.

In this study, the apparent lack of effect of enoximone on levels of cyclic AMP in rat isolated cardiomyocytes are most simply understood by considering a high degree of selectivity of the compound for the PDE III isoform with little or no action on the PDE IV isoform; therefore, although enoximone inhibits the PDE III isoenzyme, cyclic AMP could still be hydrolysed by the PDE IV isoenzyme.

The contractile effects of HN-10200, sulmazole, and IBMX were clearly manifested in a concentration-dependent manner, whereas the effects of these compounds on the accumulation of cyclic AMP were demonstrated only at maximal concentrations, in the presence of forskolin. It could be asked whether the comparative responses, in terms of the accumulation of cyclic AMP and contractile activity, are a consequence of the biochemical and functional differences produced not as a result of PDE inhibition but as a consequence of stimulation with forskolin. Contrary to this premise, it has been shown that the increase in production of cyclic AMP by forskolin is excessive relative to the changes in cardiac contractile function (Silver, 1989). Concentrations of both forskolin and isoprenaline which produce an equivalent positive contractile effect have been shown to affect the accumulation of cyclic AMP differently; that is, forskolin increases the levels of cyclic AMP to a much greater extent than isoprenaline (Seamon & Daly, 1981; Buschmans *et al.*, 1985; Waldeck & Widmark, 1985; Hubbard *et al.*, 1991). In this study, the effect of forskolin on contractile activity in isolated cardiomyocytes was concentration-dependent, reaching maximum stimulation at a concentration of 10^{-5} M, whereas, the effect of forskolin on accumulation of cyclic AMP was only significantly increased at concentrations $> 10^{-5}$ M. Moreover, in the absence of forskolin to stimulate adenylate cyclase in the isolated cells, the various selective PDE inhibitors, even at maximal concentrations, did not increase levels of cyclic AMP, whereas clear concentration-dependent effects were demonstrated with the same compounds on ventricular cell shortening (results not shown).

Consequently, it would appear that not all of the effect on myocardial contractile proteins in the presence of the PDE inhibitors, HN-10200, sulmazole and IBMX, is coupled to cyclic AMP. Furthermore, the differences between sulmazole, HN-10200 and IBMX on the accumulation of cyclic AMP was not correlated with the differences in contractile effect; in other words, the more potent effect of IBMX, than HN-10200 and sulmazole, on levels of cyclic AMP was not found to produce any greater increase in the potency or efficacy in terms of the contractile effect. This difference in the order of potency between the biochemical effects on levels of cyclic AMP and contractile function suggest that all three compounds involve both cyclic AMP-dependent and cyclic AMP-independent mechanisms of action in rat isolated ventricular cardiomyocytes. Positive contractile effects were evident in a dose-dependent manner from 10^{-8} M to 10^{-3} M concentrations, whereas no increases in the accumulation of cyclic AMP were found until maximal concentrations (10^{-3} M) of the respective inhibitors.

This evidence is in agreement with findings reported by Kitada *et al.* (1987) who observed that the PDE III inhibitor, MCI-154 (10^{-7} M–10^{-4} M) did not increase the level of cyclic AMP significantly during induction of the positive inotropic action. As such, this response must be largely elicited through cyclic AMP-independent mechanisms. Also a relatively small contribution of the cyclic AMP system to the contractile effect has been reported for other PDE inhibitors, such as pimobendan and UD-CG 212 Cl (Scholz & Meyer, 1986).

Alternative mechanisms involving a direct action of Ca^{2+} influx can contribute to the positive inotropic actions of PDE inhibitors. The effects of milrinone (PDE III inhibitor) in the mammalian heart may be mediated by stimulation of sarcolemmal Ca^{2+} channels, so that there is increased entry of extracellular Ca^{2+} into the cell (Holmberg & Williams, 1991). Other inotropic mechanisms of compounds with PDE inhibiting properties, and which are independent of cyclic AMP include: increase in the Ca^{2+} sensitivity of the troponin C regulatory site in cardiac myofilaments (Herzig *et al.*, 1981; Solaro & Ruegg, 1982; Ruegg, 1986; Bethke *et al.*, 1988; Fujino *et al.*, 1988; Kitada *et al.*, 1989), stimulation of the Na^+/Ca^{2+} exchange mechanism (Alousi & Johnson, 1986; Sutko *et al.*, 1986; Brasch & Iven, 1991), Na^+ influx through Na^+ channels (Grima *et al.*, 1988; Rapundalo *et al.*, 1988; Haleen *et al.*, 1989), activation of membrane Ca-ATPase (Mylotte *et al.*, 1985) and the Ca^{2+}-release channel of the sarcoplasmic reticulum (Holmberg & Williams, 1991). It has been proposed that mechanisms which produce positive contractile effects on cardiac muscle with a lesser effect on intracellular cyclic AMP may be advantageous, since an increase in cyclic AMP may lead not only to positive inotropic but also positive chronotropic and possibly arrhythmogenic effects (Scholz & Meyer, 1986). Both sulmazole and HN-10200 have been shown to produce an increase in the sensitivity of the contractile proteins (Solaro & Ruegg, 1982; Kuhberger *et al.*, 1991).

Unlike all the other PDE inhibitors investigated in this study, Ro 20-1724 did not increase the contractile response in rat cardiomyocytes. Many investigators have demonstrated little effect of PDE IV inhibitors on contraction *in vitro* (Reeves *et al.*, 1987). Reasons for such responses are most widely believed to be the result of compartmentalization of cyclic AMP in other cellular compartments irrelevant to the contractile action. Interaction between PDE IV inhibitors and compounds such as forskolin, isoprenaline or PDE III inhibitors, which increase levels of cyclic AMP by different mechanisms, has been shown to enhance their respective contractile effects (Weishaar *et al.*, 1987; Harris *et al.*, 1989; Katano & Endoh, 1990; Shahid *et al.*, 1990; Nicholson *et al.*, 1991). Results obtained from isolated ventricular cardiomyocytes, however, did not indicate any such increase in contraction in the presence of forskolin. Likewise, under similar experimental conditions, Ro 20-1724 did not have any effect in the presence of isoprenaline in isolated cardiomyocytes (Piper *et al.*, 1989). Moreover, no further increase in the contractile activity of sulmazole was observed in the presence of Ro 20-1724. Ro 20-1724 was shown to decrease the contractile response at the highest concentrations (10^{-4} M) investigated which may be due to blockade of the slow inward Ca^{2+}current (Azuma *et al.*, 1983; Weishaar *et al.*, 1986; Bethke *et al.*, 1988).

The positive contractile effect of the novel cardiotonic agent, HN-10200, is not mediated by stimulation of the α-adrenoceptor, β-adrenoceptor or histamine receptors or stimulation of adenylate cyclase in isolated ventricular cardiomyocytes. These results are in accordance with data obtained from experiments using guinea-pig, pig and human cardiac tissues (Kuhberger *et al.*, 1991). However, HN-10200 has been shown to exert an inhibitory effect on cardiac PDE isoenzymes (Kuhberger *et al.*, 1991). HN-10200 produced a

similar effect to that of sulmazole on accumulation of cyclic AMP (Figure 3d), both in terms of efficacy and potency. However, HN-10200 produced a more potent and effective response on contractile activity than sulmazole or IBMX, both in the presence and absence of forskolin. Therefore, it may be concluded that other mechanisms make a greater contribution to the contractile response of this compound. Kuhberger *et al.* (1991) reported a weak Ca^{2+}-sensitizing effect in ventricular skinned muscle fibres of pig.

In summary, the cellular actions of the novel cardiotonic agent, HN-10200, on accumulation of cyclic AMP, are similar to those of sulmazole which has been reported to weakly inhibit PDE III activity but may be less selective at high concentrations of the compound. In contrast, enoximone, which is a potent and selective inhibitor of the PDE III isoenzyme, did not produce any apparent increase in levels of cyclic AMP in isolated ventricular cardiomyocytes, probably due to the fact that the PDE IV isoenzymes present were still capable of hydrolysing the elevated levels of cyclic AMP. Furthermore, it was clear that enoximone, in addition to HN-10200, sulmazole, and IBMX (compounds with PDE III activity), involve cyclic AMP-independent mechanisms of action, whilst Ro 20-1724 (a selective PDE IV inhibitor) increased accumulation of cyclic AMP without effects on contractile function in rat isolated cardiomyocytes, adding support to the theory of compartmentalization of cyclic AMP by PDE IV inhibitors.

This work was supported by the Harold McCauley Fund for Cardiovascular Research. The authors wish to thank Dr Rosenhow (Hafslund Nycomed, Linz, Austria) for the supply of HN-10200, and Dr Humphreys (Boehringer, Ingelheim, Berkshire, U.K.) for sulmazole.

References

AHN, H.S., EARDLEY, D., WATKINS, R. & PRIOLI, N. (1986). Effects of several newer cardiotonic drugs on cardiac cyclic AMP metabolism. *Biochem. Pharmacol.*, **35**, 1113–1121.

ALOUSI, A.A. & JOHNSON, D.C. (1986). Pharmacology of the bipyridines: amrinone and milrinone. *Circulation*, **73**, III-10–III-24.

AZUMA, J., HARADA, H., SAWAMURA, A., HASEGAWA, H., KISHIMOTO, S. & SPERELAKIS, N. (1983). Concentration-dependent effect of trapidil on slow action potential in cardiac muscle. *J. Mol. Cell. Cardiol.*, **15**, 43–52.

BEAVO, J.A. (1988). Multiple isoenzymes of cyclic nucleotide phosphodiesterase. *Adv. Second Mess. Phosphoprot. Res.*, **22**, 1–38.

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isoenzymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 130–155.

BETHKE, T., BRUNKHORST, D., VAN DER LEYEN, H., MEYER, W., NIGBUR, R. & SCHOLZ, H. (1988). Mechanism of action and cardiotonic activity of a new phosphodiesterase inhibitor, the benzimidazole derivative adibendan (BM 14.478), in guinea-pig hearts. *Naunyn-Schmied. Arch. Pharmacol.*, **337**, 576–582.

BLINKS, J.R. & ENDOH, M.D. (1984). Sulmazol (AR-L 115 BS) alters the relation between [Ca2+] and tension in living canine ventricular muscle. *J. Physiol.*, **353**, 63.

BODE, D.C., KANTER, J.R. & BRUNTON, L.L. (1991). Cellular distribution of phosphodiesterase isoforms in rat cardiac tissue. *Circ. Res.*, **68**, 1070–1079.

BRISTOW, M.R., GINSBURG, R., STROSBERG, A., MONTGOMERY, W. & MINOBE, W. (1984). Pharmacology and inotropic potential of forskolin in the human heart. *J. Clin. Invest.*, **74**, 212–223.

BRASCH, H. & IVEN, H. (1991). Inotropic and electrophysiological effects of BDF 9148, a congener of DPI 210-106, in guinea-pig atria and papillary muscles. *Br. J. Pharmacol.*, **103**, 1939–1945.

BUSCHMANS, E., HEARSE, D.J. & MANNING, A.S. (1985). Forskolin: effects on cyclic AMP and contractile function in the isolated rat and guinea pig heart. *Can. J. Cardiol.*, **1**, 385–394.

COLUCCI, W.S., WRIGHT, R.F. & BRAUNWALD, E. (1986). New positive inotropic agents in the treatment of congestive heart failure. *N. Engl. J. Med.*, **314**, 290–299.

ENDOH, M. & BLINKS, J.R. (1987). Inhibition of isoproterenol-induced increase in peak calcium transients by theophylline in the isolated canine ventricular muscle. *J. Muscle Res. Cell Motility*, **8**, 468.

ENDOH, M., YANAGISAWA, T., MORITA, T. & TAIRA, N. (1985). Differential effects of sulmazole (AR-L 115 BS) on contractile force and cyclic AMP levels in canine ventricular muscle: comparison with MDL 17,043. *J. Pharmacol. Exp. Ther.*, **234**, 267–273.

ENDOH, M., YANAGISAWA, T. & TAIRA, N. (1980). Dissociation of cyclic AMP and contractile responses to isoprenaline: effects of dihydropyridine derivative, nicardipine (YC-93), on canine ventricular muscle. *Eur. J. Pharmacol.*, **67**, 225–233.

ENDOH, M., YANAGISAWA, T., TAIRA, N. & BLINKS, J.R. (1986). Effects of new inotropic agents on cyclic nucleotide metabolism and calcium transients in canine ventricular muscle. *Circulation*, **73** (Suppl. III), 117–133.

EVANS, D.B. (1986). Modulation of cyclic AMP: mechanism for positive inotropic action. *J. Cardiovasc. Pharmacol.*, **8** (Suppl. 9), S22–S29.

FUJINO, K., SPERELAKIS, N. & SOLARO, R.J. (1988). Sensitization of dog and guinea-pig heart myofilaments to calcium activation and the inotropic effect of pimobendan: comparison with milrinone. *Circ. Res.*, **63**, 911–922.

GEORGE, E.E., ROMANO, F.D. & DOBSON, J.G. (1991). Adenosine and acetylcholine reduce isoproterenol-induced protein phosphorylation of rat myocytes. *J. Mol. Cell. Cardiol.*, **23**, 749–764.

GRIMA, M., FREYSS BEGUIN, M., MILLANVOYE-VAN-BRUSSEL, E., DECKER, N. & SCHWARTZ, J. (1988). Effects on the sodium channel of some new cardiotonic drugs. *J. Cardiovasc. Pharmacol.*, **12**, 255–263.

HALEEN, S.J., STEFFEN, R.P. & WEISHAAR, R.E. (1989). Species differences in the positive inotropic response to DPI 201-106, a novel cardiotonic agent. *Can. J. Physiol. Pharmacol.*, **67**, 1460–1463.

HARRIS, A.L., CONNELL, M.J., FERGUSON, E.W., WALLACE, A.M., GORDON, R.J., PAGANI, E.D. & SILVER, P.J. (1989). Role of low Km cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **251**, 199–203.

HARTMANN, A., SAEED, M., SUTSCH, G. & BING, R.J. (1986). Phosphodiesterasehemming als neues positiv-inotropes. *Dtsch. Med. Wochenschr.*, **111**, 1971.

HERZIG, J.W., FEILE, K. & RUEGG, J.C. (1981). Activating effects of AR-L 115 BS on the Ca2+ sensitive force, stiffness and unloaded shortening velocity (Vmax) in isolated contractile structures from mammalian heart muscle. *Arzneimittelforsch/Drug Res.*, **31**, 188–191.

HOHL, C.M. & LI, Q. (1991). Compartmentation of cyclic AMP in adult canine ventricular myocytes. *Circ. Res.*, **69**, 1369–1379.

HOLMBERG, S.R.M. & WILLIAMS, A.J. (1991). Phosphodiesterase inhibitors and the cardiac sarcoplasmic reticulum calcium release channel: differential effects of milrinone and enoximone. *Cardiovasc. Res.*, **25**, 537–545.

HORACKOVA, M. & MAPPLEBECK, C. (1988). Electrical, contractile, and ultrastructural properties of adult rat and guinea-pig ventricular myocytes in long-term primary cultures. *Can. J. Physiol.*, **67**, 740–750.

HSIEH, C.P., KARIYA, T., DAGE, R.C. & RUBERG, S.J. (1987). Effects of enoximone and isobutylmethylxanthine on contractile tension and cyclic nucleotide levels in isolated blood-perfused dog papillary muscle. *J. Cardiovasc. Pharmacol.*, **9**, 230–236.

HUBBARD, J.W., CONWAY, P.G., NORDSTROM, L.C., HARTMANN, H.B., LEBENDINSKY, Y., O'MALLEY, G.J. & KOSLEY, R.W. (1991). Cardiac adenylate cyclase activity, positive chronotropic and inotropic effects of forskolin analogues with either low, medium or high binding site affinity. *J. Pharmacol. Exp. Ther.*, **256**, 621–627.

KATANO, Y. & ENDOH, M. (1990). Differential effects of Ro 20-1724 and isobutylxanthine on the basal force of contraction and beta-adrenoceptors-mediated response in the rat ventricular myocardium. *Biochem. Biophys. Res. Commun.*, **167**, 123–129.

KELSO, E.J., MCDERMOTT, B.J. & SILKE, B. (1992). The role of phosphodiesterase inhibitors in regulating cyclic AMP and contractile function in isolated ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.*, **24**, S101.

KITADA, Y., KOBAYASHI, M., NARIMATSU, A. & OHIZUMA, Y. (1989). Potent stimulation of myofilament force and adenosine-triphosphatase activity of canine cardiac muscle through a direct enhancement of troponin C, Ca2+ binding by MCI-154, a novel cardiotonic agent. *J. Pharmacol. Exp. Ther.*, **250**, 272–277.

KITADA, Y., NARIMATSU, A., SUZUKI, R., ENDOH, M. & TAIRA, N. (1987). Does the positive inotropic action of a novel cardiotonic agent MCI-154, involve mechanisms other than cyclic AMP. *J. Pharmacol. Exp. Ther.*, **243**, 639–645.

KUHBERGER, E., STIMMEDER, D., STROßNIG, H. & PRUSS, T.P. (1991). Pharmacological in vitro characterization of the new vasorelaxant and positive inotropic agent HN-10200. *Cardiovasc. Drugs Ther.*, **5**, P23.

LINDNER, E. & METZGER, H. (1983). The action of forskolin on muscle cells is modified by hormones, calcium ions and calcium antagonists. *Arzneim. Forsch.*, **33**, 1436–1441.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol in reagent. *J. Biol. Chem.*, **193**, 265–266.

MILLAR, B.C., PIPER, H.M. & MCDERMOTT, B.J. (1988). The antiadrenergic effect of neuropeptide Y on the ventricular cardiomyocyte. *Naunyn Schmied. Arch. Pharmacol.*, **338**, 426–429.

MULLER, B., LUGNIER, C. & STOCLET, J.C. (1990). Involvement of rolipram-sensitive cyclic AMP phosphodiesterase in the regulation of cardiac contraction. *J. Cardiovasc. Pharmacol.*, **16**, 796–803.

MYLOTTE, K.M., CODY, V., DAVIS, P.J., DAVIS, F.B., BLAS, S.D. & SCHOENL, M. (1985). Milrinone and thyroid hormone stimulates myocardial membrane Ca-ATPase activity and share structural homologies. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7974–7978.

NICHOLSON, C.D., CHALLISS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.*, **12**, 19–27.

OHNISHI, S.T. & BARR, J.K. (1978). A simplified method of quantitating proteins using the biuret and phenol reagents. *Anal. Biochem.*, **86**, 193–197.

PARSONS, W.J., RAMKUMAR, V. & STILES, G.L. (1988). The new cardiotonic agent sulmazole is an A1 adenosine receptor antagonist and functionally blocks the inhibitory regulator Gi. *Mol. Pharmacol.*, **33**, 441–448.

PIPER, H.M., MILLAR, B.C. & MCDERMOTT, B.J. (1989). The negative inotropic effect of neuropeptide Y on the ventricular cardiomyocyte. *Naunyn Schmied. Arch. Pharmacol.*, **340**, 333–337.

PIPER, H.M., PROBST, I. & SCHWARTZ, P. (1982). Culturing of calcium stable adult cardiac myocytes. *J. Mol. Cell. Cardiol.*, **14**, 397–412.

POWELL, T., TATHAM, P.E.R. & TWIST, V.W. (1984). Cytoplasmic free calcium measured by Quin 2 fluorescence in isolated ventricular myocytes at rest and during potassium depolarization. *Biochem. Biophys. Res. Commun.*, **122**, 1012–1020.

POWELL, T. & TWIST, V.W. (1976). A rapid technique for the isolation and purification of adult 'cardiac muscle cells having respiratory control and a tolerance to calcium. *Biochem. Biophys. Res. Commun.*, **72**, 327–333.

PRIGENT, A.F., FOUGIER, S., NEMOZ, G., ANKER, G., PACHECO, H., LUGNIER, C., LEBEC, A. & STOCLET, J.C. (1988). Comparison of cyclic nucleotide phosphodiesterase isoforms from rat heart and bovine aorta. *Biochem. Pharmacol.*, **37**, 3671–3681.

RAPUNDALO, S.T., LATHROP, D.A., HARRISON, S.A., BEAVO, J.A. & SCHWARTZ, A. (1988). Cyclic AMP-dependent and cyclic AMP-independent actions of a novel cardiotonic agent, OPC-8212. *Naunyn Schmied. Arch. Pharmacol.*, **338**, 692–698.

REEVES, M.L., LEIGH, B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 535–541.

RODGER, I.W. & SHAHID, M. (1984). Forskolin, cyclic nucleotides and positive inotropism in isolated papillary muscles of the rabbit. *Br. J. Pharmacol.*, **81**, 151–159.

RUEGG, J.C. (1986). Effects of new inotropic agents on calcium sensitivity of contractile proteins. *Circulation*, **73**, III-78–III-84.

RUEGG, J.C. & MORANO, I. (1989). Calcium-sensitivity modulation of cardiac myofibrillar proteins. *J. Cardiovasc. Pharmacol.*, **14**, S20–S23.

SCHMITZ, W., VAN DER LEYEN, H., MEYER, W., NEUMANN, J. & SCHOLZ, H. (1989). Phosphodiesterase inhibition and positive inotropic effects. *J. Cardiovasc. Pharmacol.*, **14**, S11–S14.

SCHOLZ, H. & MEYER, W. (1986). Phosphodiesterase-inhibiting properties of newer inotropic agents. *Circulation*, **73**, III-99–III-108.

SEAMON, K.B. & DALY, J.W. (1981). Activation of adenylate cyclase by the diterpine forskolin does not require the guanine nucleotide regulatory protein. *J. Biol. Chem.*, **256**, 9799–9801.

SHAHID, M., WILSON, M., NICHOLSON, C.D. & MARSHALL, R.J. (1990). Species-dependent differences in the properties of particulate cyclic nucleotide phosphodiesterase from rat and rabbit ventricular myocardium. *J. Pharm. Pharmacol.*, **42**, 283–284.

SILVER, P.J. (1989). Biochemical aspects of inhibition of cardiovascular low (Km) cyclic adenosine monophosphate phosphodiesterase. *Am. J. Cardiol.*, **63**, 2A–8A.

SILVER, P.J., HARRIS, A.L., CANNIFF, P.C., LEPORE, R.E., BENTLEY, R.G., HAMEL, L.T. & EVANS, D.B. (1989). Phosphodiesterase isoenzyme inhibition, activation of the cyclic AMP system, and positive inotropy mediated by Milrinone in isolated guinea-pig cardiac muscle. *J. Cardiovasc. Pharmacol.*, **13**, 530–540.

SOLARO, R.J. & RUEGG, J.C. (1982). Stimulation of Ca2+ binding and ATPase activity of cardiac myofibrils by AR-L 115BS, a novel cardiotonic agent. *Circ. Res.*, **51**, 290–294.

STROßNIG, H. & PRUSS, T.P. (1991). Effects of the new vasodilating and positive inotropic agent, HN-10200, in the anaesthetized and conscious rat and dog. *Cardiovasc. Drugs Ther.*, **5**, P165.

SUTKO, J.L., KENYON, J.L. & REEVES, J.P. (1986). Effects of amrinone and milrinone on calcium influx into the myocardium. *Circulation*, **73** (Suppl. III), 52–58.

THOMPSON, W.J. (1991). Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. *Pharmacol. Ther.*, **51**, 13–33.

TOVEY, K.C., OLDHAM, K.G. & WHELAN, J.A. (1974). A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. *Clin. Chim. Acta*, **56**, 221–234.

VERRIJK, R., VLEEMING, W., VAN ROOIJ, H.H., WEMER, J. & PORSIUS, A.J. (1989). Positive inotropic effect of milrinone, sulmazole and AR-L100 in isolated normal and infarcted hearts of the rat. *Arch. Int. Pharmacodyn.*, **297**, 7–17.

WALDECK, B. & WIDMARK, E. (1985). Comparison of the effects of forskolin and isoprenaline on tracheal, cardiac and skeletal muscles from guinea-pig. *Eur. J. Pharmacol.*, **112**, 349–353.

WEISHAAR, R.E., BURROWS, S.D., KOBYLARZ, D.C., QUADE, M.M. & EVANS, D.B. (1986). Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. *Biochem. Pharmacol.*, **35**, 787–800.

WEISHAAR, R.E., CAIN, M.H. & BRISTOL, J.A. (1985). A new generation of phosphodiesterase inhibitors: multiple molecular forms of phosphodiesterase and the potential for drug selectivity. *J. Med. Chem.*, **28**, 537–545.

WEISHAAR, R.E., KOBYLARZ-SINGER, D.C. & KAPLAN, H.R. (1987). Subclasses of cyclic AMP phosphodiesterase in cardiac muscle. *J. Mol. Cell. Cardiol.*, **19**, 1025–1036.

WILLIAMS, A.J. & HOLMBERG, R.M. (1990). Sulmazole (AR-L 115BS) activates the sheep cardiac muscle sarcoplasmic reticulum calcium-release channel in the presence and absence of calcium. *J. Mem. Biol.*, **115**, 167–178.

(Received December 21, 1992
Revised July 28, 1993
Accepted August 12, 1993)

Br. J. Pharmacol. (1993), **110**, 1395–1400 © Macmillan Press Ltd, 1993

Comparison of haemoconcentration induced by big endothelin-1 and endothelin-1 in mice

Hiroshi Okumura, Naoki Ashizawa, Fujio Kobayashi, Koshi Arai, Rieko Asakura, Nami Ashikawa & [1]Akihiro Matsuura

Pharmaceutical Res. Labs., Sapporo Breweries Ltd., 10 Okatohme, Yaizu, Shizuoka, 425 Japan

1 The profile of haemoconcentration induced by big endothelin-1(big ET-1), a precursor of endothelin-1 (ET-1), was compared with that induced by endothelin-1 in mice.

2 ET-1(1.5 nmol kg^{-1}, i.v.) increased haematocrit in mice, which reached a maximum at 5 min and then returned to the control value within 30 min after the administration, this occurred at the same time as changes in the plasma immunoreactive endothelin-1 and rat atrial natriuretic peptide (rANP)-like activities (IR-ET-1 and IR-rANP, respectively).

3 Big ET-1(2.5–15 nmol kg^{-1}, i.v.) also caused a significant and dose-dependent increase in haematocrit, that lasted over 3 h although elevated plasma IR-ET-1 and IR-rANP had almost been restored to the initial levels within 10 min after big ET-1 injection.

4 A metalloproteinase inhibitor, phosphoramidon (10 mg kg^{-1}, i.v.), which inhibits the activity of endothelin converting enzyme (ECE), delayed the onset of big ET-1-induced haemoconcentration, but failed to alter the maximal value and the duration of the haemoconcentration.

5 Pretreatment with phosphoramidon (10 mg kg^{-1}, i.v.) did not affect the big ET-1-induced change in plasma IR-ET-1, while significant delay of the disappearance of plasma IR-rANP and significant suppression of a sustained increase in tissue IR-ET-1 were observed.

6 These results suggest that ET-1, not in plasma but in tissue, plays an important role in the pathogenesis of big ET-1-induced long-lasting haemoconcentration, in which unknown factors besides rANP are involved.

Keywords: Big endothelin-1; endothelin-1; haemoconcentration; immunoreactive endothelin-1 like activity; plasma; lung; mice

Introduction

In 1988, Yanagisawa *et al.* isolated and characterized a potent vasoconstrictor peptide from cultured porcine endothelial cells. Now referred to as endothelin-1 (ET-1), this peptide plays an important role in humoral control of the circulation as well as in cardiovascular, central nervous, bronchopulmonary and renal systems (Yanagisawa *et al.*, 1988; Brenner *et al.*, 1989; Rubanyi, 1992). ET-1 has been found to induce the release of prostacyclin amd endothelium-derived relaxing factor (EDRF) (de Nucci *et al.*, 1988), aldosterone (Goetz *et al.*, 1988; Miller *et al.*, 1989) and atrial natriuretic peptide (ANP) (Fukuda *et al.*, 1988), which would influence the regulation of vascular tone and water balance. These facts indicate that a part of the biological activity caused by ET-1 is mediated by the consecutive release of these biologically active substances.

ET-1 causes an increase in haematocrit, namely haemoconcentration, not by urinary fluid loss but by vascular leakage (Goetz *et al.*, 1988; King *et al.*, 1989; Lopez-Farre *et al.*, 1989). It is well-known that ANP also causes haemoconcentration *in vitro* and *in vivo* (Almeida *et al.*, 1986). Recently, Valentin *et al.* (1991) reported that the haemoconcentration and the increase in plasma ANP concentration induced by ET-3 were completely attenuated by anti-ANP antiserum in anaesthetized rats. These studies suggest that haemoconcentration induced by ET-3 is due to endogenous ANP release.

It is well accepted that the conversion of big endothelin-1(big ET-1) to ET-1 by endothelin converting enzyme (ECE) is requisite for all of the biological responses induced by big ET-1, because biological activities of big ET-1 were reported to be much weaker than those of ET-1 (Pons *et al.*, 1991). Our previous paper also demonstrated that big ET-1-induced sudden death and elevation of the plasma ET-1 level were effectively blocked by a metalloproteinase inhibitor phosphoramidon (Matsuura *et al.*, 1992). From these results, we expected that the haemoconcentration induced by big ET-1 would be similar to that caused by ET-1. In this study, we have investigated whether big ET-1 causes haemoconcentration similar to ET-1 in mice and in addition have evaluated whether phosphoramidon inhibits haemoconcentration via inhibition of both ECE and the production of ANP by measuring plasma and tissue immunological ET-1 and ANP (plasma alone).

Methods

Male ICR mice weighing 30–40 g were used in all experiments. The volume of all treatments in all experiments was set at 0.05 ml 10 g^{-1} body weight.

Haemoconcentration

In the dose-dependent studies, ET-1(0–3 nmol kg^{-1}), big ET-1(0–15 nmol kg^{-1}) and rANP (0–30 μg kg^{-1}) dissolved in saline solution were given intravenously to mice, 5, 20 and 3 min prior to blood collection, respectively. The blood collection was carried out under terminal anaesthesia with ether. Blood samples (ca. 40 μl) were collected from the orbital sinus using glass capillaries precoated with heparin. These blood sampling times were employed as a result of our preliminary studies (data not shown). The haematocrit was measured by spinning blood at 12,000 r.p.m. for 5 min and is shown as a percentage. In the time course studies, ET-1(2.5 nmol kg^{-1}), big ET-1(10 nmol kg^{-1}) or rANP (10 μg kg^{-1}) were given intravenously to mice, and blood collections from different animals were carried out at the appointed time to measure the haematocrit as already described. Phosphoramidon (10 mg kg^{-1}) dissolved in saline was given intravenously to the mice 10 min before big ET-1 injection.

[1] Author for correspondence.

Measurement of plasma immunoreactive ET-1 like ativity (IR-ET-1) and immunoreactive rANP like activity (IR-rANP)

In ICR mice, blood samples (ca. 0.5 ml) were collected by cardiac puncture under terminal anaesthesia with ether from different animals at 0, 3, 5, 10 and 20 min after ET-1(2.5 nmol kg^{-1}), big ET-1(10 nmol kg^{-1}) or rANP (10 μg kg^{-1}) injection. The blood samples were put in chilled tudes containing aprotinin (300 KIU ml^{-1}) and EDTA (2 mg ml^{-1}) and centrifuged at 2000 *g* for 15 min at 4°C; then the plasma sample diluted ten times with phosphate-buffered saline (PBS) was added to the microtest plate.

IR-ET-1 was measured by sandwich-enzyme immunoassay (EIA) as described previously (Matsuura *et al.*, 1992). Briefly, EIA was employed using a rabbit IgG anti ET-1(15–21)-coated microtest plate. After blocking with 1% bovine serum albumin (BSA), the plate was reacted with standard ET-1 or reaction mixture followed by a reaction with rabbit IgG F(ab')2-HRP anti-ET-1. The plate was washed with washing buffer (PBS, containing 0.1% Triton X-100), and then the bound enzyme activity was measured with *o*-phenylenediamine as a chromogen. The assay showed no cross reactivity with big ET-1 at a concentration of 100 nM and the plasma sample (10% concentration) obtained from mice without big ET-1 treatment did not affect the EIA.

IR-rANP was measured by sandwich-EIA using the alpha-hANP Test-Wako kit. Briefly, blood samples were collected and prepared as described above. With 0.1 M phosphate buffer solution and the anti-hANP mouse mono-clonal antibody conjugated bead, the plasma sample or standard rANP was added to the test tube. After incubation at 4°C for 20 h, the tube was washed with distilled water followed by a reaction with peroxidase conjugated anti rabbit alpha-hANP antibody. The bound enzyme activity was measured with *o*-phenylenediamine as a chromogen.

Measurement of IR-ET-1 in the lung

Ten minutes after phosphoramidon (10 mg kg^{-1}, i.v.) pretreatment, big ET-1 at a dose of 10 nmol kg^{-1}, i.v. was administered to ICR mice. Mice were killed by dislocation of the neck at 0, 3, 5, 10 and 20 min after big ET-1 injection, and the lungs were immediately removed and frozen on dry ice. The amount of ET-1 in the lung tissues was measured by the same EIA method as that used for the plasma concentration of ET-1 with the following pretreatment, which involved minor modification of the methods previously described (Shibouta *et al.*, 1990; Watanabe *et al.*, 1991). The tissues were homogenized in 1.4 ml of chloroform:methanol (2:1) at 4°C for 2 min. Then, 0.35 ml of water was added to the homogenates, followed by centrifugation at 2500 × *g* for 25 min. The aqueous phase was stored at − 80°C. The tissue extracts were acidified with acetic acid to a final concentration of 3% and were applied to a Sep-Pak C-18 cartridge. The immunoreactive ET-1 fraction was eluted with methanol and was concentrated to dryness for the EIA.

Materials

ET-1, big ET-1, rANP, phosphoramidon and aprotinin were supplied by Peptide Institute (Osaka, Japan); EDTA, Triton-X100 and the alpha-hANP Test-Wako kit by Wako (Osaka, Japan), rabbit IgG anti ET-1(15–21) and rabbit IgG F(ab')2-HRP anti ET-1 by International Reagents Corp. (Kobe, Japan); BSA and *o*-phenylendiamine by Sigma (St. Louis, U.S.A.). All other chemicals were of analytical grade.

Data analysis

Data are expressed as means ± s.e.mean from *n* animals. Where a difference was found across the groups, an unpaired Student's *t* test or one-way analysis of variance with the Barferroni modification was used. *P* values less than 0.05 were regarded as statistically significant.

Results

Intravenous administration of ET-1(1.5–2.5 nmol kg^{-1}), big ET-1(2.5–15 nmol kg^{-1}) and rANP (3–100 μg kg^{-1}) to mice significantly increased haematocrit values in a dose-dependent manner (Figure 1).

ET-1(1.5 and 2.5 nmol kg^{-1}, i.v.) caused an immediate increase in haematocrit with maximum response at 3–5 min after ET-1 injection; then the haematocrit declined (Figure 2a), especially it fully returned to the initial level at 1.5 nmol kg^{-1}. On the other hand, the intravenous administration of big ET-1(1.5–15 nmol kg^{-1}) to mice caused a long-lasting increase in haematocrit (Figure 2b), continuing beyond 3 h after big ET-1 injection. Even at a minimum dose of 1.5 nmol kg^{-1}, big ET-1 caused longer-lasting haemoconcentration than that caused by the same dose of ET-1.

The time course of changes in plasma IR-ET-1 and IR-rANP induced by ET-1 (2.5 nmol kg^{-1}, i.v.) are shown in Figures 3a and 3b, respectively. Plasma IR-ET-1 reached a maximum at 3 min after ET-1 administration and then rapidly returned to its initial value (Figure 3a), but plasma IR-rANP reached a maximum at 5 min and then slowly returned in correspondence to the change in hematocrit (Figure 3b).

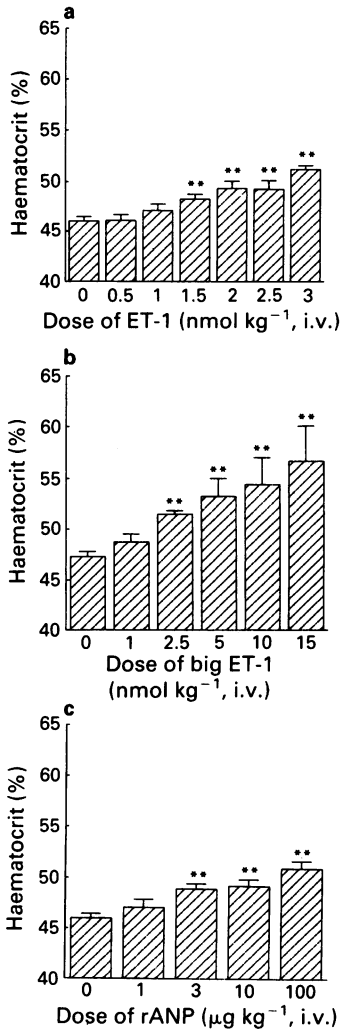


Figure 1 Dose-dependency of changes in haematocrit induced by endothelin-1(ET-1)(a), big endothelin-1(big ET-1)(b) and rat atrial natriuretic peptide (rANP)(c) in mice. The animals were given ET-1(0.5 to 3 nmol kg^{-1}), big ET-1(1 to 15 nmol kg^{-1}), rANP (1 to 100 μg kg^{-1}) or saline intravenously, and orbital sinus blood collections were carried out in different animals under terminal anaesthesia at 5 min, 20 min or 3 min, respectively. Values are means ± s.e.mean of 6–8 animals. **Significantly different from the control value ($P<0.01$).

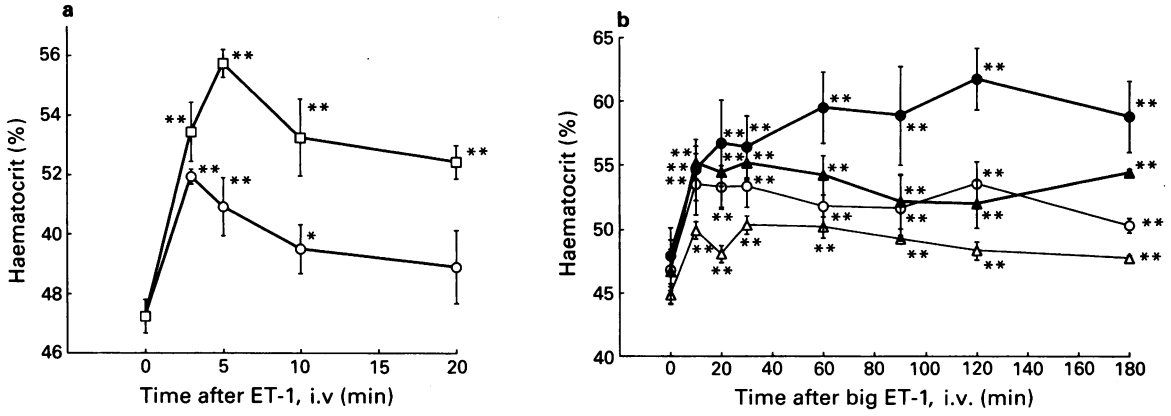


Figure 2 Time course of changes in haematocrit induced by endothelin-1(ET-1)(a) and big endothelin-1(big ET-1)(b) in mice.The animals were given ET-1 at doses of 1.5 (○) and 2.5 nmol kg^{-1} (□) or big ET-1 at doses of 1.5(△), 5(○), 10(▲) and 15(●) nmol kg^{-1} intravenously. Orbital sinus blood collections were carried out in different animals under terminal anaesthesia at 3, 5, 10 and 20 min for ET-1 and at 10, 20, 30, 60, 120 and 180 min for big ET-1. Values are means ± s.e.mean of 6–8 animals. *Significantly different from saline group ($P<0.05$), **$P<0.01$.

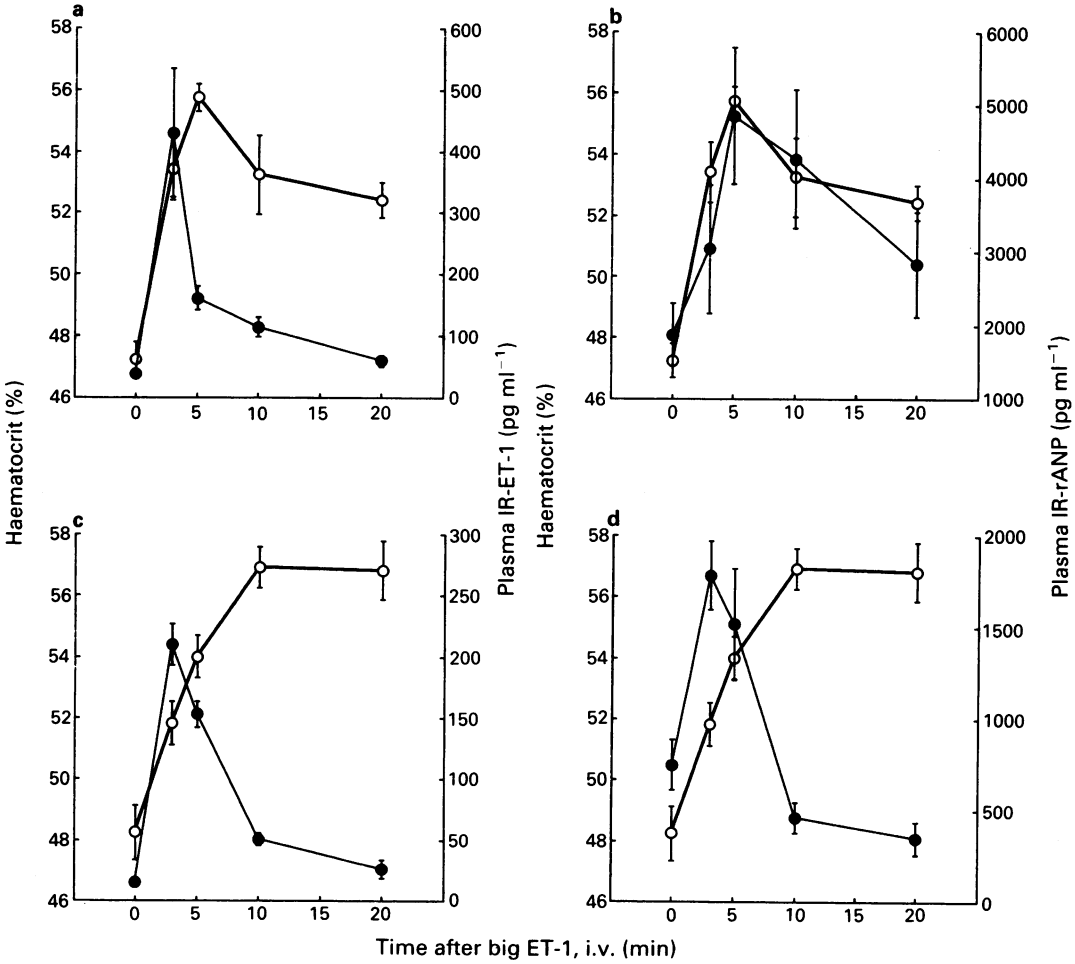


Figure 3 Relationship between haematocrit (○) and plasma immunoreactive endothelin-1 like activity (IR-ET-1)(●) changes induced by endothelin-1(ET-1)(a), big endothelin-1(big ET-1)(c) and that between haematocrit (○) and plasma immunoreactive rat atrial natriuretic peptide-like activity (IR-rANP)(●) changes induced by ET-1(b) and big ET-1(d) in mice. Haematocrit and plasma IR-ET-1/rANP were measured in separate experiments. In the haematocrit study, orbital sinus blood was collected at 0, 3, 5, 10 and 20 min. In the plasma IR-ET-1/rANP study, cardiac puncture for blood collection was carried out at 0, 3, 5, 10 and 20 min after ET-1 or big ET-1 injection. Both blood sampling procedures were carried out in different animals under terminal anaesthesia. Values are means ± s.e.mean of 7 animals.

Big ET-1(10 nmol kg^{-1}) also caused significant increases in plasma IR-ET-1 and plasma IR-rANP (Figures 3c,d). Both changes reached their peaks within 5 min and then rapidly returned to the initial levels within 10 min after big ET-1 injection. Elevation of haematocrit induced by big ET-1, however, was remarkably long-lasting, independent of changes in plasma IR-ET-1 and IR-rANP.

Pretreatment with phosphoramidon at a dose of 10 mg kg^{-1}, i.v., significantly attenuated big ET-1-induced haemoconcentration within 10 min after big ET-1 injection (Figure 4). At 20 min after big ET-1 injection, there was no difference in haematocrit between control and phosphoramidon-treated mice. Effects of phosphoramidon on the changes in plasma IR-ET-1 and IR-rANP induced by big ET-1 were also examined in the same experiment (Figures 5a,b). Although phosphoramidon failed to affect the change in plasma IR-ET-1 (Figure 5a), plasma IR-rANP remained significantly higher than the control 10–20 min after big ET-1 injection (Figure 5b) probably due to its inhibition of endopeptidases, which hydrolyze rANP.

In another experiment, the effect of phosphoramidon on the change in lung tissue IR-ET-1 was measured. Intravenous administration of big ET-1(10 nmol kg^{-1}) to mice caused long-lasting increases in lung tissue IR-ET-1 (Figure 6). Pretreatment with phosphoramidon at a dose of 10 mg kg^{-1}, i.v., 10 min prior to big ET-1 administration significantly inhibited the increase in the tissue IR-ET-1 at least for 20 min after big ET-1 injection.

Discussion

It is well known that ET-1 induces a sustained contraction of isolated blood vessels *in vitro* and causes a potent long-lasting pressor response *in vivo*. Although big ET-1, a precursor of ET-1, has 100 fold less contractile activity compared to ET-1 *in vitro*, it causes pressor activity almost equivalent to that of ET-1 *in vivo* (Kashiwabara *et al.*, 1989; Matsumura *et al.*, 1990), because big ET-1 is readily converted to ET-1 by specific ECE, existing extensively in the endothelium of the blood vessels. Several studies also revealed that phosphoramidon, a metalloproteinase inhibitor,

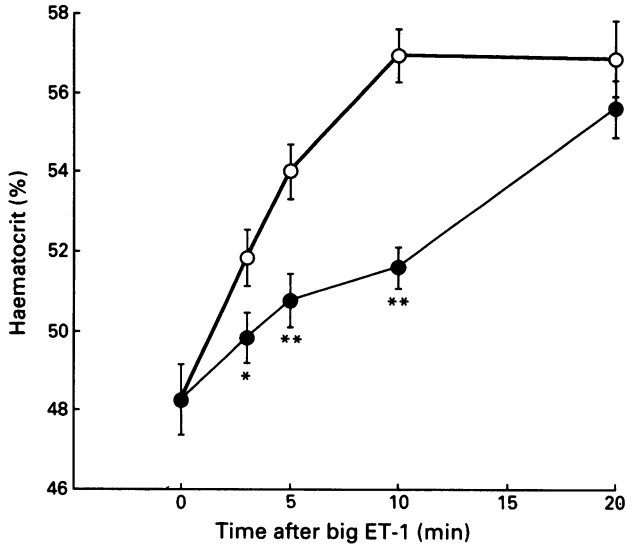


Figure 4 The effect of phosphoramidon on the haemoconcentration induced by big endothelin-1 (big ET-1) in mice. The animals were pretreated intravenously with phosphoramidon at a dose of 10 mg kg^{-1} (●) or saline (○) at 10 min before i.v. bolus injection of big ET-1(10 nmol kg^{-1}). Orbital sinus blood collections were carried out in different animals under terminal anaesthesia at 3, 5, 10 or 20 min after big ET-1 injection. Values are means ± s.e.mean of 6–8 animals. Significantly different from saline group: $*P<0.05$; $**P<0.01$.

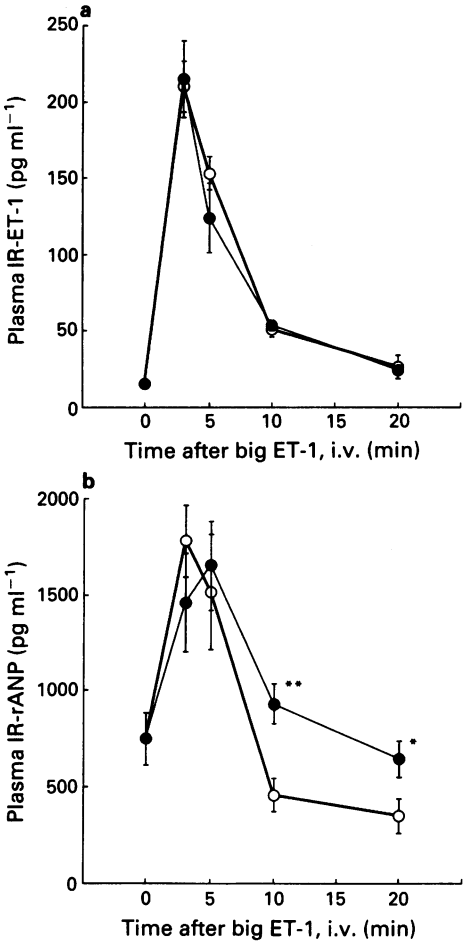


Figure 5 The effect of phosphoramidon on the change in plasma immunoreactive endothelin-1 like activity (IR-ET-1)(a) and plasma immunoreactive rat atrial natriuretic peptide like activity (IR-rANP) (b) induced by big endothelin-1 (big ET-1) in mice. The animals were pretreated intravenously with phosphoramidon at a dose of 10 mg kg^{-1} (●) or saline (○) at 10 min before i.v. bolus injection of big ET-1(10 nmol kg^{-1}). Cardiac puncture for blood collection was carried out in different animals under terminal anaesthesia at 0, 3, 5, 10 and 20 min after big ET-1 injection. Values are means ± s.e.mean of 7 animals. Significantly different from saline group; $*P<0.05$; $**P<0.01$.

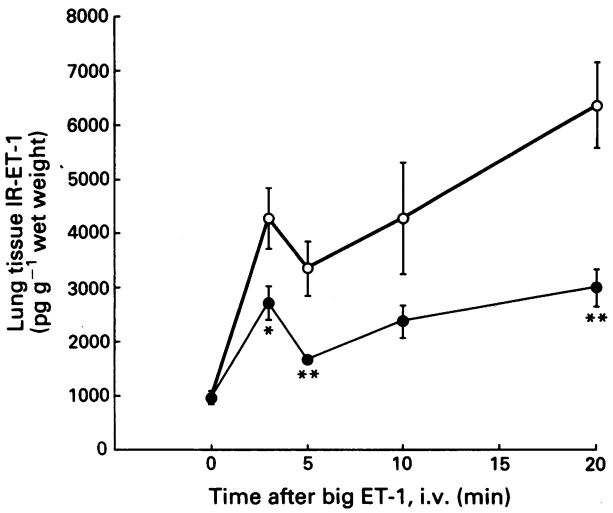


Figure 6 The effect of phosphoramidon on the change in tissue immunoreactive endothelin-1 like activity (IR-ET-1) induced by big endothelin-1 (big ET-1) in mice. The animals were pretreated intravenously with phosphoramidon at a dose of 10 mg kg^{-1} (●) or saline (○) at 10 min before i.v. bolus injection of big ET-1(10 nmol kg^{-1}). The mice were killed by dislocation of the neck at 0, 3, 5, 10 and 20 min after big ET-1 injection. Values are means ± s.e.mean of 7 animals. Significantly different from saline group; $*P<0.05$; $**P<0.01$.

blocked big ET-1-induced responses, such as hypertension (Matsumura *et al.*, 1990; McMahon *et al.*, 1991) and sudden death (Matsuura *et al.*, 1992) accompanying the inhibition of ET-1 formation. Since it was strongly believed that all of the biological activities by big ET-1 require the conversion of big ET-1 to ET-1, we supposed that the profiles of haemoconcentration induced by big ET-1 would be similar to that by ET-1. Therefore, we have investigated haemoconcentration induced by big ET-1 and compared it with that induced by ET-1 in mice.

In the first experiment, both ET-1 and big ET-1 dose-dependently increased haematocrit in mice as expected. Haemoconcentration induced by big ET-1 even at a minimum dose of 1.5 nmol kg^{-1}, however, was extremely long-lasting beyond our expectation, while ET-1-induced haemoconcentration rapidly recovered at the same dose. What causes the difference between the two responses?

It was reported that hypertension induced by ET-1 was attended by ANP release from the atrium in conscious dogs (Goetz *et al.*, 1988). ANP is released from the cardiac atria by pressure-induced atrial distension and/or volume expansion (Lang *et al.*, 1985; Ledsome *et al.*, 1985), which are positively affected by ET-1. Furthermore, ET-1 was a potent releaser of ANP from cultured neonatal rat atrial cardiocytes *in vitro* probably due to Ca^{2+} influx through the voltage-dependent Ca^{2+}-channel as well as activation of protein kinase C by ET-1 (Fukuda *et al.*, 1988). In our study, the change in haematocrit induced by ET-1 was coincident not with the change in plasma IR-ET-1 (Figure 3a) but with the change in plasma IR-rANP (Figure 3b), which was observed with a delay from IR-ET-1 changes. These results suggest that ET-1 administered exogenously causes a rise in plasma ET-1 concentration, resulting in the subsequent release of rANP from the atrium. Released rANP then enhances vascular permeability and finally leads to haemoconcentration.

In the present study, big ET-1 also provoked haemoconcentration which was characterized by a long duration of action. Even at 20 min after big ET-1 administration, the haematocrit value remained much higher than the normal value, while plasma IR-ET-1 and IR-rANP had already returned to their control levels. This provided the possibility that other factors besides rANP might be involved in haemoconcentration induced by big ET-1 in mice.

In addition to ANP, there are many endogenous bio-active substances conceived as mediators of haemoconcentrations induced by ET-1. Goetz *et al.* (1988) reported that intravenous administration of ET-1 (0.72 nmol kg^{-1} h^{-1}) increased plasma levels of vasopressin, renin, aldosterone, noradrenaline, adrenaline and ANP in conscious dogs. Platelet activating factor (PAF) was also reported to be involved in the increase in haematocrit induced by ET-1; i.e., pretreatment with the specific PAF receptor antagonist, WEB 2086 (1 mg kg^{-1}, i.v.) or BN 52021 (10 mg kg^{-1}, i.v.) partially attenuated the ET-1 (1 nmol kg^{-1})-induced rise in haematocrit by about 50 and 30%, respectively, but they failed to improve ET-1-induced hypertension in conscious rats (Filep *et al.*, 1991). Additionally, the thromboxane A_2 receptor antagonist, BM-13505, also partially attenuated vascular permeability enhanced by ET-1 without any influence on pressor responses in conscious rats (Sirois *et al.*, 1992). These results suggested that the mechanism of haemoconcentration induced by ET-1 might be highly complex and involve many endogenous factors different from those involved in inducing pressor responses.

Recently, Hisaki *et al.* (1993) reported that phosphoramidon did not influence the amount of IR-ET-1 in the perfusate during perfusion of big ET-1 but attenuated it in the lung tissue using the rat isolated perfused lung. Therefore, they proposed that the major functional conversion of big ET-1 into ET-1 occurred in the lung tissue. In our present study, exogenous administration of big ET-1 caused a continuous increment in lung tissue IR-ET-1 at least for 20 min after the injection. Pretreatment with phosphoramidon at a dose which did not affect plasma IR-ET-1 (10 mg kg^{-1}, i.v.) significantly decreased the increment in tissue IR-ET-1. Thus the suppression of big ET-1-induced haemoconcentration than that in plasma. Accordingly it was suggested that since pulmonary tissues contain a large number of blood vessels in which ECE existed in endothelial cells formation in tissue is more important for prevention of haemoconcentration than that in plasam. Accordingly it was suggested that since pulmonary tissues contain a large number of blood vessels in which ECE existed in endothelial cells (Ohnaka *et al.*, 1990; Okada *et al.*, 1990), the change in the amount of lung tissue IR-ET-1 was partly involved in the mechanism of big ET-1 induced haemoconcentration.

The precise mechanisms of the long-lasting haemoconcentration induced by big ET-1 are still unclear. Several explanations could be presented as follows: big ET-1-induced haemoconcentration depended on the tissue level of ET-1 converted from big ET-1, which was trapped and sequestered in the endothelial cells of the vessels. The gradual and sustained conversion of big ET-1 to ET-1 in the endothelial cells would lead to the long-lasting haemoconcentration. In addition to ET-1 and rANP, other endogenous factors must participate secondarily in the effects of big ET-1 on haematocrit.

In conclusion, big ET-1 caused long-lasting haemoconcentration in mice with a transient rise in plasma IR-ET-1 and rANP and sustained elevation of tissue level of IR-ET-1. Phosphoramidon alleviated the haemoconcentration by the inhibition of the increase in IR-ET-1 of pulmonary tissue. These facts indicate that long-lasting haemoconcentration induced by big ET-1 may be attributable to the tissue level of ET-1 and/or to other endogenous factors besides ET-1 and rANP.

We express our thanks to Ms M. Takahashi and Ms Y. Aoshima for their technical assistance.

References

ALMEIDA, F.A., SUZUKI, M. & MAACK, T. (1986). Atrial natriuretic factor increases hematocrit and decreases plasma volume in nephrectomized rats. *Life Sci.*, **39**, 1193–1199.

BRENNER, B.M., TROY, J.L. & BALLERMANN, B.J. (1989). Endothelium-dependent vascular responses; mediators and mechanisms. *J. Clin. Invest.*, **84**, 1373–1378.

DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9797–9800.

FILEP, J.G., SIROIS, M.G., ROUSSEAU, A., FOURNIER, A. & SIROIS, P. (1991). Effects of endothelin-1 on vascular permeability in the conscious rat: interactions with platelet-activating factor. *Br. J. Pharmacol.*, **104**, 797–804.

FUKUDA, Y., HIRATA, Y., YOSHIMI, H., KOJIMA, T., KOBAYASHI, Y., YANAGISAWA, M. & MASAKI, T. (1988). Endothelin is a potent secretagogue for atrial natriuretic peptide in cultured rat atrial myocytes. *Biochem. Biophys. Res. Commun,*. **155**, 167–172.

GOETZ, K.L., WANG, B.C., MADWED, J.B., ZHU, J.L. & LEADLEY, Jr. R.J. (1988). Cardiovascular, renal and endocrine responses to intravenous endothelin in conscious dogs. *Am. J. Physiol.*, **255**, R1064–1068.

HISAKI, K., MATSUMURA, Y, MAEKAWA, H., FUJITA, K., TAKAOKA, M. & MORIMOTO, S. (1993). Phosphoramidon inhibits the conversion of big endothelin-1 to endothelin-1 in isolated perfused rat lung. *Jpn. J. Pharmacol.*, **61**, Suppl. I, 135P.

KASHIWABARA, T., INAGAKI, Y., OHTA, H., IWAMATSU, A., NOMIZU, M., MORITA, A. & NISHIKORI, K. (1989). Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect in vivo. *FEBS Lett.*, **247**, 73–76.

KING, A.J., BRENNER, B.M. & ANDERSON, S. (1989). Endothelin: a potent renal and systemic vasoconstrictor peptide. *Am. J. Physiol.*, **256**, F1051–1058.

LANG, R.E., THOLKEN, H., GANTEN, D., LUFT, F.C,. RUSKOAHO, H. & UNGER, T. (1985). Atrial natriuretic factor – a circulating hormone stimulated by volume loading. *Nature*, **314**, 264–266.

LEDSOME, J.R., WILSON, N., COURNEYA, A. & RANKIN, A.J. (1985). Release of atrial natriuretic peptide by atrial distension. *Can. J. Physiol. Pharmacol.*, **63**, 739–742.

LOPEZ-FARRE, A., MONTANES, A.I., MILLAS, I. & LOPEZ-NOVOA, J. (1989). Effect of endothelin on renal function in rats. *Eur. J. Pharmacol,*. **163**, 187–189.

MATSUMURA, Y., HISAKI, K., TAKAOKA, M. & MORIMOTO, S. (1990). Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur. J. Pharmacol.*, **185**, 103–106.

MATSUURA, A., OKUMURA, H., ASHIZAWA, N. & KOBAYASHI, F. (1992). Big endothelin-1-induced sudden death is inhibited by phosphoramidon in mice. *Life Sci.*, **50**, 1631–1638.

MCMAHON, E.G., PALOMO, M.A., MOORE, W.M., MCDONALD, J.F. & STERN, M.K. (1991). Phosphoramidon blocks the pressor activity of porcine big endothelin-1-(1–39) *in vivo* and conversion of big endothelin-1-(1–39) to endothelin-1-(1–21) *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 703–707.

MILLER, W.L., REDFIELD, M.M. & BURNETT, Jr. J.C. (1989). Integrated cardiac, renal and endocrine actions of endothelin. *J. Clin. Invest.*, **83**, 317–320.

OHNAKA, K., TAKAYANAGI, R., YAMAUCHI, T., OKAZAKI, H., OHASHI, M., UMEDA, F. & NAWATA, H. (1990). Identification and characterization of endothelin converting activity in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun.*, **168**, 1128–1136.

OKADA, K., MIYAZAKI, Y., TAKADA, J., MATSUYAMA, K., YAMAKI, T. & YANO, M. (1990). Conversion of big endothelin-1 by membrane-bound metalloendopeptidase in cultured bovine endothelial cells. *Biochem. Biophys. Res. Commun,*. **171**, 1192–1198.

PONS, F., TOUVAY, C., LAGENTE, V., MENCIA-HUERTA, J.M. & BRAQUET, P. (1991).Bronchopulmonary and pressor activities of endothelin-1 (ET-1), ET-2, ET-3 and big ET-1 in guinea pig. *J. Cardiovasc. Pharmacol.*, **17**, Suppl. 7, S326–328.

RUBANYI, G.M. (1992). Potential physiological and pathological significance of endothelins. *Drugs of the Future*, **17**, 915–936.

SHIBOUTA, Y., SUZUKI, N., SHINO, A., MATSUMOTO, H., TERASHITA, Z., KONDO, K. & NISHIKAWA, K. (1990). Pathophysiological role of endothelin in acute renal failure. *Life Sci.*, **46**, 1611–1618.

SIROIS, M.G., FILEP, J.G., ROUSSEAU, A., PLANTE, G.E. & SIROIS, P. (1992). Endothelin-1 enhances vascular permeability in conscious rats: role of thromboxane A_2. *Eur. J. Pharmacol.*, **214**, 119–125.

VALENTIN, J.-P., GARDNER, D.G., WIEDEMANN, E. & HUMPHREYS, M.H. (1991). Modulation of endothelin effects on blood pressure and hematocrit by atrial natriuretic peptide. *Hypertension*, **17**, 864–869.

WATANABE, T., SUZUKI, N., SHIMAMOTO, N., FUJINO, M. & IMADA, A. (1991). Contribution of endogenous endothelin to the extension of myocardial infarct size in rats. *Circ. Res.*, **69**, 370–377.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAY ASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

(Received May 24, 1993
Revised July 12, 1993
Accepted August 2, 1993)

Br. J. Pharmacol. (1993), **110**, 1401–1406 © Macmillan Press Ltd, 1993

Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells

[1]Anwar R. Baydoun, [2]Richard G. Bogle, Jeremy D. Pearson & Giovanni E. Mann

Vascular Biology Research Centre, Biomedical Sciences Division, King's College, Campden Hill Road, London W8 7AH

1 Effects of dexamethasone on induction of nitric oxide (NO) synathase and L-arginine transport by lipopolysaccharide (LPS) were examined in a murine cultured macrophage cell line J774. Metabolism of L-arginine to L-citrulline and subsequent changes in intracellular amino acids pools were correlated with changes in nitrite production.

2 Despite a high intracellular concentration of arginine in activated J774 cells, LPS (1 $\mu g\,ml^{-1}$, 8 h) induced a 2.4 fold increase in arginine transport. Treatment of cells with cycloheximide (1 $\mu g\,ml^{-1}$) inhibited the time-dependent (1–8 h) induction of NO synthase and arginine transport mediated by LPS.

3 Induction of NO synthase by LPS (1 $\mu g\,ml^{-1}$, 24 h) alone was accompanied by a marked increase in arginine utilisation leading to decreased intracellular arginine levels and elevated intracellular and extracellular L-citrulline levels. These changes were further enhanced in the presence of interferon-γ (IFN-γ, 100 units ml^{-1}, 24 h).

4 Dexamethasone (1 μM) abolished the increases in both nitrite and citrulline production induced by LPS alone but only partially reversed the combined effects of LPS and IFN-γ. In contrast, treatment of cells with dexamethasone (10 μM) had no effect on the LPS-mediated induction of arginine transport or the decrease in intracellular arginine concentration.

5 We conclude that induction of arginine transporter activity in LPS-stimulated J774 cells involves *de novo* synthesis of carrier proteins, which increases transport of exogenous arginine during enhanced NO production. Moreover, the intracellular signalling pathways mediating induction of arginine transport and of NO synthase by LPS in activated macrophages diverge, since only the latter is sensitive to dexamethasone.

Keywords: Macrophage; nitric oxide; L-arginine transport; bacterial lipopolysaccharide; dexamethasone; interferon-γ; cycloheximide; intracellular amino acid concentrations; L-arginine; L-citrulline; L-ornithine

Introduction

Metabolism of arginine to nitric oxide (NO) in macrophages is mediated by a Ca^{2+}/calmodulin-insensitive NO synthase enzyme, which is induced following activation of these cells with bacterial endotoxin and/or cytokines (Hibbs *et al.*, 1987; 1988; Iyengar *et al.*, 1987; Stuehr & Marletta, 1987; Marletta *et al.*, 1988). Expression of this enzyme is time-dependent, known to involve *de novo* protein synthesis and can be inhibited by glucocorticoids such as dexamethasone (Di Rosa *et al.*, 1990; Assreuy & Moncada, 1992). The sustained production of NO is critically dependent on the presence of extracellular arginine (Drapier & Hibbs, 1988; Granger *et al.*, 1990; Takema *et al.*, 1991; Bogle *et al.*, 1992a; Assreuy & Moncada, 1992).

We and another group have recently shown that activation of either a murine macrophage cell line J774 or murine peritoneal macrophages with LPS results in a marked increase in arginine uptake (Bogle *et al.*, 1992a; Sato *et al.*, 1992). In both studies uptake was mediated by a carrier resembling the cationic amino acid transporter system y^+ (White, 1985). We found that the V_{max} for arginine uptake doubled when J774 cells were activated, with no significant alteration in K_t (0.14 mM), suggesting that enhanced uptake of arginine reflects increased expression of carrier protein (Bogle *et al.*, 1992a). Sato *et al.* (1992), did not perform comparative kinetic studies, but stated that increased arginine uptake could be blocked by treatment with cycloheximide. We also demonstrated that inhibition of arginine uptake in activated J774 cells by ornithine or lysine (alternative system y^+ substrates) resulted in a concentration-dependent inhibition of NO production (Bogle *et al.*, 1992a). Activation of macrophages by LPS seems to involve induction of the system y^+ transporter, and enhanced arginine transport appears to be essential for these cells to synthesize NO, despite several reports that activated macrophages apparently contain adequate intracellular levels of arginine (Hecker *et al.*, 1990; Bogle *et al.*, 1992b; Assreuy & Moncada, 1992).

In the present study we have therefore determined whether enhanced arginine transport in J774 cells is dependent on new protein synthesis and investigated whether similar signal transduction pathways regulate induction of arginine transport and NO synthase. We have also examined in more detail alterations in intracellular amino acid pool sizes in activated J774 cells, to gain further information about the relative roles of intracellular and extracellular arginine in NO production.

Methods

Cell culture

The murine monocyte/macrophage cell line, J774, was obtained from the European Collection of Animal Cell Cultures (E.C.A.C.C., Wiltshire). J774 cells were maintained in continuous culture in T75 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 0.4 mM L-arginine and supplemented with 4 mM glutamine, penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\,ml^{-1}$) and 10% foetal calf serum. Cells were harvested by gentle scraping and passaged every 3–6 days by dilution of a suspension of the cells 1:10 in fresh medium.

[1] Author for correspondence.

[2] Present address: Department of Pharmacology & Clinical Pharmacology, St. George's Hospital Medical School, London SW17 0RE.

Measurement of L-arginine transport

J774 cells were plated at a density of 10^5 cells per well in 96-well microtiter plates and allowed to adhere for 2 h. Thereafter medium was replaced with either fresh DMEM or DMEM containing lipopolysaccharide (LPS, 1 μg ml^{-1}) alone or with LPS plus either cycloheximide (1 μM) or dexamethasone (10 μM) for specified time periods (1–8 h). After each incubation, cells were rinsed twice with a modified HEPES-buffered Krebs solution of the following composition (mM): NaCl 131, KCl 5.5, $MgCl_2$ 1, $CaCl_2$ 2.5, $NaHCO_3$ 25, NaH_2PO_4 1, D-glucose 5.5, HEPES 20, pH 7.4, 37°C.

L-[^{3}H]-arginine (1 μCi ml^{-1}, 0.25 mM) was added to the monolayers and transport measured over the next 5 min, since in our previous study uptake of L-arginine was shown to be linear for 10 min (Bogle *et al.*, 1992a). Incubations were terminated by placing the plates on melting ice and rinsing cells three times with 200 μl ice-cold Dulbecco's phosphate buffered saline (pH 7.4) containing 10 mM unlabelled L-arginine. In some experiments an extracellular reference tracer D-[^{14}C]-mannitol was included in the incubation medium. The efficiency of the washing procedure was confirmed by a recovery in the cell lysate of $<0.01\%$ of D-[^{14}C]-mannitol applied. Cell protein was determined with the BioRad reagent and cell number was estimated in a haemocytometer. Radioactivity in formic acid digests of the cells was determined by liquid scintillation counting. Uptake was expressed in units of nmol/10^6 cells h^{-1}.

Measurement of nitrite production as an assay of NO release

NO production by J774 cells was assayed by measuring the accumulation of nitrite in the culture medium using the Griess reaction (Green *et al.*, 1982). J774 cells were seeded into 96-well microtiter plates (10^5 cells/well) and exposed to LPS (1 μg ml^{-1}) or LPS and IFN-γ (100 units ml^{-1}) for 1–24 h. In some experiments cycloheximide (1 μM) or dexamethasone (1 μM) was added to the culture medium. After a specified incubation time, an aliquot (100 μl) of the medium was removed, mixed with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthyl-ethylenediamine dihydrochloride/2.5% H_3PO_4) and then incubated at room temperature for 10 min. Absorbance was read at 560 nm using a Multiskan II plate reader (Titertek). Nitrite concentration in the medium was determined with sodium nitrite used as a standard.

Analysis of intracellular amino acid concentrations in J774 cells

J774 cells were seeded into 24 well plates (10^6 cells/well) and allowed to adhere for 2 h. Thereafter the medium was replaced with either fresh medium or medium containing LPS and/or other compounds. Following a 24 h incubation period, the medium was collected, centrifuged at 10,000 *g* for 5 min and stored at − 70°C for subsequent analysis by high performance liquid chromatography (h.p.l.c.). Cells were rinsed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), lysed with 0.5 ml methanol and cell lysates stored at − 70°C for h.p.l.c. analysis. The following were added to each standard or deproteinised sample (20 μl): 80 μl borate buffer (0.2 M, pH 9), 20 μl internal standard (0.1 mM homoserine in distilled water), 20 μl iodoacetic acid reagent (0.74 g iodoacetic acid, 0.62 g boric acid in 100 ml water; pH 9.5), and 20 μl *o*-phthaldialdehyde reagent (80 mg dissolved in 2 ml ethanol and 0.2 ml 2-mercaptoethanol in 100 ml distilled water). The tube was vortex-mixed and 1 min later a 50 μl aliquot of this mixture was injected into a Hypersil Ultratechsphere ODS-5 μm h.p.l.c. column (Jones Chromatography, Mid-Glamorgan) attached to a Beckman 112 h.p.l.c. system (Beckman RIIC Ltd., Bucks.).

Amino acids were separated by a two-solvent gradient system delivered to the column at a flow rate of 2 ml min^{-1} (Baydoun *et al.*, 1990). Upon elution amino acid derivatives were detected by use of a Gilson Spectra-Glo Fluorescence detector (Gilson Medical Laboratories Inc., Wisconsin, U.S.A.) at an excitation wavelength of 388 nm and emission cut off filter of 425 nm. Amino acid concentrations were calculated from peak areas with reference to the internal standard homoserine and the area of an amino acid in a standard mixture containing 0.1 mM concentrations of each amino acid. Peak areas were integrated using a Shimadzu CR1B integrator (Shimadzu Corporation, Tokyo, Japan). Intracellular amino acid concentrations are expressed in mmol l^{-1}, where the volume is the intracellular water space (1 ± 0.1 pl, $n = 4$) measured as described by Kletzien *et al.* (1975).

Measurement of L-leucine incorporation into protein

Protein synthesis was estimated by monitoring incorporation of L-[^{3}H]-leucine into J774 cell protein. Cells monolayers were incubated with L-[^{3}H]-leucine (5 μCi ml^{-1}) for 8 h, rinsed twice with phosphate buffered saline, and then 200 μl trichloroacetic acid (5%) were added to precipitate cellular protein. Pellets were washed with methanol and formic acid digests were transferred to scintillation vials for liquid scintillation counting.

Materials

All reagents for cell culture, except foetal calf serum (Globe Laboratories Ltd), were from Gibco. LPS extracted from

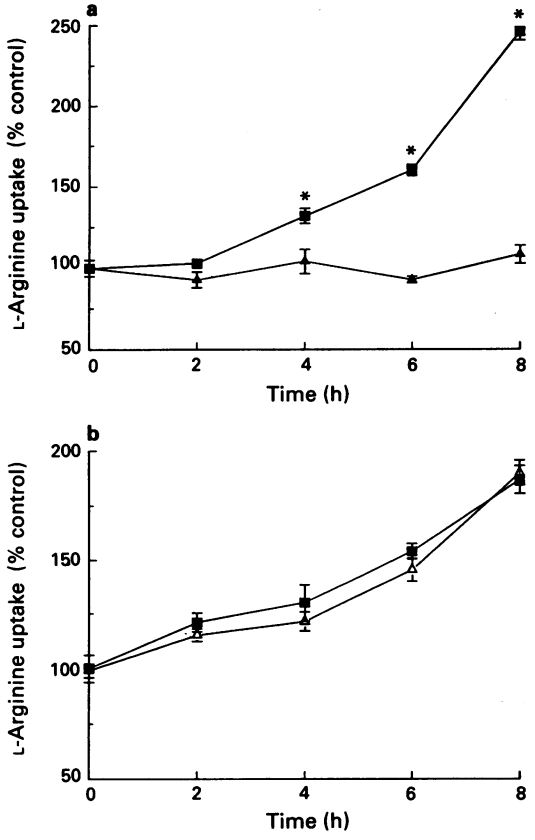


Figure 1 Effects of cycloheximide or dexamethasone on arginine uptake in J774 cells activated with lipopolysaccharide (LPS). Cells were stimulated with LPS (1 μg ml^{-1}) for increasing time periods (0–8 h) (a) in the absence (■) or presence of cycloheximide (▲, 1 μM) and (b) in the absence (■) or presence of dexamethasone (△, 10 μM). Uptake of L-[^{3}H]-arginine (0.25 mM) was then measured over a 5 min interval during incubation of cells with a HEPES-buffered amino acid-free Krebs solution (see Methods). Values denote the mean ± s.e. of replicate measurements in 3 separate experiments. *$P < 0.05$, Student's unpaired *t* test.

Escherichia coli (serotype 055:B5) was obtained from Difco, Michigan, U.S.A. IFN-γ (specific activity 100,000 units ml^{-1}; Holland Biotechnologies) was a gift from Wellcome Research Laboratories, Beckenham. Dexamethasone (Sigma, U.K.) was dissolved in 100% ethanol and diluted for use in culture medium at a final ethanol concentration of 0.01%. Other chemicals were from Sigma or BDH and of the highest grade obtainable. The radioactive tracers L-[2,3-^{3}H]-arginine (53 Ci $mmol^{-1}$), D-[1-^{14}C]-mannitol (50 mCi $mmol^{-1}$) and L-[3,4,5-^{3}H]-leucine (147 Ci $mmol^{-1}$) were obtained from NEN, Dreieich, Germany.

Statistics

All values are means ± s.e. of n experiments. Statistical analysis was performed using Student's t test with $P<0.05$ considered statistically significant.

Results

Effects of cycloheximide or dexamethasone on arginine transport and NO synthase in LPS-activated J774 cells

J774 cells were incubated with LPS (1 μg ml^{-1}) in the presence or absence of either cycloheximide (1 μM) or dexamethasone (10 μM) for up to 8 h and transport of arginine was then measured over a 5 min interval. Incubation of cells with LPS for 8 h increased transport from a basal value of 10.3 ± 1.8 to 24.3 ± 2.1 nmol per 10^6 cell h^{-1} ($n = 3$) and nitrite production to a maximum of 11.2 ± 1.4 nmol per 10^6 cells 8 h^{-1} ($n = 3$). Treatment with cycloheximide (1 μM, 8 h), a concentration which reduced the incorporation of L-[^{3}H]-leucine into cell protein by 85 ± 2% ($n = 10$), prevented induction of NO synthase, and abolished the time-dependent increase in arginine uptake without reducing uptake below control levels (Figure 1a). Exposure of J774 cells to cycloheximide for longer than 8 h caused cell loss from the culture wells.

As shown in Figure 1b, induction of arginine transport by LPS (1 μg ml^{-1}) was unaffected by co-incubation of J774 cells with a maximum concentration of dexamethasone (10 μM). In contrast, dexamethasone treatment resulted in substantial inhibition of NO synthase, reducing nitrite production from 11.2 ± 1.4 to 2.0 ± 0.3 nmol per 10^6 cells 8 h^{-1} ($n = 3$), confirming previous findings in cultured J774 cells (Di Rosa *et al.*, 1990; Assreuy & Moncada, 1992).

Amino acid concentrations in activated J774 cells

Chromatographic analyses of cell lysates obtained from untreated J774 cells revealed a high concentration of arginine with only trace amounts of citrulline (Table 1). Following activation of cells with LPS (1 μg ml^{-1}, 24 h), intracellular citrulline levels increased 1.5 fold. It should be noted that this increase dramatically underestimates the total production of citrulline, since there was a 5 fold accumulation of this amino acid in the extracellular medium (Table 2). Stimulation of cells with LPS (1 μg ml^{-1}) and IFN-γ (100 units ml^{-1}) resulted in greater increases in intracellular and extracellular concentrations of citrulline. Treatment with IFN-γ alone had no significant effect on intracellular citrulline (Table 1) and did not induce nitrite production (data not shown).

Although dexamethasone (1 μM, 24 h) completely inhibited the elevation of intracellular citrulline induced by LPS, it inhibited by only one third the production of nitrite and citrulline induced by a combination of LPS and IFN-γ (Figure 2). Increases in citrulline and nitrite accumulation following activation (Figure 2) were accompanied by parallel decreases in intracellular arginine levels (Table 1). LPS (1 μg ml^{-1}) alone reduced cellular arginine concentrations by 26%, whilst addition of IFN-γ (100 units ml^{-1}) and LPS reduced the intracellular pool by 57%, and also decreased medium concentrations of arginine by 56% (Table 2). These

Table 1 Effects of lipopolysaccharide (LPS), interferon-γ (IFN-γ) and dexamethasone on intracellular arginine, citrulline and ornithine concentrations in J774 cells

Condition	*Intracellular concentration* (mM) *L-Arginine*	*L-Citrulline*	*L-Ornithine*
Control	3.50 ± 0.30	0.11 ± 0.01	0.25 ± 0.05
LPS (1 μg ml^{-1})	2.61 ± 0.06*	0.17 ± 0.02*	0.31 ± 0.05
IFN-γ (100 units ml^{-1})	2.42 ± 0.36	0.08 ± 0.01	0.18 ± 0.01
LPS + IFN-γ (100 units ml^{-1})	1.50 ± 0.18*	0.35 ± 0.03*	0.58 ± 0.16
LPS + dexamethasone (1 μM)	2.69 ± 0.16*	0.10 ± 0.02	0.65 ± 0.18
LPS + IFN-γ (100 units ml^{-1}) + dexamethasone (1 μM)	1.77 ± 0.18*	0.23 ± 0.02*	0.51 ± 0.12

J774 cells were cultured to confluence in 24-well plates (10^6 cells/well) and treated for 24 h with combinations of LPS, IFN-γ and dexamethasone. Amino acids in lysates from control and treated cells were measured by reversed phase h.p.l.c. and the free amino acid concentrations expressed relative to an intracellular water space of 1 pl/cell. Values denote the mean ± s.e. of 4 separate experiments. *$P<0.05$, Student's unpaired t test.

Table 2 Effects of lipopolysaccharide (LPS), interferon-γ (IFN-γ) and dexamethasone on arginine, citrulline and ornithine concentrations in culture medium

Condition	*Culture medium concentration* (mM) *L-Arginine*	*L-Citrulline*	*L-Ornithine*
Control	0.40 ± 0.10	0.02 ± 0.005	0.05 ± 0.01
LPS (1 μg ml^{-1})	0.38 ± 0.06	0.10 ± 0.01*	0.08 ± 0.02
IFN-γ (100 units ml^{-1})	0.43 ± 0.05	0.06 ± 0.005	0.07 ± 0.01
LPS + IFN-γ (100 units ml^{-1})	0.17 ± 0.01*	0.32 ± 0.03*	0.06 ± 0.01
LPS + dexamethasone (1 μM)	0.33 ± 0.04	0.04 ± 0.005	0.05 ± 0.01
LPS + IFN-γ (100 units ml^{-1}) + dexamethasone (1 μM)	0.23 ± 0.04*	0.21 ± 0.01*	0.08 ± 0.02

Details as in legend to Table 1. Culture medium (DMEM containing 10% foetal calf serum) from control and treated cells were deproteinised and free amino acid concentrations were determined by reversed phase h.p.l.c. Values are the mean ± s.e. of measurements in 4 experiments. *$P<0.05$, Student's unpaired t test.

changes in arginine concentration were unaffected by dexamethasone, despite inhibition of nitrite and citrulline production, demonstrating that arginine is utilised by other pathways activated by LPS/IFN-γ. The fact that intracellular ornithine levels tended to increase in the presence of LPS/IFN-γ suggests increased metabolism via arginase. Furthermore, dexamethasone had no significant effect on the production of ornithine in LPS/IFN-γ activated cells (Table 1).

It is worth noting that treatment of J774 cells with either LPS (1 μg ml^{-1}) or IFN-γ (100 units ml^{-1}) alone for 24 h reduced the total intracellular amino acid pool by 33% or 44%, respectively (Table 3). These changes in intracellular amino acid concentrations were enhanced further when cells were incubated with LPS and IFN-γ for 24 h (Table 3). Dexamethasone had no significant effects on LPS/IFN-γ induced changes in the intracellular amino acid pool or medium concentrations.

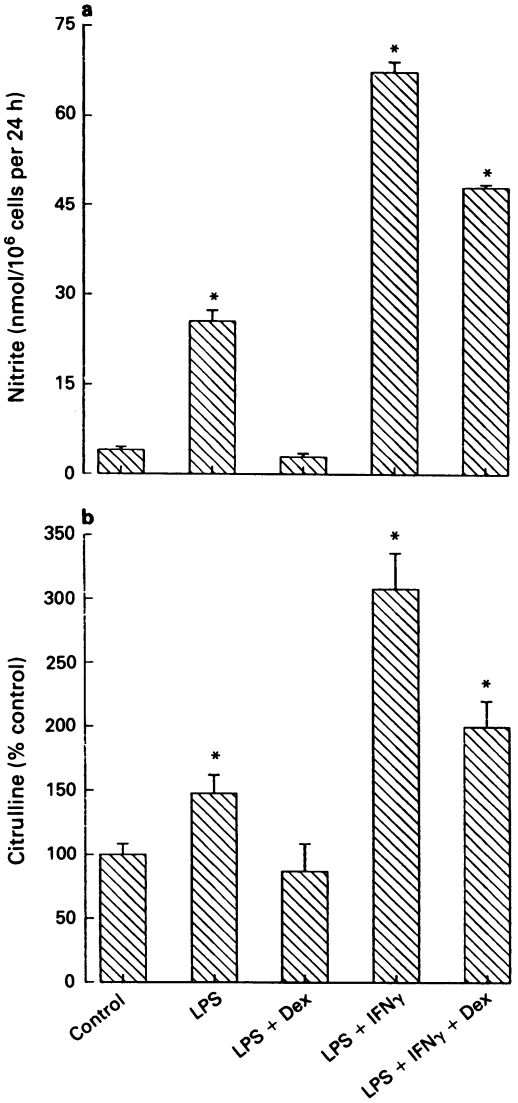


Figure 2 Effects of dexamethasone on nitrite and citrulline production in J774 cells activated with lipopolysaccharide (LPS) or LPS plus interferon-γ (IFN-γ). Cells were incubated with combinations of LPS (1 μg ml^{-1}), IFN-γ (100 units ml^{-1}) and dexamethasone (1 μM) for 24 h. Nitrite accumulation in the medium (a) was determined using the Griess reaction and intracellular citrulline concentrations by reversed-phase h.p.l.c. (b). Values denote the mean ± s.e. of measurements in 4 separate experiments. *$P<0.05$ Student's unpaired t test.

Discussion

The present results demonstrate that LPS induces arginine transport activity, in addition to NO synthase, in the murine macrophage cell line J774. More importantly, our findings show that although induction of both these processes depends on *de novo* protein synthesis, each is regulated differentially by glucocorticoids. Activation of cells by LPS in the presence of dexamethasone resulted in a complete inhibition of nitrite and citrulline production, whereas induction of arginine transport and changes in intracellular arginine concentrations were unaffected. Moreover, addition of IFN-γ further increased LPS-stimulated nitrite production (Figure 2), but has no additional effect on arginine transport (Bogle *et al.*, 1992a).

Uptake of arginine in macrophages occurs via a carrier-mediated system which has been identified as y^+ (Bogle *et al.*, 1992a; Sato *et al.*, 1992). System y^+ operates as a facilitated diffusion carrier which accumulates arginine against a substantial concentration gradient (~10 fold in J774 cells, see Tables 1 and 2). It is driven by the membrane potential (Bussolati *et al.*, 1987) and in macrophages, as in other cell types, is sensitive to *trans*-stimulation (White, 1985; Sato *et al.*, 1991). These two mechanisms, in addition to synthesis of new carrier protein, could in principle contribute to the enhanced rates of arginine uptake in activated cells. As LPS either has no effect on or depolarizes the membrane potential (Larsen *et al.*, 1985; Henry-Toulme *et al.*, 1990) and amino acid levels are decreased in activated J774 cells (Tables 1 and 3), these effects would reduce rather than enhance uptake of arginine in LPS-stimulated macrophages.

Induction of NO synthase in macrophages is strongly inhibited by glucocorticoids such as dexamethasone and hydrocortisone (Di Rosa *et al.*, 1990), probably acting at the level of transcription of mRNA. We confirmed that dexamethasone substantially inhibited nitrite and citrulline production in LPS-activated J774 cells (Figure 2). Interestingly, the synergistic actions of LPS/IFN-γ on nitrite and citrulline production were only partially inhibited by dexamethasone. It has recently been shown that LPS/IFN-γ act synergistically at the level of transcription of NO synthase mRNA (Lors-

Table 3 Effects of lipopolysaccharide (LPS), interferon-γ (IFN-γ) and dexamethasone on total amino acid concentrations in J774 cells and culture medium

Condition	*Total amino acid concentration* (mM) *J774 cells*	*Medium*	*Cell/Medium*
Control	86.4 ± 9.9	12.7 ± 0.3	6.8
LPS (1 μg ml^{-1})	58.2 ± 4.0*	15.7 ± 1.4	3.7
IFN-γ (100 units ml^{-1})	48.7 ± 6.0*	16.4 ± 1.3	3.0
LPS + IFN-γ (100 units ml^{-1})	32.5 ± 2.6*	16.4 ± 0.8	2.0
LPS + dexamethasone (1 μM)	60.3 ± 2.6*	13.2 ± 1.3	4.6
LPS + IFN-γ (100 units ml^{-1}) + dexamethasone (1 μM)	28.1 ± 2.7*	14.7 ± 1.7	1.9

Details as in legends to Tables 1 and 2. Cell to medium ratios were estimated from the mean amino acid concentrations. All other values denote the mean ± s.e. of measurements in 4 experiments. *$P<0.05$, Student's unpaired t test.

bach *et al.*, 1993). Our result therefore indicates that transcriptional modulation by dexamethasone may be less effective when IFN-γ-induced promoters are present.

Induction of arginine transport by LPS was unaffected by treatment of J774 cells with a concentration of dexamethasone that abolished nitrite production. This observation suggests that the system y^+ gene is not sensitive to glucocorticoids. Dexamethasone and other natural steroids have been reported to enhance hormone-stimulated transport of neutral amino acids (see review, Guidotti *et al.*, 1978), but we are unaware of any studies reporting the effects of dexamethasone on system y^+. Our results also show that production of NO does not serve as an autocrine loop to induce arginine transport. We have previously shown that arginine uptake by endothelial cells is rapidly and transiently upregulated during agonist-stimulated release of NO via constitutive NO synthase; however, this was also predominantly independent of NO production (Bogle *et al.*, 1991).

There is controversy as to whether citrulline generated by NO synthase in activated macrophages can be recycled to arginine, as reported intially in endothelial cells (Hecker *et al.*, 1990). Although LPS-activated rat peritoneal macrophages apparently recycle citrulline to arginine (Wu & Brosnan, 1992), studies with murine peritoneal macrophages suggest that citrulline only accumulates in the extracellular medium (Benninghoff *et al.*, 1991). Our current experiments have shown that citrulline accumulates both intracellularly and in the culture medium of activated J774 cells, and in preliminary experiments we have found that J774 cells transport and metabolise citrulline to arginine which can then be used for NO synthesis (Bogle *et al.*, 1992b).

In activated J774 cells, transported arginine may be metabolised via pathways other than inducible NO synthase. In activated murine macrophages only 30% of cellular L-arginine metabolism occurs via NO synthase (Granger *et al.*, 1990), though there is disagreement as to whether the flux of arginine to ornithine via arginase is concomitantly enhanced in activated macrophages (Benninghoff *et al.*, 1991; Sato *et al.*, 1992). We found increases in intracellular ornithine in J774 cells generating nitrite (Table 1) with negligible changes in extracellular ornithine (Table 2). Studies with selective inhibitors of ornithine decarboxylase, the main metabolic route for ornithine metabolism leading to polyamine synthesis, could be designed to test the importance of this pathway in the induction of system y^+. However, we believe it is more likely that induction of arginine transport by LPS is regulated independently of the intracellular metabolism of arginine. Investigation of the regulatory sequence of the system y^+ gene, recently identified (Kim *et al.*, 1991; Wang *et al.*, 1991), will be needed to substantiate this hypothesis.

Finally, it should be noted that the K_m of arginine for NO synthase ($<10\,\mu M$) is much lower than for arginase (6.8–18 mM; Kaysen & Strecker, 1973), and thus alterations in the flux of arginine via arginase should have minimal effects on the flux through NO synthase. In addition, we wish to emphasize that despite a fall of about 50% in the intracellular arginine pool in LPS/IFN-γ-activated J774 cells synthesizing NO, this pool size is theoretically well in excess of the amount required for maximal rates of NO production. Nonetheless, the rate of NO synthesis in these cells is directly related to the rate of entry of exogenous arginine (Bogle *et al.*, 1992a; Assreury & Moncada, 1992). We conclude that extracellular arginine is preferentially used to generate NO in activated macrophages, and that the intracellular pathways mediating induction of transport and NO synthase are regulated differentially by dexamethasone.

A.R.B. and G.E.M. gratefully acknowledge support from the British Heart Foundation (1890453). R.G.B. was the recipient of a SERC/CASE Award with Wellcome Research Laboratories, Beckenham, U.K. We thank Dr P.W. Emery for use of his h.p.l.c. apparatus and gratefully acknowledge Dr Salvador Moncada and Dr David Morgan for their helpful discussion of this work.

References

ASSREUY, J. & MONCADA, S. (1992). A perfusion system for the long term study of macrophage activation. *Br. J. Pharmacol.*, **107**, 317–321.

BAYDOUN, A.R., EMERY, P.W., PEARSON, J.D. & MANN, G.E. (1990). Substrate dependent regulation of intracellular amino acid concentrations in cultured bovine aortic endothelial cells. *Biochem. Biophys. Res. Commun.*, **173**, 940–948.

BENNINGHOFF, B., LEHMANN, V., ECK, H.-P. & DRÖGE, W. (1991). Production of citrulline and ornithine by interferon-γ treated macrophages. *Int. Immunol.*, **3**, 413–417.

BOGLE, R.G., BAYDOUN, A.R., MONCADA, S., PEARSON, J.D. & MANN, G.E. (1992a). L-arginine transport is increased in macrophages generating nitric oxide. *Biochem. J.*, **284**, 15–18.

BOGLE, R.G., BAYDOUN, A.R., PEARSON, J.D. & MANN, G.E. (1992b). Citrulline can restore the ability of activated macrophages to generate nitric oxide in the absence of extracellular arginine. *Br. J. Pharmacol.*, **107**, 286P.

BOGLE, R.G., COADE, S.B., MONCADA, S., PEARSON, J.D. & MANN, G.E. (1991). Bradykinin and ATP stimulate L-arginine uptake and nitric oxide release in vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **180**, 926–932.

BUSSOLATI, O., LARIS, P.C., NUCCI, F.A., DALL'ASTRA, V., LONGO, N., GUIDOTTI, G.G. & GAZZOLA, C.C. (1987). Dependence of L-arginine accumulation on membrane potential in cultured human fibroblasts. *Am. J. Physiol.*, **253**, C391–C397.

DI ROSA, M., RADOMSKI, M., CARNUCCIO, R. & MONCADA, S. (1990). Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. *Biochem. Biophys. Res. Commun.*, **172**, 1246–1252.

DRAPIER, J.-C. & HIBBS, J.B. Jr. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells in L-arginine dependent inhibition of mitochondrial iron-sulphur enzymes in the macrophage effector cells. *J. Immunol.*, **140**, 2829–2838.

GRANGER, D.L., HIBBS, J.B. Jr., PERFECT, J.R. & DURACK, D.T. (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J. Clin. Invest.*, **85**, 264–273.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.

GUIDOTTI, G.G., BORGHETTI, A.F. & GAZZOLA, G.C. (1978). The regulation of amino acid transport in animal cells. *Biochem. Biophys. Acta.*, **515**, 329–366.

HECKER, M., SESSA, W.C., HARRIS, H.J., ANGGARD, E.E. & VANE, J.R. (1990). The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 8612–8616.

HENRY-TOULME, N., SARTHOU, P. & BOLARD, J. (1990). Early membrane potential and cytoplasmic calcium changes during mitogenic stimulation of WEHI 231 cell line by polyene antibiotics, lipopolysaccharide and anti-immunoglobulin. *Biochim. Biophys. Acta*, **1051**, 285–292.

HIBBS, J.B. Jr., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.

HIBBS, J.R. Jr., VAVRIN, Z. & TAINTOR, R.R. (1987). L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.*, **138**, 550–565.

IYENGAR, R., STUEHR, D.J. & MARLETT, M.A. (1987). Macrophage synthesis of nitrite, nitrate and N-nitrosamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 6369–6373.

KAYSEN, G.A. & STRECKER, H.J. (1973). Purification and properties of arginase of rat kidney. *Biochem. J.*, **133**, 779–788.

KIM, J.W., CLOSS, E.I., ALBRITTON, L.M. & CUNNINGHAM, J.M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature*, **352**, 725–728.

KLETZIEN, R.F., PARIZA, M.W., BECKER, J.E. & POTTER, V.R. (1975). A method using 3-O-methyl-D-glucose and phloretin for the determination of intracellular water space of cells in monolayer culture. *Anal. Biochem.*, **68**, 537–544.

LARSEN, N.E., ENELOW, R.I., SIMONS, E.R. & SULLIVAN, R. (1985). Effect of bacterial endotoxin on the transmembrane electrical potential and plasma membrane fluidity of human monocytes. *Biochim. Biophys. Acta*, **815**, 1–8.

LORSBACH, R.B., MURPHY, W.J., LOWENSTEIN, C.J., SNYDER, S.H. & RUSSELL, S.W. (1993). Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. *J. Biol. Chem.*, **268**, 1908–1913.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.

SATO, H., FUJIWARA, M. & BANNAI, S. (1992). Effect of lipopolysaccharide on transport and metabolism of arginine in mouse peritoneal macrophages. *J. Leuk. Biol.*, **52**, 161–164.

SATO, H., ISHII, T., SUGITA, Y. & BANNAI, S. (1991). Induction of cationic amino acid transport activity in mouse peritoneal macrophages by lipopolysaccharide. *Biochim. Biophys. Acta*, **100**, 46–52.

STUEHR, D.J. & MARLETTA, M.A. (1987). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon-γ. *J. Immunol.*, **139**, 518–525.

TAKEMA, M., INABA, K., UNO, K., KAKIHARA, K.-I., TAWARA, K. & MURAMATSU, S. (1991). Effect of L-arginine on the retention of macrophage tumoricidal activity. *J. Immunol.*, **146**, 1928–1933.

WANG, H., KAVANAUGH, M.P., NORTH, R.A. & KABAT, D. (1991). Cell-surface receptor for ecotropic murine retroviruses is a basic amino acid transporter. *Nature*, **352**, 729–731.

WHITE, M.F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochim. Biophys. Acta*, **822**, 355–374.

WU, G. & BROSNAN, J.T. (1992). Macrophages can convert citrulline into arginine. *Biochem. J.*, **281**, 45–48.

(Received July 15, 1993
Revised August 12, 1993
Accepted August 13, 1993)

Br. J. Pharmacol. (1993), **110**, 1407–1412 © Macmillan Press Ltd, 1993

Pharmacological characterization of the metabotropic glutamate receptor inhibiting D-[^{3}H]-aspartate output in rat striatum

Grazia Lombardi, Marina Alesiani, Patrizia Leonardi, Giovanna Cherici, *Roberto Pellicciari & [1]Flavio Moroni

Dipartimento di Farmacologia Preclinica e Clinica 'Mario Aiazzi Mancini', Università di Firenze, Firenze and *Istituto di Chimica Farmaceutica e Tecnica Farmaceutica, Università di Perugia, Perugia, Italy

1 The effects of several agonists of the metabotropic glutamate receptor (mGluR) were studied in adult rat striatal slices by measuring (i) KCl (30 mM)-induced output of previously taken up D-[^{3}H]-aspartate (Asp), (ii) forskolin (30 μM)-induced adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation and (iii) phophoinositide (PI) hydrolysis.

2 K^+-induced efflux of D-[^{3}H]-Asp was inhibited by the following mGluR agonists: (1S,3S,4S)-(carboxycyclopropyl)glycine (L-CCG-I), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) and quisqualic acid (Quis). 2-Amino-4-phosphonobutyrate (L-AP4) was inactive up to 300 μM. The maximal inhibition of D-[^{3}H]-Asp output was 60 ± 8%. The EC_{50}s of mGluR agonists were: 0.5 μM for L-CCG-I, 100 μM for 1S,3R-ACPD and 100 μM for Quis.

3 Forskolin-induced cyclic AMP accumulation was also inhibited by mGluR agonists. The maximal inhibition was 50 ± 4% and was obtained at a concentration of 10 μM for L-CCG-I and 100 μM for 1S,3R-ACPD. The EC_{50}s for this inhibition were: 0.9 μM for L-CCG-I and 20 μM for 1S,3R-ACPD. Quis (300 μM) inhibited cyclic AMP accumulation by approximately 20%. L-AP4 slightly potentiated cyclic AMP accumulation.

4 PI hydrolysis was stimulated by mGluR agonists. The most potent compound was Quis (100 μM), which increased inositol phosphate formation up to 2.2 fold over control values. Its EC_{50} was 15 μM. L-CCG-I and 1S,3R-ACPD increased inositol phosphate formation by approximately 1.8 fold and their EC_{50} values were 30 and 25 μM, respectively. L-AP4 did not affect PI hydrolysis.

5 In conclusion, mGluR agonists that reduce D-[^{3}H]-Asp output have a pharmacological profile similar to that of mGluR agonists inhibiting cyclic AMP accumulation. L-CCG-I appears to be a relatively selective agonist for the mGluR receptor which inhibits D-[^{3}H]-Asp efflux and cyclic AMP accumulation, while Quis appears to act preferentially on the mGluR receptor linked to the metabolism of PIs.

Keywords: Metabotropic glutamate receptors; excitatory amino acid release; (2S,3S,4S)-(carboxycyclopropyl) glycine (L-CCG-I); (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD); quisqualic acid; cyclic AMP; synaptic plasticity, phosphoinositide hydrolysis

Introduction

Molecular cloning has revealed the existence of at least six metabotropic glutamate receptors (mGluRs; mGluR1 to mGluR6) (Houamed *et al.*, 1991; Masu *et al.*, 1991; Tanabe *et al.*, 1992; Nakanishi, 1992). These six subtypes can be classified into three different groups according to their sequence similarities: one group consists of mGluR1 and mGluR5, which are preferentially linked, through G proteins, to phosphoinositide hydrolysis (PI); the second group includes mGluR2 and mGluR3, which are coupled to the inhibition of forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation; the third group includes mGluR4 and mGluR6, is thought to be similarly linked to the inhibition of adenylate cyclase. The pharmacological characterization of these receptors is far from being completed because of the lack of selective agonists and antagonists for each receptor subtype. Experiments using transfected cells appear to be particularly useful for this goal (Aramori & Nakanishi, 1991; Hayashi *et al.*, 1992). To date, most of the literature data on the role of the mGluRs in brain function has been obtained by using (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), the prototype agonist of mGluRs (Aronica *et al.*, 1991; Anwyl, 1991; Baskys & Malenka, 1992; Schoepp & Conn, 1993). Different steroisomers of 1S,3R-ACPD seem to be useful for the classification of mGluRs (Cartmell *et al.*, 1993b).

It has been shown recently that appropriate concentrations of 1S,3R-ACPD, in the presence of arachidonic acid, enhance glutamate exocytosis from cerebrocortical synaptosomes (Herrero *et al.*, 1992). This observation has been considered particularly important for the understanding of the overall function of glutamatergic synapses and the role that mGluRs may play in the mechanisms of synaptic potentiation and neuronal plasticity (Nicholls, 1992). In fact, the importance of mGluR activation for the stimulation of neurotransmitter release and for the induction and maintenance of long term potentiation has been repeatedly stressed (Anwyl, 1991; Bortolotto & Collingridge, 1992; Zheng & Gallagher, 1992). However, using electrophysiological approaches, it has also been shown that 1S,3R-ACPD and quisqualic acid (Quis), a non-selective mGluR agonist, cause a synaptic inhibition that has been ascribed to the decrease of transmitter release from glutamatergic terminals (Lovinger, 1991; Calabresi *et al.*, 1992; Rannie & Shinnick-Gallagher, 1992). These observations were particularly well-defined in the rat striatum, a brain structure enriched in glutamatergic presynaptic terminals which originate primarily in the frontal cortex and are known to modulate the function of several neuronal populations (Wood *et al.*, 1979).

In order to understand the role of mGluRs in brain func-

[1] Author for correspondence at Department of Pharmacology, University of Florence, Viale G.B. Morgagni 65, 50134 Florence, Italy.

tion and to set up simple models for the identification of new mGluR agonists and antagonists, we performed experiments aimed at evaluating how mGluR stimulation could affect excitatory amino acid release from brain slices. We here show that mGluR agonists significantly inhibited the depolarization-induced output of D-[^{3}H]-aspartate (Asp) from rat striatal slices. After evaluating the actions of several proposed mGluR agonists on D-[^{3}H]-Asp efflux, on the inhibition of cyclic AMP accumulation and on PI metabolism, we noticed that the pharmacological properties of the mGluR type controlling transmitter output were different from those of the receptor stimulating PI metabolism but similar to the profile of the mGluR modulating cyclic AMP accumulation. Thus, we propose that glutamate release may be inhibited by presynaptic glutamate receptors modulating cyclic AMP formation in a manner that may be compared to the control of noradrenaline output exerted by α_2-auto-adrenoceptors (Langer, 1980; Starke, 1987).

Methods

Preparation of rat striatal slices

Male Wistar rats (Nossan strain, Milan, Italy), weighing 180–200 g were used. After decapitation, their striata were rapidly removed and placed into ice-cold Krebs-bicarbonate buffer (in mM: NaCl 122, KCl 3.1, $MgSO_4$ 1.2, KH_2PO_4 0.4, $CaCl_2$ 1.3, $NaHCO_3$ 25 and glucose 10). Transverse slices (350 μm thick) were prepared with a McIlwain tissue chopper and then left to stand, dipped into Krebs-bicarbonate solution gassed with 95% O_2/5% CO_2, for 1 h at 37°C in order to allow functional recovery.

Studies on D-[^{3}H]-Asp release

Recovered slices were incubated for 45 min at 37°C in oxygenated Krebs solution containing D-[^{3}H]-Asp (final concentration 50 nM) and subsequently washed for 30 min by changing the incubation medium twice. The labelled slices were transferred (two per chamber) to Perspex chambers (0.3 ml volume) (see Beani *et al.*, 1978; Moroni *et al.*, 1981) and superfused with gassed (95% O_2/5% CO_2) Krebs solution at 37°C. Preliminary experiments showed that after 30 min of superfusion, a quasi steady state efflux of tritium was reached. Different mGluR agonists were added 5 min before slices were challenged for 5 min with a 30 mM KCl-containing buffer (with isomolar reduction of NaCl) (see Figure 1 for experimental details). The superfusion solutions were directly collected in counting vials. Instagel was added and the radioactivity was counted in a Packard (Tri Carb 1500) liquid scintillation analyzer. Tritium output was calculated as the percentage of total radioactivity (c.p.m. tritium in the perfusate/c.p.m. tritium in the slices at the beginning of the respective 5 min superfusion period).

Measurements of cyclic AMP formation

Striatal slices (two per tube) were placed for 30 min in 500 μl of oxygenated (95% O_2/5% CO_2) Krebs solution at 37°C. After this time, 5 μl of either forskolin (final concentration, 30 μM) or vehicle, 3-isobutyl-1-methylxanthine (IBMX, final concentration 1 mM), and 5 μl of a solution containing mGluR agonists were added. Tubes were then placed in a shaking water bath for 15 min. The incubation was terminated by adding 0.75 ml of ice-cold 12 mM disodium EDTA solution to each tube. The samples were immediately homogenized in a Tetronix Tissuemixer and then boiled for 10 min. After centrifugation the supernatants were frozen at − 80°C until cyclic AMP was measured. Cyclic AMP levels were determined with a cyclic AMP radioimmunoassay kit.

Studies on PI hydrolysis

Slices prepared as previously described were incubated for 2 h with [^{3}H]-inositol (20 μCi ml^{-1}). They were then washed in 50 ml of freshly oxygenated buffer and transferred to test-tubes (two slices each) with 500 μl of drug-containing medium and gently stirred in the presence of 10 mM LiCl by bubbling 95% O_2/5% CO_2. After 15 min at 37°C, the reaction was stopped by the addition of 1.88 ml of chloroform/methanol (1:2). The phases were separated by adding 0.65 ml of chloroform and 0.65 ml of water and, after brief sonication, by centrifuging the tubes at 800 *g* for 10 min. The upper phase (2 ml), which contained the water-soluble [^{3}H]-inositol phosphates (inositol monophosphate, IP; inositol 1,4-bisphosphate, IP_2 inositol 1,4,5-trisphosphate, IP_3) was transferred to test-tubes and water (3 ml) was added. The inositol phosphates were then separated on Dowex AG 1-X 8 anion exchange resins (formate form, 100–200 mesh) according to Berridge & Irvine (1984), as previously described (Pellegrini-Giampietro *et al.*, 1988). The radioactivity in portions (8 ml) of these fractions was determined by scintillation counting. Calculations were performed on the sum of IP, IP_2 and IP_3 (d.p.m. mg^{-1} protein).

Protein assay

The protein content of the samples was measured according to Lowry *et al.* (1951).

Materials

(1S,3S,4S)-(Carboxycyclopropyl)glycine (L-CCG-I) was synthesized as described by Pellicciari *et al.* (1986, 1988, 1991). In those papers the active compounds were named cyclopropylglutamate stereoisomers (CGA), to stress the analogy with glutamate; (1S,3S,4S)-CGA corresponded to L-CGA B and is here named L-CCG-I. The nomenclature used by Shinozaki *et al.* (1989) and by Tocris Neuramin (Bristol, U.K.) has now been introduced in order to ensure unambiguous assignment of the molecule. 1S,3R-ACPD, Quis and 2-amino-4-phosphonobutyrate (L-AP4) were purchased from Tocris Neuramin (Bristol, U.K.). D-[^{3}H]-Asp (10–30 Ci mmol^{-1}) and [^{3}H]-cyclic AMP radioimmunoassay kit (25 Ci mmol^{-1}) were from Amersham (Amity PG, Milan, Italy). *myo* 2-[^{3}H]-N-inositol (10–20 Ci mmol^{-1}) was from New England Nuclear (Du Pont de Nemours, Milan, Italy). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), and Dowex AG-1-X 8 anion exchange resin (100–200 mesh) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The scintillation fluid (Instagel) was from Packard (Groningen, The Netherlands). All other reagents were of analytical grade and obtained from Merck (Darmstadt, Germany).

Statistical analysis

Statistical significance in all experiments was evaluated by performing the analysis of variance. The EC_{50}s were defined as the concentration which caused 50% of the maximal effects. Preliminary experiments showed that the maximal effects obtainable were: inhibition of 50% of cyclic AMP formation; inhibition of 60% of D-[^{3}H]-Asp release; accumulation of inositol phosphates of 2.2 times over controls for Quis and 1.8 times for L-CCG-I and 1S,3R-ACPD (see Figures 3, 4 and 5).

Results

Effects of mGluR agonists on K$^+$-induced D-[^{3}H]-Asp striatal output

In striatal slices prelabelled with D-[^{3}H]-Asp and superfused for a 50 min period, application of buffer containing 30 mM

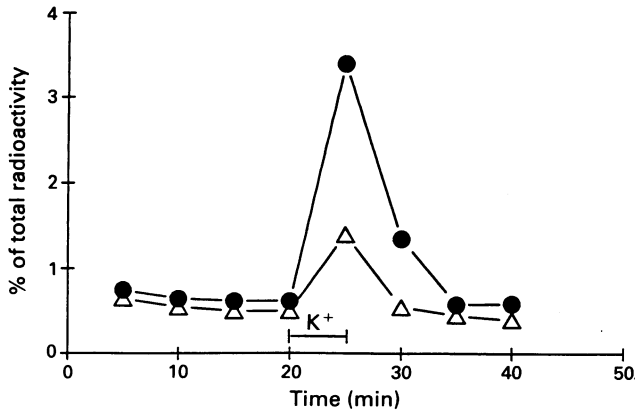


Figure 1 Striatal slices were incubated with D-[^{3}H]-aspartate (D-[^{3}H]-Asp) for 45 min at 37°C and subsequently washed twice prior to being placed in the superfusion chambers. They were then superfused for 30 min before starting the collection of the samples. After the collection of 3 samples the slices were challenged for 5 min with a 30 mM KCl-containing solution (with isomolar reduction of NaCl). The slices contained $150 \pm 20 \times 10^3$ c.p.m. mg^{-1} protein at the beginning of the collection of superfusates. Each point is the percentage of total radioactivity (radioactivity present in the perfusate/radioactivity present in the slices at the beginning of the respective 5 min superfusion period). At least 5 duplicate experiments were performed; s.e.mean were within 15% of the reported values. (●) Normal Krebs; (△) Ca^{2+}-free Krebs.

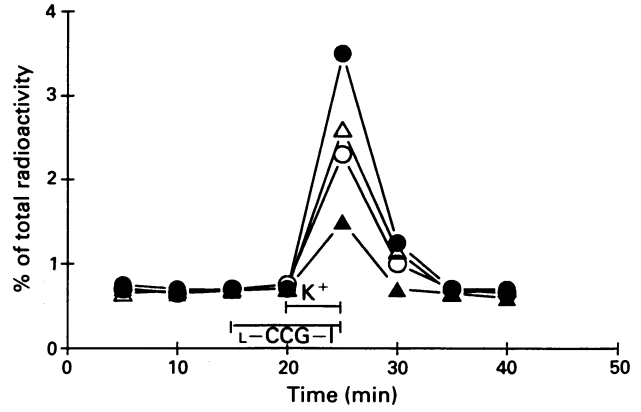


Figure 2 Dose-dependent effects of (1S,3S,4S)-(carboxycyclopropyl) glycine (L-CGG-I) on K^+-induced D-[^{3}H]-aspartate (D-[^{3}H]-Asp) output. Different concentrations of L-CCG-I were added to the superfusion fluid 5 min before KCl (30 mM). Each point represents the mean obtained in at least 5 different experiments conducted in duplicate (see Figure 1 for the experimental protocol). (●) Controls; (△) L-CCG-I 0.1 μM; (○) L-CCG-I 0.3 μM; (▲) L-CCG-I 1 μM.

KCl increased D-[^{3}H]-Asp output by 3.5 ± 0.7 times. This output was reduced by $50 \pm 7\%$ when $CaCl_2$ was omitted from the solution and EDTA (1 mM) was added (Figure 1). When L-CCG-I was present in the superfusion solution 5 min before and during the K^+-induced depolarization, the output of radioactivity was reduced in a dose-dependent manner. The maximum effect ($60 \pm 8\%$ over control values) was obtained at 1 μM L-CCG-I (Figure 2). 1S,3R-ACPD (30–300 μM) and Quis (100–300 μM) had similar effects (Figure 3), while L-AP4 did not affect tritium output. The EC_{50}s for the inhibition of this output were: 0.5 μM for L-CCG-I; 100 μM for 1S,3R-ACPD, and 100 μM for Quis.

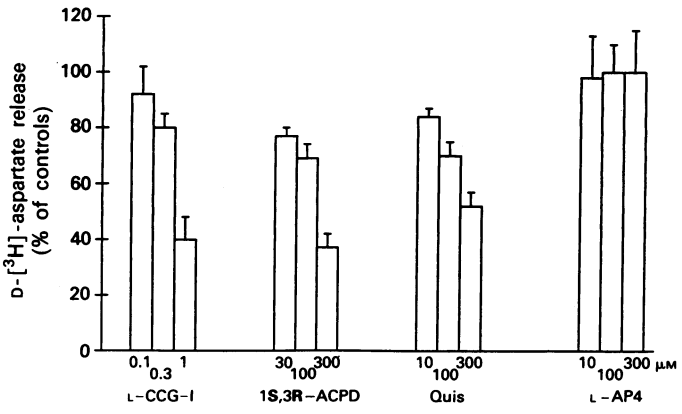


Figure 3 Different concentrations of mGluR agonists (L-CCG-I, 1S-3R-ACPD, Quis, L-AP4) were added 5 min before depolarization of striatal slices with 30 mM of KCl (see Figure 1 for details). Each column represents radioactivity output expressed as percentage of controls and represents the mean ± s.e.mean of at least 5 experiments run in duplicate. For abbreviations, see text.

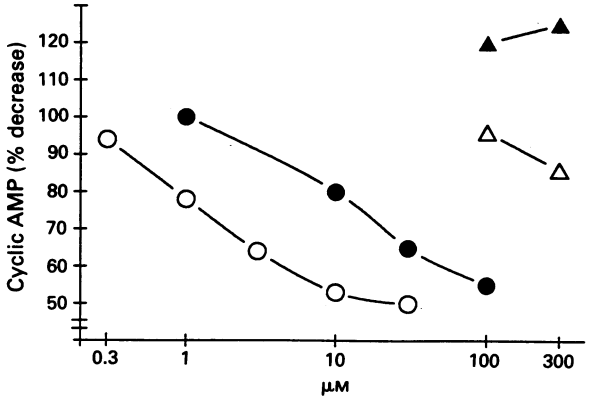


Figure 4 Striatal slices were incubated for 15 min at 37°C with various concentrations of L-CCG-I, 1S,3R-ACPD, Quis and L-AP4 in the presence of 30 μM forskolin and 1 mM IBMX. The content of cyclic AMP of each sample was measured by radioimmunoassay. Data are the means of 5 experiments run in triplicate and are expressed as percentage over the forskolin-stimulated values in each experiment, s.e.mean, were consistently within 10% of the reported values. (○) L-CCG-I; (●) 1S,3R-ACPD; (△) Quis; (▲) L-AP4. For abbreviations, see text.

Effects of mGluR agonists on forskolin-stimulated cyclic AMP accumulation

The addition of forskolin (30 μM) to striatal slices incubated in the presence of 1 mM IBMX increased their content in cyclic AMP from 4.7 ± 0.2 to 102 ± 10 pmol mg^{-1} protein. When agonists of mGluRs were added, the stimulation was significantly reduced. Figure 4 shows that L-CCG-I (10 μM) reduced the effect of forskolin by approximately 50%. This was the maximal inhibition we could achieve. Similar effects were evoked by larger concentrations of 1S,3R-ACPD (100 μM), while Quis was less active. The EC_{50}s for this effect were: 0.9 μM for L-CCG-I, 20 μM for 1S,3R-ACPD, and 300 μM for Quis. Conversely, L-AP4 slightly potentiated forskolin-stimulated cyclic AMP accumulation.

The effects of mGluR agonists on the stimulation of PI hydrolysis

The addition of mGluR agonists to striatal slices increased PI metabolism in a concentration-dependent manner. Figure 5

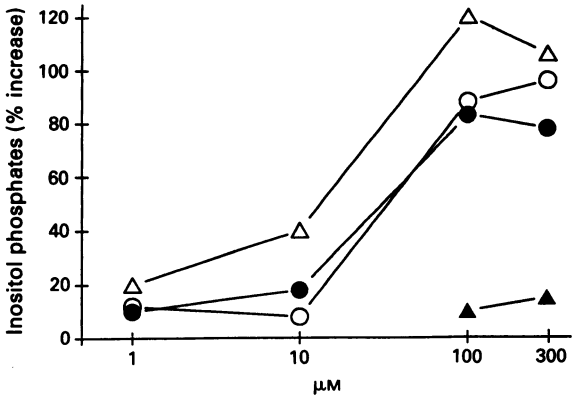


Figure 5 Slices prelabelled with [^{3}H]-inositol were exposed to different concentrations of mGluR agonists (Quis, 1S,3R-ACPD, L-CGG-I and L-AP4) for 15 min in the presence of 10 mM LiCl. Data are the means of at least 3 experiments run in triplicate and are expressed as percentage increases over basal values in each experiment. The basal value was 15910 ± 1267 d.p.m. mg^{-1} protein and represent the sum of radioactivity found in the fractions corresponding to [^{3}H]-IP, [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$. (○) L-CCG-I; (●) 1S,3R-ACPD; (△) Quis; (▲) L-AP4. For abbreviations, see text.

shows that Quis (100 μM) increased 2.2 fold the formation of inositol phosphates. At the same concentration, the stimulation evoked by L-CCG-I and 1S,3R-ACPD was approximately 1.8 fold. L-AP4 was inactive. The EC_{50}s were: 15 μM for Quis, 25 μM for 1S,3R-ACPD, and 30 μM for L-CCG-I.

Discussion

Studies on the characterization of the physiological and pharmacological properties of mGluRs have been impeded by the lack of selective agonists and antagonists. In fact, the commonly used agonsits 1S,3R-ACPD or Quis do not distinguish among the different receptor subtypes and the recently described antagonists derived from phenylglycine seem to have partial agonist properties and are not completely characterized (Ito *et al.*, 1992; Eaton *et al.*, 1993; Birse *et al.*, 1993). Other compounds such as L-Asp-β-hydroxamate seem to have mixed agonist-antagonist activity on the stimulation of PI metabolism (Porter *et al.*, 1992). In a similar manner, 2-amino-3-phosphonopropionate (L-AP3), once considered a selective mGluR antagonist (Schoepp & Johnson 1989), has been demonstrated to have agonist-antagonist activity leading to rapid desensitization of the receptor (Lonart *et al.*, 1992). In mammalian cells expressing the cloned mGluR subtypes, Hayashi *et al.* (1992) noticed that L-CCG-I, another proposed agonist of mGluRs (Nakagawa *et al.*, 1990; Ishida *et al.*, 1990; Kudo *et al.*, 1991), had an EC_{50} of 0.3 μM when studied on mGluR2 mediating the inhibition of forskolin-induced cyclic AMP accumulation and of 50 μM when studied on mGluR1 mediating increase of phosphoinositide metabolism. This could suggest that L-CCG-I is a relatively selective agonist for the subtype of mGluR negatively linked to the cyclase system. This receptor subtype has been described in different brain slice preparations (Schoepp *et al.*, 1992; Cartmell *et al.*, 1992; Manzoni *et al.*, 1992; Casabona *et al.*, 1992). In rat striatal slices L-CCG-I had an EC_{50} of 0.9 μM for the inhibition of cyclic AMP accumulation and of 30 μM for the stimulation of PI metabolism thus confirming, in a different model, data obtained in transfected cells (Hayashi *et al.*, 1992). Furthermore, L-CCG-I significantly inhibited K^+-induced output of D-[^{3}H]-Asp. This output, once considered as a gross index of synaptic transmitter release (Potashner, 1978; Notman *et al.*, 1984; Arqueros *et al.*, 1985) is now regarded with some suspect because D-[^{3}H]-Asp penetrates poorly into the synaptic vescicles (see: McMahon & Nicholls, 1991). The apparent calcium dependency of D-[^{3}H]-Asp release under our experimental conditions and preliminary results obtained by measuring the effects of mGluR agonists on the K^+ (30 mM)-evoked release of endogenous glutamate from striatal slices suggest that we are studying actions on the depolarization-induced output of synpatic transmitter. However, the possibility that mGluR agonists affect the cytoplasmic output of glutamate (with an action on the excitatory amino acid carrier ?) and not the exocytotically released transmitter cannot be ruled out by the present experiments. Since the EC_{50} of L-CCG-I for the inhibition of D-[^{3}H]-Asp efflux and that for the blockade of cyclic AMP accumulation are in the same range and in view of the fact that the orders of potency of mGluR agonists for both actions were comparable, it is reasonable to propose that the receptor involved might be the same. Receptors inhibiting cyclic AMP accumulation and modulating transmitter release have been widely studied in the adrenergic system and selective agonists for these receptors are now useful therapeutic tools (Langer, 1980; Starke, 1987; Schoffelmeer *et al.*, 1988).

Glutamate receptors capable of inhibiting D-[^{3}H]-Asp output have previously been reported (McBean & Roberts, 1981) and a number of studies have demonstrated presynaptic glutamate receptors stimulated by L-AP4 that inhibit synaptic excitation (Koerner & Cotman, 1981; Gannon *et al.*, 1989; Forsythe & Clements, 1990; Trombley & Westbrook, 1992). L-AP4 has recently been proposed as a prototype agonist of mGluR4 (Thomsen *et al.*, 1992), one of the mGluR types negatively linked to adenylate cyclase and inhibition of transmitter release (Nakanishi, 1992). Since L-AP4 binding sites have been described in the striatum (Butcher *et al.*, 1983), we also tested L-AP4 in our experiments. Our results indicate that L-AP4 (100–300 μM) did not inhibit D-[^{3}H]-Asp release and slightly stimulated forskolin-induced cyclic AMP accumulation. Therefore, the receptor subtype involved in the control of transmitter release in the rat striatum does not appear to be mGluR4. This observation is in line with *in situ* hybridization studies suggesting that mGluR4 receptors are neither present in the striatum nor in the cortex where the major glutamatergic input to the striatum originates (Tanabe *et al.*, 1993). However, L-AP4 inhibits the forskolin-induced cyclic AMP accumulation in the guinea-pig cortex (Cartmell *et al.*, 1993a) and it is not clear whether or not this inhibition is associated with a decrease of transmitter output.

1S,3R-ACPD and Quis inhibited D-[^{3}H]-Asp output and forskolin-induced cyclic AMP accumulation at concentrations very similar to those stimulating PI hydrolysis (this paper) and potentiating transmitter release, in the presence of arachidonic acid, in other brain areas (Herrero *et al.*, 1992). It has been demonstrated that arachidonic acid release from striatal neurones is promoted by the concerted action of Quis on both ionotropic and metabotropic receptors (Dumuis *et al.*, 1990), nevertheless in striatal slices Quis diminished D-[^{3}H]-Asp output. The demonstration that similar concentrations of 1S,3R-ACPD, a prototype agonist of the mGluRs, reduced D-[^{3}H]-Asp output in the striatum (this study) and increased glutamate release in the neocortex (Herrero *et al.*, 1992) may offer an explanation for the contradictory results thus far reported on the pharmacological and toxic effects of this mGluR agonist. In fact, 1S,3R-ACPD has been shown both to reduce and increase synaptic transmission and to cause and prevent excitotoxic neuronal damage. Different receptors are probably responsible for the numerous effects of this compound. For instance, Koh *et al.* (1991) reported that 1S,3R-ACPD could protect cortical neuronal cultures from the toxic action of N-methyl-D-aspartate. Similar results have been obtained in the retina (Siliprandi *et al.*, 1992) and in the mouse hippocampus (Chiamulera *et al.*, 1992) *in vivo*. Conversely, Sacaan & Schoepp (1992) described an excitotoxic action for this mGluR agonist in the rat hippocampus. The possible interaction of 1S,3R-ACPD with different

mGluR subtypes and the differential expression of these receptors in different species and brain areas could easily explain these apparently contradictory observations.

The acceptable selectivity of LCCG-I for the mGluR receptor linked to the inhibition of cyclic AMP and the inhibition of transmitter output shows that it is possible to obtain compounds that significantly reduce transmitter release at glutamatergic synapses by acting on a specific mGluR subtype. Experiments along this line are in progress in our laboratory. The availability of selective agonists for specific mGluR subtypes could help in understanding the overall control of the function of glutamatergic synapses and could be useful in pathological situations linked to excitotoxic events such as epilepsy, neurodegeneration and brain ischaemia (see: Guidotti, 1990; Meldrum 1990).

Supported by the University of Florence and by the National Research Council (C.N.R.). We thank Dr A Cozzi for technical assistance.

References

ANWYL, R. (1991). The role of the metabotropic receptor in synaptic plasticity. *Trends Pharmacol. Sci.*, **12**, 324–326.

ARAMORI, I. & NAKANISHI, S. (1992). Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. *Neuron*, **8**, 757–765.

ARONICA, E., FREY, V., WAGNER, M., SCHROEDER, H., KRUG, M., RUTHRICH, H., CATANIA, M.V., NICOLETTI, F. & REYMANN, K.G. (1991). Enhanced sensitivity of 'metabotropic' glutamate receptors after induction of long-term potentiation in rat hippocampus. *J. Neurochem*, **57**, 376–383.

ARQUEROS, L., ABARCA, J. & BUSTOS, G. (1985). Release of D-^{3}H-aspartic acid from the rat striatum. *Biochem. Pharmacol.*, **34**, 1217–1224.

BASKYS, A. & MALENKA, C.R. (1991). Trans-ACPD depresses synaptic transmission in the hippocampus. *Eur. J. Pharmacol.*, **193**, 131–132.

BEANI, L., BIANCHI, C., GIACOMELLI, A. & TAMBERI, F. (1978). Noradrenaline inhibition of acetylcholine release from guinea-pig brain. *Eur. J. Pharmacol.*, **48**, 179–193.

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol trisphosphate, a novel second messagger in cellular signal transduction. *Nature*, **312**, 315–317.

BIRSE, E.F., EATON, S.A., JANE, D.E., JONES, P.L.S., PORTER, R.H.P., POOK, P.C.K., SUNTER, D.C., UDVARHELYI, P.M., WHARTON, B., ROBERTS, P.J., SALT, T.E. & WATKINS, J.C. (1993). Phenylglycine derivatives as new pharmacological tools for investigating the role of metabotropic glutamate receptors in the central nervous system. *Neuroscience*, **52**, 481–488.

BORTOLOTTO, A.Z. & COLLINGRIDGE, L.G. (1992). Activation of glutamate metabotropic receptors induces long-term potentiation. *Eur. J. Pharmacol.*, **214**, 297–298.

BUTCHER, S.P., COLLINS, J.F. & ROBERTS, P.J. (1983). Characterization of the binding of DL-[^{3}H]-2-amino-4-phosphonobutyrate to L-glutamate-sensitive sites on rat brain synaptic membranes. *Br. J. Pharmacol.*, **80**, 355–364.

CALABRESI, P., MERCURI, N.B. & BERNARDI, G. (1992). Activation of quisqualate metabotropic receptors reduces glutamate and GABA-mediated synaptic potentials in the rat striatum. *Neurosci. Lett.*, **139**, 41–44.

CARTMELL, J., KEMP, J.A., ALEXANDER, S.P., HILL, S.J. & KENDALL, D.A. (1992). Inhibition of forskolin-stimulated cyclic AMP formation by 1-aminocyclopentanetrans-1,3-dicarboxylate in guinea-pig cerebral cortical slices. *J. Neurochem.*, **58**, 1964–1966.

CARTMELL, J., CURTIS, A.R., KEMP, S.A., ALEXANDER, S.P.H. & KENDALL, D.A. (1993a). Inhibition of A_{2b} adenosine receptor-elicited and forskolin-stimulated cyclic AMP accumulation by L-2-amino-4-phosphonobutyrate (L-AP4). *Br. J. Pharmacol.*, **108**, 268P.

CARTMELL, J., CURTIS, A.R., KEMP, S.A., KENDALL, D.A. & ALEXANDER, S.P.H. (1993b). Subtypes of metabotropic excitatory amino acid receptor distinguished by stereoisomers of the rigid glutamate analogue 1-aminocyclopentane-1,3-dicarboxylate. *Neurosci. Lett.*, **153**, 107–110.

CASABONA, G., GENNAZZANI, A.A., DI STEFANO, M., SORTINO, M.A. & NICOLETTI, F. (1992). Developmental changes in the modulation of cyclic AMP formation by the metabotropic glutamate receptor agonist 1S,3R-aminocyclopentane-1,3-dicarboxylic acid in brain slices. *J. Neurochem.*, **59**, 1161–1163.

CHIAMULERA, C., ALBERTINI, P., VALERIO, E. & REGGIANI, A. (1992). Activation of metabotropic receptors has a neuroprotective effect in a rodent model of focal ischaemia. *Eur. J. Pharmacol.*, **216**, 335–336.

DUMUIS, A., DOMAGARIK, J.P.P., SEBBEN, M. & BOCKAERT, J. (1990). Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors. *Nature*, **347**, 182–184.

EATON, S.A., JANE, D.E., JONES, P.L.S.J., PORTER, R.H.P., POOK, P.C.K., SUNTER, D.C., UDVARHELYI, P.M., ROBERTS, P., SALT, T.E. & WATKINS, J.C. (1993). Competitive antagonism at metabotropic glutamate receptors by (S)-4-carboxyphenylglycine and (RS)-α-methyl-4-carboxyphenylglycine. *Eur. J. Pharmacol.*, **244**, 195–197.

FORSYTHE, I.D. & CLEMENTS, J.D. (1990). Presynaptic glutamate receptors depress excitatory monosynaptic transmission between mouse hippocampal neurones. *J. Physiol.*, **429**, 1–16.

GANNON, R.L., BATY, L.T. & TERRIAN, D.M. (1989). L(+)-2-amino-4-phosphonobutyrate inhibits the release of both glutamate and dynorphin from guinea pig but not rat hippocampal mossy fiber synaptosomes. *Brain Res.*, **495**, 151–155.

GUIDOTTI, A. (1990). *Neurotoxicity of Excitatory Amino Acids.* FIDIA Research Foundation Symposium Series. Vol. 4. New York: Raven Press.

HAYASHI, Y., TANABE, Y., ARAMORI, I., MASU, M., SHIMAMOTO, K., OHFUNE, Y. & NAKANISHI, S. (1992). Agonist analysis of 2-(carboxycyclopropyl)glycine isomers for cloned metabotropic glutamate receptor subtypes expressed in Chinese hamster ovary cells. *Br. J. Pharmacol.*, **107**, 539–543.

HERRERO, I., MIRAS-PORTUGAL, M.T. & SANCHEZ-PRIETO, J. (1992). Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. *Nature*, **360**, 163–166.

HOUAMED, K.M., KUIJPER, J.L., GILBERT, T.L., HALDEMAN, B.A., O'HARA, P.J., MULVIHILL, E.R., ALMERS, W. & HAGEN, F.S. (1991). Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from rat brain. *Science*, **252**, 1318–1321.

ISHIDA, M., AKAGI, H., SHIMAMOTO, K., OHFUNE, Y. & SHINOZAKI, H. (1990). A potent metabotropic glutamate receptor agonist: electrophysiological actions of a conformationally restricted glutamate analogue in the rat spinal cord and Xenopus oocytes. *Brain Res.*, **537**, 311–314.

ITO, I., KOHDA, A., TANABE, S., HIROSE, E., HAYASHI, M., MITSUNAGA, S. & SUGIYAMA, H. (1992). 3,5-dihydroxyphenylglycine: a potent agonist of metabotropic glutamate receptors. *Neuropharmacol. Neurotoxicol.*, **3**, 1013–1016.

KOERNER, J.F. & COTMAN, C.W. (1981). L-2-Amino-phosphonobutyric acid selectively inhibits perforant path synapses from lateral entorhinal cortex. *Brain Res.*, **216**, 192–198.

KOH, J.-Y., PALMER, E. & COTMAN, C.W. (1991). Activation of the metabotropic glutamate receptor attenuates N-methyl-D-aspartate neurotoxicity in cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 9431–9435.

KUDO, Y., AKITA, K., ISHIDA, M. & SHINOZAKI, H. (1991). A significant increase in intracellular Ca^{++} concentration induced by (2S,3R,4S)-2-(carboxycyclopropyl)glycine, a new potent NMDA agonist, in cultured rat hippocampal neurons. *Brain Res.*, **567**, 342–345.

LANGER, S.Z. (1980). Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.*, **32**, 337–362.

LONART, G., ALAGARSAMY, S., RAVULA, R., WANG, J. & JOHNSON, K.M. (1992). Inhibition of the phospholipase C-linked metabotropic glutamate receptor by 2-amino-3-phosphonopropionate is dependent on extracellular calcium. *J. Neurochem.*, **59**, 772–775.

LOVINGER, D.M. (1991). Trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) decreases synaptic excitation in rat striatal slices through a presynaptic action. *Neurosci. Lett.*, **129**, 17–21.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 256–275.

MANZONI, O., PREZEAU, L., SLADECZEK, F. & BOCKAERT, J. (1992). Trans-ACPD inhibits cAMP formation via a pertussis toxin-sensitive G-protein. *Eur. J. Pharmacol.*, **225**, 357–358.

MASU, M., TANABE, Y., TSUCHIDA, K., SHIGEMOTO, R. & NAKANISHI, S. (1991). Sequence and expression of a metabotropic glutamate receptor. *Nature*, **349**, 760–765.

MCBEAN, G.J. & ROBERTS, P.J. (1981). Glutamate-preferring receptors regulate the release of D-[3H]-aspartate from rat hippocampal slices. *Nature*, **291**, 593–594.

MCMAHON, H.T. & NICHOLLS, D.G. (1991). The bioenergetics of neurotransmitter release. *Biochim. Biophys. Acta*, **1059**, 243–264.

MELDRUM, B. (1990). Protection against ischaemic neuronal damage by drugs acting on excitatory neurotransmission. *Cerebrovasc. Brain Metab. Rev.*, **2**, 27–57.

MORONI, F., BIANCHI, C., TANGANELLI, S., MONETI, G. & BEANI, L. (1981). The release of γ-aminobutyric acid, glutamate and acetylcholine from striatal slices: a mass-fragmentographic study. *J. Neurochem.*, **36**, 1691–1697.

NAKAGAWA, Y., SAITOH, K., ISHIHARA, T., ISHIDA, M. & SHINOZAKI, H. (1990). (2S,3S,4S)-(carboxycyclopropyl)glycine is a novel agonist of metabotropic glutamate receptors. *Eur. J. Pharmacol.*, **184**, 205–206.

NAKANISHI, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science*, **258**, 597–603.

NICHOLLS, D.G. (1992). A retrograde step forward. *Nature*, **360**, 106–107.

NOTMAN, H., WHITNEY, R. & JHAMANDAS, K. (1984). Kainic acid evoked release of D-[^{3}H]aspartate from rat striatum in vitro: characterization and pharmacological modulation. *Can. J. Physiol. Pharmacol.*, **62**, 1070–1077.

PELLEGRINI-GIAMPIETRO, D.E., RUGGIERO, M., GIANNELLI, S., CHIARUGI, V. & MORONI, F. (1988). Morphine withdrawal in vitro: potentiation of agonist-dependent polyphosphoinositide breakdown. *Eur. J. Pharmacol.*, **149**, 297–306.

PELLICCIARI, R., CURINI, M., NATALINI, B. & CECCHERELLI, P. (1986). Preparation of cyclopropylglutamic analogs. *IX International Symposium on Medicinal Chemistry*, p.118.

PELLICCIARI, R., NATALINI, B., MARINOZZI, M., CHIORRI, C., LONTHORN, T.H., MONAHAN, J.B. & SNYDER, J.P. (1991). 3,4-cyclopropylglutamates as N-methyl-D-aspartate receptor ligands. In *Excitatory Amino Acids*, ed. Meldrum, B.S., Moroni, F., Simon, R.P. & Woods, J.H. pp. 405–413. New York: Raven Press.

PELLICCIARI, R., NATALINI, B., MARINOZZI, M., SELVI, L., CHIONI, C., MONAHAN, J.B., LANTHORN, T.H. & SNYDER, J. (1988). 3,4-cyclopropylglutamate as conformationally restricted agonist of the NMDA receptor. In *Frontiers in Excitatory Amino Acid Research*. ed. Cavalheiro, E.A., Lehman, J. & Turski, L. pp. 67–70. New York: A. Liss.

PORTER, R.H., BRIGGS, R.S.J. & ROBERTS, P.J. (1992). L-Aspartate-β-hydroxamate exhibits mixed agonist-antagonist activity at the glutamate metabotropic receptor in rat neonatal cerebrocortical slices. *Neurosci. Lett.*, **144**, 87–89.

POTASHNER, S.J. (1978). Effects of tetrodotoxin, calcium and magnesium on the release of amino acids from slices of guinea pig cerebral cortex. *J. Neurochem.*, **31**, 187–195.

RANNIE, G.D. & SHINNICK-GALLAGHER, P. (1992). Trans-ACPD and L-APB presynaptically inhibit excitatory glutamatergic transmission in the basolateral amygdala (BLA). *Neurosci. Lett.*, **139**, 87–91.

SACAAN, A.I. & SCHOEPP, D.D. (1992). Activation of hippocampal metabotropic excitatory amino acid receptors leads to seizures and neuronal damage. *Neurosci. Lett.*, **139**, 77–82.

SCHOEPP, D.D. & CONN, P.J. (1993). Metabotropic glutamate receptors in brain function and pathology. *Trends Pharmacol. Sci.*, **14**, 13–20.

SCHOEPP, D.D. & JOHNSON, B.G. (1989). Inhibition of excitatory amino acid-stimulated phosphoinositide hydrolysis in the neonatal rat hippocampus by 2-amino-3-phosphonopropionate. *J. Neurochem.*, **53**, 1865–1870.

SCHOEPP, D.D., JOHNSON, B.G. & MONN, J.A. (1992). Inhibition of cyclic AMP formation by a selective metabotropic glutamate receptor agonist. *J. Neurochem.*, **58**, 1184–1186.

SCHOFFELMEER, A.N.M., HOGENBOOM, F. & MULDER, A.H. (1988). Sodium dependent ^{3}H-noradrenaline release from rat neocortical slices in the absence of extracellular calcium: presynaptic modulation by μ-opioid receptor and adenylate cyclase activation. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 548–552.

SHINOZAKI, H., ISHIDA, M., SHIMAMOTO, K. & OHFUNE, Y. (1989). Potent NMDA-like actions and potentiation of glutamate responses by conformational variants of a glutamate analogue in the rat spinal cord. *Br. J. Pharmacol.*, **98**, 1213–1224.

SILIPRANDI, R., LIPARTITI, M., FADDA, E., SAUTTER, J. & MANEV, H. (1992). Activation of the glutamate metabotropic receptor protects retina against N-methyl-D-aspartate toxicity. *Eur. J. Pharmacol.*, **219**, 173–174.

STARKE, K. (1987). Presynaptic α-autoreceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 73–146.

TANABE, Y., MASU, M., ISHII, T., SHIGEMOTO, R. & NAKANISHI, S. (1992). A family of metabotropic glutamate receptors. *Neuron*, **8**, 169–179.

TANABE, Y., NOMURA, A., MASU, M., SHIGEMOTO, R., MIZUNO, N. & NAKANISHI, S. (1993). Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4. *J. Neurosci.*, **13**, 1372–1378.

THOMSEN, C., KRISTENSEN, P., MULVIHILL, E., HALDEMAN, B. & SUZDAK, P.D. (1992). L-2-amino-4-phosphonobutyrate (L-AP4) is an agonist at the type IV metabotropic glutamate receptor which is negatively coupled to adenylate cyclase. *Eur. J. Pharmacol.*, **227**, 361–362.

TROMBLEY, P.Q. & WESTBROOK, G.L. (1992). L-AP4 inhibits calcium currents and synaptic transmission via a G-protein-coupled glutamate receptor. *J. Neurosci.*, **12**, 2043–2050.

WOOD, P.L., MORONI, F., CHENEY, D.L. & COSTA, E. (1979). Cortical lesions modulate turnover rates of acetylcholine and γ-aminobutyric acid. *Neurosci. Lett.*, **12**, 349–354.

ZHENG, F. & GALLAGHER, J.P. (1992). Metabotropic glutamate receptors are required for the induction of long-term potentiation. *Neuron*, **9**, 163–172.

(Received May 24, 1993
Revised August 11, 1993
Accepted August 13, 1993)

Br. J. Pharmacol. (1993), **110**, 1413–1420 © Macmillan Press Ltd, 1993

Different muscarinic receptor subtypes mediating the phasic activity and basal tone of pig isolated intravesical ureter

[1]M. Hernández, U. Simonsen, D. Prieto, L. Rivera, P. García, E. Ordaz & A. García-Sacristán

Departamento de Fisiología, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain

1 We have studied the effects of muscarinic cholinoceptor agonists and specific antagonists on both phasic activity and basal tone of the isolated intravesical ureter of the pig by means of isometric techniques *in vitro*.

2 Acetylcholine in the presence and absence of physostigmine increased both phasic activity and basal tone of ureteral strips in a concentration-dependent manner. Moreover carbachol, methacholine and oxotremorine-M increased both contractile parameters while bethanechol and McN-A-343 evoked only increases in tone without affecting the frequency of the phasic contractions.

3 The nicotinic receptor blocker, hexamethonium (10^{-6}–10^{-4} M), failed to modify the contractions evoked by a single dose of carbachol (10^{-5} M), whilst the muscarinic antagonist, atropine inhibited both phasic and tonic responses.

4 The muscarinic M_1 (pirenzepine), M_2 (AF-DX 116 and methoctramine), M_3 (4-DAMP, HHSiD and *p*-F-HHSiD), and putative M_4 receptor (tropicamide) antagonists significantly reversed increases in both frequency of phasic activity and baseline tone induced by a submaximal dose of carbachol (10^{-5} M). The pIC_{50} values for inhibition of the induced phasic activity were: atropine (10.16) > 4-DAMP (9.12) > HHSiD (8.22) = methoctramine (7.98) = *p*-F-HHSiD (7.88 > tropicamide (7.62) = pirenzepine (7.53) = AF-DX 116 (7.45) and for inhibition of basal tone were: atropine (10.73) > 4-DAMP (9.32) > HHSiD (8.65) = pirenzepine (8.43) = *p*-F-HHSiD (8.38) > methoctramine (7.79) > tropicamide (7.53) > AF-DX 116 (7.04).

5 The antagonist profile indicates that an M_1 receptor mediates the tonic response while the phasic activity could involve either both M_2 and M_3 or an M_4 muscarinic receptor. These results suggest that different muscarinic receptor subtypes mediate the phasic and tonic contractile activity induced by a submaximal concentration of carbachol in the porcine intravesical ureter.

Keywords: Intravesical ureter of pig; muscarinic receptors; phasic activity; tone

Introduction

Autonomic receptors play an important role in the regulation of distal ureteral function. Support for the contention that the autonomic nervous system exerts an influence on the ureter can be derived from the demonstration of a rich supply of tyrosine hydroxylase (TH) and acetylcholinesterase-positive nerve fibres, which are distributed throughout the distal ureter forming dense neuromuscular, subepithelial and perivascular plexuses (Schulman, 1985; Prieto *et al.*, 1989; 1990), in contrast with proximal portions of ureter and kidney pelvis where evidence for cholinergic innervation is scarce (Barajas & Wang, 1983; Prieto *et al.*, 1990).

Furthermore, *in vitro* studies of the sheep ureterovesical junction have demonstrated the presence of functionally active adrenoceptors (Rivera *et al.*, 1992a) and we have recently demonstrated that noradrenaline modulates both phasic and tonic contractile activity of the pig intravesical ureter, through specific populations of adrenoceptors belonging to α_1-, β_1- and β_2-subtypes, with a possible involvement of α_2-receptors in the maintenance of ureteral tonus (Hernández *et al.*, 1992). Also acetylcholine induced contractions of the sheep ureterovesical junction (Rivera *et al.*, 1992b) and evoked increases in both phasic activity and basal tone of the isolated ureteral component of the ureterovesical junction. However, the role of cholinergic innervation in the control of motor activity of the ureter at the ureterovesical junction and its functional significance in the regulation of the bladder filling and emptying at micturition is not well understood. It is also largely unknown which postjunctional muscarinic cholinoceptor subtypes are involved in the modulation of the activity.

Five unique genes coding for muscarinic receptors have been cloned and denoted m1, m2, m3, m4 and m5 (Bonner *et al.*, 1987; Peralta *et al.*, 1987). Moreover radioligand studies of cloned receptors show a close correlation between m1, m2, m3 gene products and the pharmacologically defined M_1, M_2 and M_3 muscarinic receptor subtypes (Buckley *et al.*, 1989; Hulme *et al.*, 1990).

The different antagonists now available make it possible to determine the muscarinic receptor subtypes present in tissues. The antagonist pirenzepine shows high affinity for M_1 receptors (Hammer & Giachetti, 1982; Eglen & Whiting, 1986; Giachetti *et al.*, 1986; Doods *et al.*, 1987), low affinity for M_2 (or cardiac receptors) and intermediate affinity for M_3 (ileal or smooth muscle receptors) (Eglen & Whiting, 1986; Giachetti *et al.*, 1986; Doods *et al.*, 1987). Thus, AF-DX 116 (11-[(2-{(diethylamino)-methyl]-1-piperidinyl)acetyl]-5, 11-dihydro-6H-pyrido-[2,3,6] [1,4]-benzodiazepine-6-one) (Eglen & Whiting, 1986; Giachetti *et al.*, 1986) and methoctramine (Melchiorre, 1988) are selective for M_2, and 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidinemethiodide) (Barlow *et al.*, 1976; Eglen & Whiting, 1986), hexahydrosiladiphenidol (Fuder *et al.*, 1985) and *p*-F-hexahydrosiladiphenidol (Lambrecht *et al.*, 1988; Duckles, 1990; Eglen *et al.*, 1990) for M_3-receptors. Recently, both tropicamide and himbacine have been used as M_4-receptor antagonists (Lazareno *et al.*, 1990; 1993).

Histochemical studies have demonstrated a rich supply of cholinergic nerves in the intravesical ureter (Prieto *et al.*, 1990), in sharp contrast to the proximal ureter. Therefore, the aim of the present study was to characterize the postjunctional muscarinic cholinoceptors involved in the modulation of the pig intravesical ureteral smooth muscle activity.

[1] Author for correspondence.

Methods

Urinary bladders with attached ureters were removed from adult pigs of either sex with no lesions in their urinary tract, selected at the local slaughterhouse immediately after the animals were killed. The bladders were placed in chilled physiological saline solution (PSS) at 4°C. The adjacent connective and fatty tissues were removed with care and longitudinal preparations (4–6 mm long and 2–3 mm wide) of the intravesical ureter were isolated from the bladder by dissection as described earlier (Hernández et al., 1992). The strips were suspended vertically in 30 ml organ baths containing PSS maintained at 37°C and gassed with 5% CO_2 in O_2, pH = 7.4. The distal end of a preparation was attached to a metal hook and the other end connected to an isometric transducer (Grass FT03C) with the signal continuously recorded on a polygraph (Grass 79E). Passive tension of 2 g was applied to the preparations and they were allowed to equilibrate for 60 min.

Experimental procedure

The contractile capacity of the preparations was challenged by exposing the preparations to 120 mM potassium-rich physiological saline solution (K^+PSS). Induced phasic activity described by frequency (number of contractions min^{-1}) and amplitude (g) of rhythmic contractions and increases in basal tone (g) were examined by single application of increasing concentrations of cholinoceptor agonists such as carbachol, methacholine, oxotremorine-M, bethanechol, acetylcholine and McN-A-343. Carbachol, acetylcholine and methacholine concentration-response curves were generated in the presence of physostigmine (10^{-6} M) to block acetylcholinesterase activity. The ureteral strips were stimulated with a single concentration of the muscarinic agonists and potassium-rich Krebs during a period of 3 and 4 min, respectively.

Due to the development of a strong tachyphylaxis of the tissue to the agonists, two consecutive concentration-response curves could not be constructed in the same preparation. However, the response to a single submaximal concentration (10^{-5} M) of carbachol was reproducible during repetitive exposures. Therefore, it was used to determine the effect of the muscarinic antagonists: atropine, pirenzepine, methoctramine, AF-DX 116, 4-DAMP, HHSiD, *p*-F-HHSiD and tropicamide. First, a control response to carbachol (10^{-5} M) in absence of antagonist was obtained. The preparations were then incubated with the antagonist for 30 min before carbachol was added. An inhibition curve of single concentrations of antagonist was constructed in a single strip. Control preparations without antagonist incubation were run in parallel to correct for tissue fatigue and time-induced changes (Kenakin, 1984).

Drugs and solutions

The composition of physiological saline solution (PSS) was (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 24.9, glucose 11, KH_2PO_4 1.2, EDTA (ethylene diamine tetraacetic acid) 0.027. The K^+PSS was PSS with KCl exchanged for NaCl on an equimolar basis. Stock solutions were prepared daily in distilled water.

The following drugs were used: AF-DX 116 (11-(2-((diethyl-amino)methyl)-1-piperidinylacetyl)-5,-11-dihydro-6H-pyrido(2,3-b)-(1,4)-benzodiazepine-6-one); pirenzepine HCl (Dr Karl Thomae GmbH, Germany); atropine sulphate (Merck, Germany); bethanechol (Sigma, U.S.A.); carbamoylcholine HCl (carbachol) (Sigma, U.S.A.); 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide) (courtesy of Dr R.B. Barlow, Bristol, U.K.); hexamethonium bromide (Serva); McN-A-343 (4-hydroxy-2-butynyl)-1-trimethylammonium *m*-chloro carbimilate chloride, Sigma); methacholine (acetyl-β-methylcholine, Sigma); methoctramine (Sigma, U.S.A.); oxotremorine-M (ICN Biochemicals); HHSiD (hexahydrosiladiphenidol, Research Biochemicals Incorporated, U.K.); *p*-F-HHSiD (*para* fluoro hexa-hydrosiladiphenidol, Research Biochemicals Incorporated, U.K.); tropicamide (Sigma, U.S.A.).

Physostigmine and *p*-F-HHSiD were dissolved in ethanol while tropicamide was dissolved in 0.1 N HCl and further diluted in distilled water. The other drugs were dissolved in distilled water. Previous experiments showed that the solvents had no effect on the preparation.

Calculations

For each concentration-response curve, the concentration required to give half-maximal response (EC_{50}) was determined by computerized iteration, fitting the responses and logarithmic concentrations to the Hill equation (Graph Pad software 3.0, San Diego, Calif., U.S.A.). Sensitivities to drugs are expressed in terms of pD_2 values, where $pD_2 = -\log EC_{50}$, the EC_{50} being the agonist concentration needed to produce half-maximal response. pIC_{50} values for the muscarinic antagonists were also calculated, as $pIC_{50} = -\log IC_{50}$, where IC_{50} is the concentration of antagonist required to cause half-maximal inhibition of the response induced by a single dose (10^{-5} M) of the agonist (Skärby & Larsson, 1987). IC_{50} values were converted to K_b values using the equation of Leff & Dougall (1993):

$$K_b = \frac{[IC_{50}]}{\left(2+\left(\frac{[A_f]}{[EC_{50}]}\right)^b\right)^{1/b} - 1}$$

A_f and b are the fixed concentration (10^{-5} M) and the slope factor of single increasing dose-response curves to carbachol, respectively.

Statistics

The results are expressed as mean ± s.e.mean. Statistical differences were calculated by Student's *t* test and one-way analysis of variance (ANOVA) with *a posteriori* Bonferroni test (Wallestein et al., 1980). Differences were considered significant with a probability level of $P < 0.05$.

Results

Agonist study

Acetylcholine (10^{-8}–3×10^{-4} M) and methacholine (10^{-8}–10^{-4} M) in the presence and absence of physostigmine (10^{-6} M) evoked increases in both phasic and tonic contractile activity, respectively (see Table 1). There was a significant difference ($P < 0.05$, paired *t* test) between the pD_2 values of tone induced by acetylcholine in the presence and absence of physostigmine (Figure 1). Moreover carbachol, methacholine and oxotremorine-M induced concentration-dependent increases in both frequency of phasic activity and basal tone of porcine intravesical ureteral strips. However, bethanechol and the putative M_1 agonist, McN-A-343 evoked only increases in the tone of ureteral preparations (Figures 2 and 3, Table 1). The concentration-response curve to McN-A-343 was bimodal with a $pD_2 = 7.21 \pm 0.14$ and $E_{max} = 0.81 \pm 0.17$ g for the first phase and $pD_2 = 5.26 \pm 0.16$ and $E_{max} = 2.02 \pm 0.22$ g for the second phase.

Table 1 shows pD_2 and E_{max} values for the different muscarinic cholinoceptor agonists, for both phasic activity and tone. The rank order of potency for the increase in tone was: McN-A-343 (first phase) > acetylcholine = oxotremorine-M > carbachol = methacholine = bethanechol = McN-A-343 (second phase), while for induced phasic activity it was: oxotremorine-M > carbachol = acetylcholine = methacholine.

Concentration-response curves for agonists could not be

Table 1 Effects of cholinoceptor agonists on porcine intravesical ureter

Agonist	n	Tone			Phasic activity		
		pD_2	E_{max}	Slope	pD_2	E_{max}	Slope
– Physostigmine							
Acetylcholine	8	5.58 ± 0.15	1.50 ± 0.08	0.70 ± 0.08	5.52 ± 0.11	12.57 ± 0.90	1.12 ± 0.06
Methacholine	6	5.46 ± 0.16	1.14 ± 0.27*	1.11 ± 0.10	5.47 ± 0.04	14.61 ± 2.33	1.36 ± 0.09
Carbachol	12	5.60 ± 0.28	1.81 ± 0.29	1.57 ± 0.28[a]	5.88 ± 0.16	14.27 ± 1.39	1.22 ± 0.17
+ Physostigmine							
Acetylcholine	8	6.72 ± 0.14*	1.61 ± 0.25	1.42 ± 0.12[a]	5.57 ± 0.15	13.91 ± 3.08	0.99 ± 0.15
Methacholine	6	5.63 ± 0.19	1.17 ± 0.18*	1.26 ± 0.06	5.53 ± 0.06	14.75 ± 4.55	1.07 ± 0.02
Carbachol	8	5.64 ± 0.35	1.83 ± 0.24	1.52 ± 0.23[a]	5.79 ± 0.21	15.11 ± 1.17	1.19 ± 0.12
Oxotremorine-M	8	6.62 ± 0.12*	2.16 ± 0.44	2.23 ± 0.23[a]	6.59 ± 0.05*	15.01 ± 3.49	1.95 ± 0.69[a]
Bethanechol	6	5.37 ± 0.13	1.76 ± 0.28	1.03 ± 0.04	–	–	–
McN-A-343	8	5.69 ± 0.16	2.02 ± 0.22	0.84 ± 0.14	–	–	–

n, number of ureters. $pD_2 = -\log EC_{50}$; EC_{50} is the effective concentration which induced 50% of the maximal response. E_{max} is the maximum effect in case of ureteral tonus (g) or phasic activity (number contractions min^{-1}). Results are expressed in absolute values as mean ± s.e.mean.

[a]Slope factor significantly different from unity.

*Significantly different parameter compared to carbachol ($P<0.05$, *a posteriori*, Bonferroni).

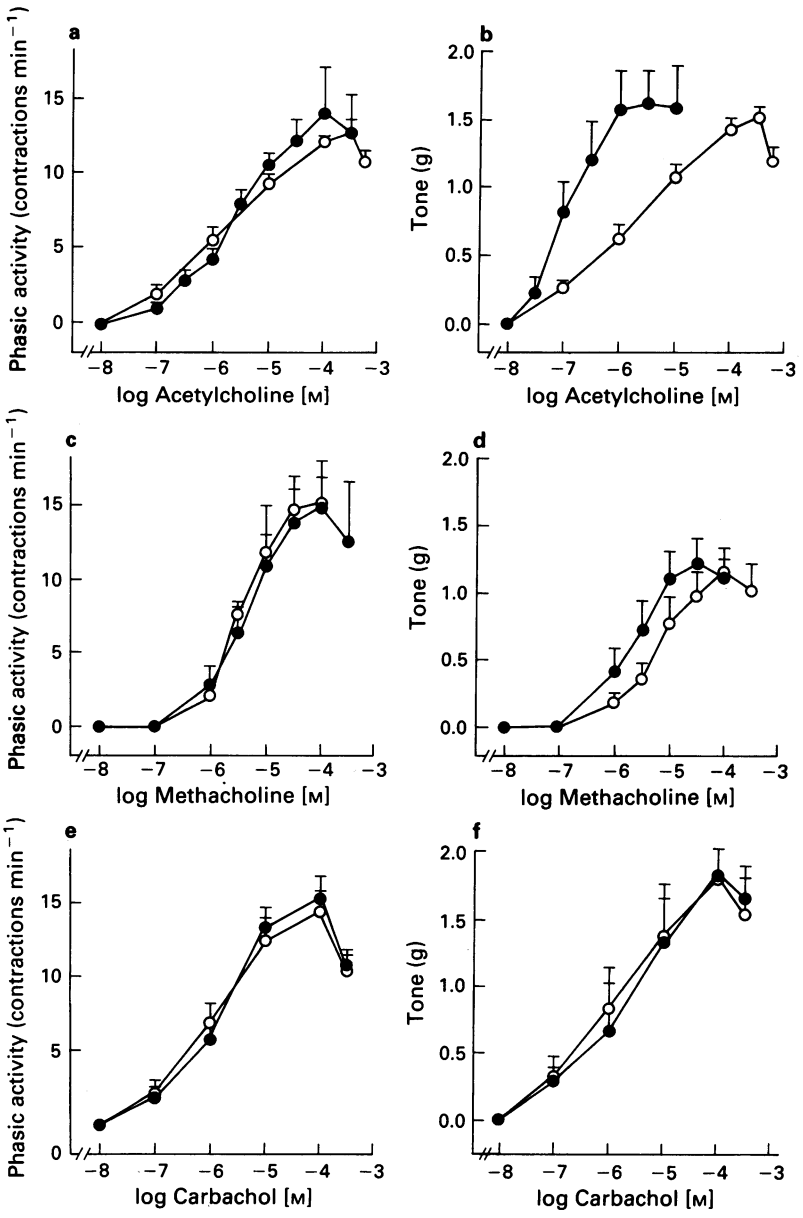


Figure 1 Concentration-response curves of porcine intravesical ureteral preparations to increasing single doses of acetylcholine (a, b), methacholine (c, d) and carbachol (e, f) in absence (○) and presence (●) of physostigmine (10^{-6} M). (a, c and e) Phasic activity, and (b, d and f) tone of the preparations. Each point represents mean (± s.e.mean, vertical bar) of 6–8 preparations. Results are expressed as absolute values.

repeated on the same preparation due to the development of strong tachyphylaxis. Thus, in a first concentration-response curve, the pD_2 and E_{max} values for carbachol on basal tone were 5.60 ± 0.28 and 1.81 ± 0.29 ($n = 12$), respectively, while in a second curve the pD_2 and E_{max} values were 5.24 ± 0.21 and 1.37 ± 0.44 ($P < 0.05$, paired t test). However, increases in both phasic activity and basal tone induced by a submaximal dose (10^{-5} M) of carbachol were reproducible during repetitive exposures. The phasic activity and tone evoked by a single dose (10^{-5} M) of carbachol on the first exposure were 12.36 ± 1.54 contractions min^{-1} and 1.36 ± 0.38 g respectively, compared to 11.21 ± 1.72 contractions min^{-1} and 1.32 ± 0.29 g respectively to the fifth exposure ($n = 24$).

Antagonist study

Treatment of ureteral strips with the selective nicotinic cholinoceptor antagonist, hexamethonium (10^{-6}–10^{-4} M) did not alter the response to 10^{-5} M carbachol. The tone was 1.42 ± 0.21 g and phasic activity 10.18 ± 1.33 contractions min^{-1} before, and tone 1.37 ± 0.39 g and phasic activity 9.43 ± 1.52 contractions min^{-1} after incubation with hexamethonium (10^{-4} M) ($n = 8$).

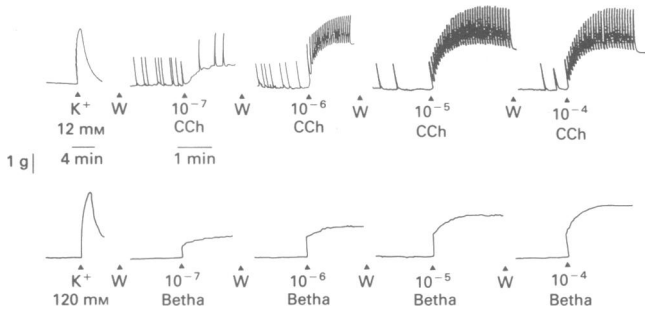


Figure 2 Traces showing the response of intravesical ureteral preparations of the pig to 120 mM K^+-PSS and increasing concentrations of carbachol (CCh, 10^{-7}–10^{-4} M) and bethanechol (Betha, 10^{-7}–10^{-4} M) added in single doses with washout (W) between each response. Numbers indicate molar concentration in the bath.

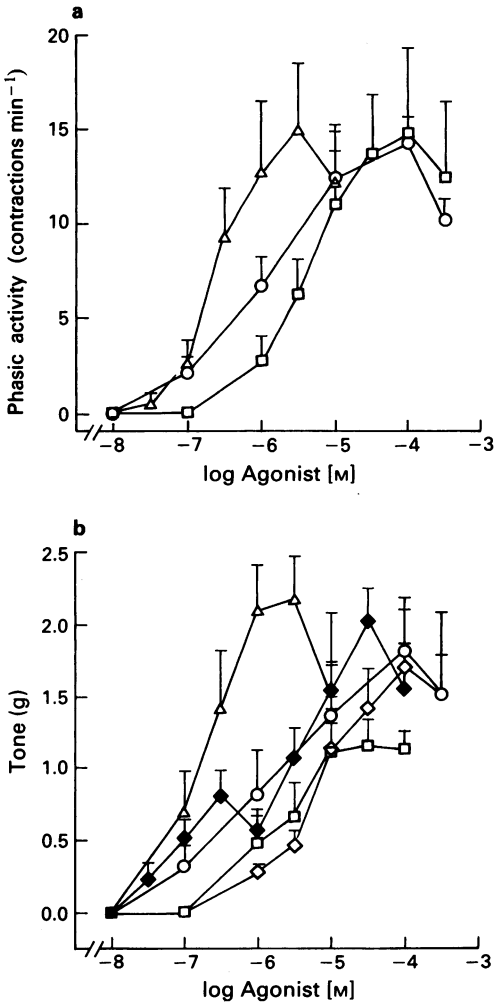


Figure 3 Concentration-response curves of porcine intravesical ureteral preparations to increasing single doses of carbachol (○), methacholine (□), oxotremorine-M (△), bethanechol (◇), and McN-A343 (◆). (a) Phasic activity, and (b) tone of the preparations. Each point represents mean (± s.e.mean, vertical bar) of 6–12 preparations. Results are expressed as absolute values.

Table 2 Effects of muscarinic cholinoceptor antagonists on tone and phasic activity induced by carbachol (10^{-5} M) in porcine intravesical ureter

		Tone			*Phasic activity*		
Antagonist	*n*	pIC_{50}	Slope	pK_B	pIC_{50}	Slope	pK_B
Atropine	8	10.73 ± 0.13	1.43 ± 0.21	10.81	10.16 ± 0.06*	1.72 ± 0.17	10.62[a]
Pirenzepine	8	8.43 ± 0.18	0.83 ± 0.09	8.60	7.53 ± 0.18*	0.86 ± 0.08	7.86
AF-DX 116	6	7.04 ± 0.22	0.77 ± 0.10	6.91	7.45 ± 0.16	1.33 ± 0.11	7.77
Methoctramine	6	7.79 ± 0.24	0.82 ± 0.06	8.12	7.98 ± 0.20	0.76 ± 0.10	8.35
4-DAMP	6	9.32 ± 0.06	0.93 ± 0.06	9.44	9.12 ± 0.09	1.14 ± 0.08	9.58
HHSiD	6	8.41 ± 0.05	1.18 ± 0.33	8.55	8.30 ± 0.12	1.26 ± 0.19	8.76
p-F-HHSiD	6	8.38 ± 0.13	1.12 ± 0.02	8.47	7.88 ± 0.15*	1.83 ± 0.18	8.28
Tropicamide	10	7.53 ± 0.09	0.68 ± 0.11	7.70	7.62 ± 0.11	0.72 ± 0.13	8.11[a]

n, number of ureters. pIC_{50} defined as the negative logarithm of the antagonist concentration that causes a 50% inhibition of the contraction induced by carbachol (10^{-5} M). Results are expressed in absolute values as mean ± s.e.mean.
*Significantly different parameter compared to pIC_{50} values for tone.
[a]Significantly different parameter compared to pIC_{50} and pK_b values of antagonist inhibiting carbachol-induced phasic activity in the same preparation ($P < 0.05$, paired t test). pK_b defined as the negative logarithm of K_b; K_b is the dissociation constant. IC_{50} values were converted to K_b values by using the equation of Leff & Dougall (1993):

$$\text{Equation of Leff \& Dougall} \quad K_b = \frac{[IC_{50}]}{\left(2 + \left(\frac{[A_f]}{[EC_{50}]}\right)^b\right)^{1/b} - 1}$$

A_f and b are the fixed concentration (10^{-5} M) and the slope factor of single increasing dose-response of carbachol, respectively. For abbreviations, see text.

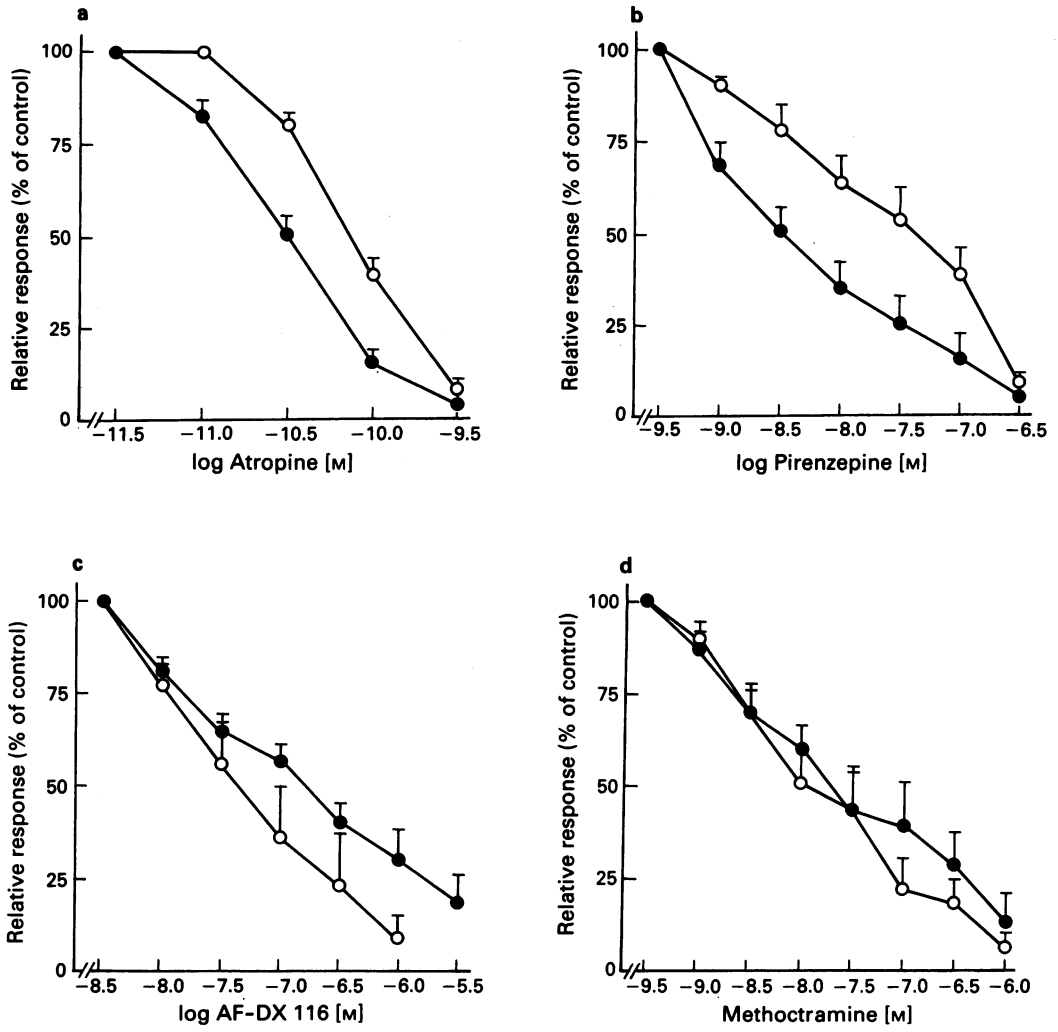


Figure 4 Effects of atropine (a), pirenzepine (b), AF-DX 116 (c) and methoctramine (d) on phasic activity (○) and tone (●) induced by carbachol (10^{-5} M). Each curve represents mean (± s.e.mean) of 6–8 strips. Results show the relative response to carbachol after incubation with increasing concentrations of atropine, pirenzepine, AF-DX 116 and methoctramine.

All the antagonists significantly inhibited both tone and phasic activity induced by a submaximal dose of carbachol (10^{-5} M). The effects of atropine, the selective M_1 antagonist, pirenzepine, and the selective M_2 antagonists AF-DX 116 and methoctramine are shown in Table 2 and Figure 4. The effects of the M_3 selective antagonists 4-DAMP, HHSiD and *p*-F-HHSiD and the M_4 selective antagonist, tropicamide are shown in Table 2 and Figure 5. Atropine and both the preferential M_1 and M_3 muscarinic receptor antagonists, pirenzepine and *p*-F-HHSiD, respectively, showed a higher inhibitory effect on tonic than phasic contractile activity while the selective M_4 antagonist, tropicamide blocked both contractile parameters induced by a submaximal dose of carbachol (Table 2). This indicates different profiles of the muscarinic receptor antagonists in inhibiting tone and frequency of contractions to 10^{-5} M carbachol in the porcine intravesical ureter.

Discussion

Functional studies have shown that autonomic neurotransmitters can affect the rate of urine transport at physiological flow rates through the canine ureter by modulating not only the peristaltic frequency but also the urine bolus volume. Thus, acetylcholine increased the peristaltic frequency, reduced the bolus volume and decreased the fluid transport (Morita *et al.*, 1987). In our study, acetylcholine in the presence of physostigmine evoked increases in both phasic activity and basal tone of pig intravesical ureteral preparations. However, preincubation of ureteral strips with physostigmine only enhanced the effect of acetylcholine on the tonic contraction of the ureter, suggesting that the muscarinic receptors involved in this response are in a different location from those enhancing the phasic activity, and are protected from bath-applied acetylcholine by acetylcholinesterase. In contrast, the neuronal uptake blocker of noradrenaline increased the phasic activity without affecting the basal tone (Hernández *et al.*, 1992), although exogenously applied noradrenaline was shown to modulate both phasic and tonic contractile activity. Although the effects of intrinsic nerve stimulation have not been studied, these results suggest that both sympathetic and parasympathetic nerves may control the transport of urine into the bladder, the sympathetics enhancing urine flow by increasing phasic activity, and the parasympathetics producing sustained contraction to prevent reflux during evacuation of the bladder as suggested earlier (Rivera *et al.*, 1992b).

Agonist study

The present study was performed in order to characterize the muscarinic receptors involved in the phasic and tonic contractile activity of the pig isolated intravesical ureter. For this

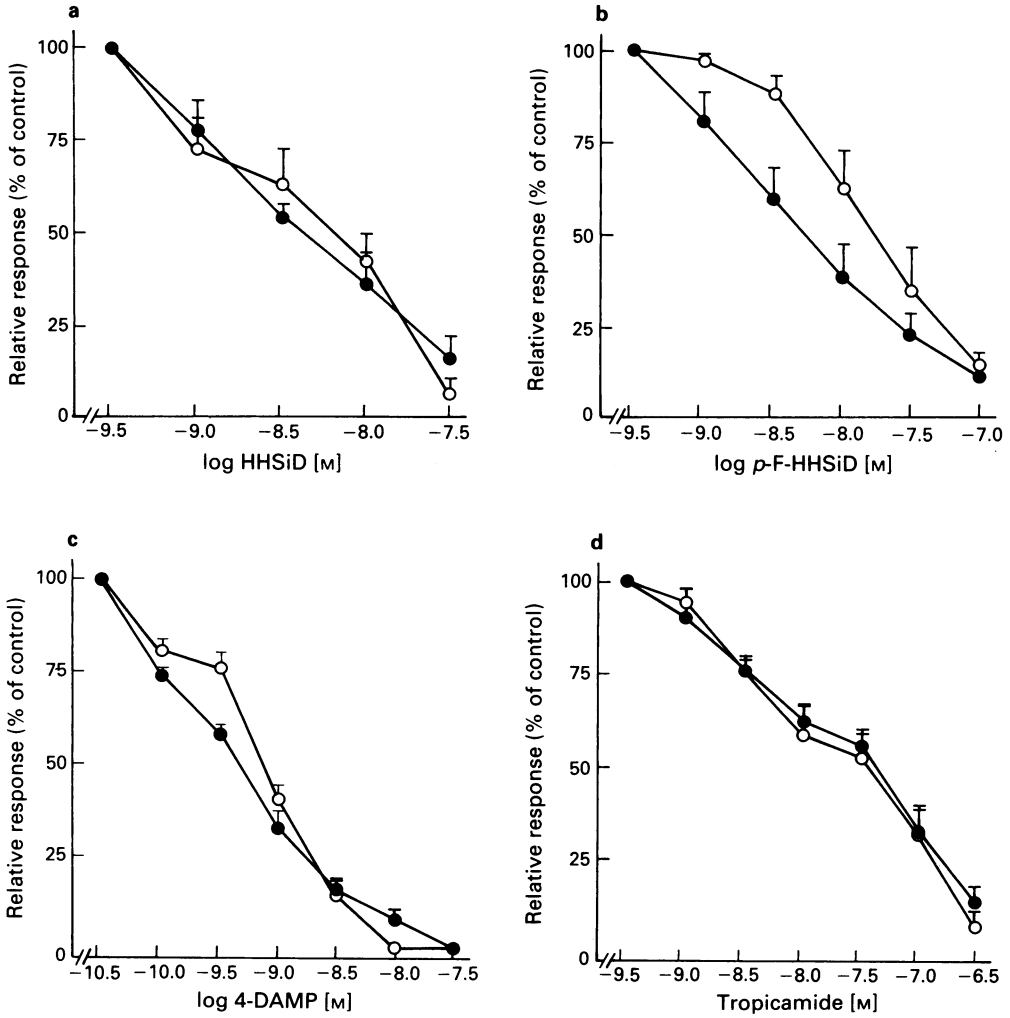


Figure 5 Effects of HHSiD (a), *p*-F-HHSiD (b), 4-DAMP (c) and tropicamide (d) on phasic activity (○) and tone (●) induced by carbachol (10^{-5} M). Each point represents mean (± s.e.mean) of 6–10 strips. Results show the relative response to carbachol after incubation with increasing concentrations of HHSiD, *p*-F-HHSiD, 4-DAMP and tropicamide. For abbreviations, see text.

purpose we used selective agonists and antagonists to determine the muscarinic receptor subtypes that mediate the phasic contractile activity and increases of basal tone of the isolated intravesical ureteral strips to carbachol.

The potency order of agonists which enhanced the phasic contractions in our preparations is similar to that obtained in the rat bladder (Grana *et al.*, 1987) and rabbit vas deferens (Eltze, 1988), both of which possess M_2-receptors. The putative M_1 (Hammer & Giachetti, 1982) and M_3 (Rattan & Goyal, 1984) agonists, McN-A-343 and bethanechol respectively, only increased the tone without producing phasic activity.

Our agonist study suggests that both M_1 and M_3 muscarinic receptor subtypes mediate the increases in the basal tone, while M_2-receptors are involved in the increases in the frequency of phasic contractions in the porcine isolated intravesical ureter in response to cholinoceptor agonists, although the selectivity of the muscarinic agonists is very low.

Antagonist study

The potencies of the antagonists are expressed as pIC_{50} values and are compared with pA_2 values obtained in other tissues. We also obtained pK_b values using the equation of Leff & Dougall (1993) with the aim of comparing these values with the pIC_{50} values and evaluating the validity of this equation for application in functional studies of ureteral strips as in other tissues which manifested tachyphylactic activity. The pK_b values obtained were not significantly different from the pIC_{50} values, which suggests that the equation of Leff & Dougall (1993) could be a good tool for antagonist study in pig isolated intravesical ureter.

Hexamethonium, a ganglionic nicotinic receptor blocker failed to modify the carbachol effect, suggesting that activation of nicotinic receptors is not involved in the response. The lack of activity of hexamethonium could be related to the absence of AChE-positive ganglion-cells in the porcine isolated intravesical ureter in contrast to the body of the urinary bladder and the sheep ureter vesical junction (Rivera *et al.*, 1991; 1992b), where there were numerous autonomic intramural ganglia mostly composed of AChE-positive neurones.

In contrast to hexamethonium, atropine inhibited with high affinity both the phasic activity and basal tone induced by carbachol in intravesical ureteral strips, suggesting that these contractile responses are mediated through muscarinic receptors.

The clearest evidence for postulating different muscarinic receptor subtypes has been obtained with the selective M_1-antagonist, pirenzepine, which differentiates muscarinic receptor subtypes through its markedly higher affinity for the M_1-receptor (Hammer *et al.*, 1980; Watson *et al.*, 1982). The pIC_{50} values obtained for this drug on both phasic activity (7.53) and tone (8.45) of the pig intravesical ureter seem to

indicate that tonic but not phasic activity could be mediated through M_1 receptors. This is supported by the range of selectivity of pirenzepine for M_1-receptors (Eglen & Whiting, 1986; Mitchelson *et al.*, 1989). Moreover, pirenzepine had a significantly higher affinity for inhibition of the tone than the phasic activity which could indicate that M_1 muscarinic receptor activation is implicated in the constriction of the ureter.

On the other hand, the selective M_2 antagonist, AF-DX 116 (Giachetti *et al.*, 1986) exhibited similar pIC_{50} values on both tone (7.04) and phasic activity (7.45) which seems to correspond with the stimulation of M_2 receptors. These results are consistent with pA_2 values found in the vas deferens (6.85–7.89) (Eltze, 1988). In order to confirm these findings we used methoctramine and obtained a pIC_{50} of 7.79 and 7.98 on tone and phasic activity, respectively, which is similar to the pA_2 value (7.8) reported for M_2 in the porcine basilar artery (Eglen & Whiting, 1990).

The high pIC_{50} values obtained for 4-DAMP on both phasic activity and basal tone (9.12 and 9.32, respectively), are very close to those reported for M_3 receptors (9.04–9.50) (Clague *et al.*, 1985; Grider *et al.*, 1987; Kurtel *et al.*, 1990). Moreover, these results are similar to those found in the guinea-pig muscularis mucosae (9.32) (Barocelli *et al.*, 1990), common bile duct (8.99) (Karahan *et al.*, 1991), in the chick and guinea-pig ileum (9.2 and 9.1 respectively) (Choo *et al.*, 1988), and in the sheep detrusor muscle and ureterovesical junction (9.26 and 9.41 respectively) (Rivera *et al.*, 1991; 1992b). However, since 4-DAMP does not clearly differentiate between M_1 and M_3 receptors (Delmendo *et al.*, 1989; Hulme *et al.*, 1990), we used *p*-F-HHSiD and HHSiD in our study. These antagonists exhibit high affinity for the M_3 receptor subtype (Lambrecht *et al.*, 1988; Duckles, 1990; Eglen *et al.*, 1990). In the present study, the pIC_{50} values (8.38 and 7.88) obtained with *p*-F-HHSiD on tone and phasic activity, respectively, seem to correspond to M_3 receptors. *p*-F-HHSiD was more potent in inhibiting the tone than phasic activity, indicating a possible involvement of M_3 receptors in contraction of the ureteral smooth muscle. These results are consistent with pA_2 values for M_3-receptors (7.50–8.22) obtained in different vascular preparations, human SH-SY5Y cells, guinea-pig ileum, and oesophageal muscularis mucosae (Duckles, 1990; Eglen *et al.*, 1990). HHSiD showed high affinity in inhibition of both phasic activity and basal tone ($pIC_{50} = 8.22$ and 8.65, respectively) consistent with the activation of M_3 receptors, such as found in the guinea-pig ileum ($pA_2 = 8.08–8.40$) (Fuder *et al.*, 1985), in the sheep detrusor muscle (8.49) (Rivera *et al.*, 1991) and ureterovesical junction (8.66) (Rivera *et al.*, 1992b).

Finally, with a muscarinic antagonist reported to have modest affinity for the M_4-receptors (Lazareno *et al.*, 1993), we obtained a pK_b value for inhibition of the phasic contractions induced with carbachol similar to the pK_b value obtained in rabbit lung (Lazareno *et al.*, 1990). This indicates that the phasic contractions induced by the cholinoceptor agonists in the pig intravesical ureter might be mediated through M_4-receptors.

The present investigation demonstrates that M_1, M_2 and M_3 muscarinic receptors could be involved in both phasic and tonic contractile activity of the pig intravesical ureter smooth muscle. These results are similar to those found in the gastrointestinal tract (Goyal, 1988), in the human lung (MacLagen & Barnes, 1990) and in the sheep ureterovesical junction (Rivera *et al.*, 1992b), where the contractile responses of muscarinic agonists are mediated through three pharmacologically defined muscarinic subtypes.

Moreover, we suggest the presence of an M_4 muscarinic receptor subtype possibly involved in the phasic activity, due to the high affinity exhibited for methoctramine, 4-DAMP, HHSiD and tropicamide in porcine ureteral strips. This antagonist profile is similar to that previously reported for M_4 receptors characterized in binding studies of NG108-15 cells and rat forebrain (Michel *et al.*, 1989; Waelbroeck *et al.*, 1990).

In summary, some of the cholinoceptor agonists evoked increases in the phasic contractions while all induced tone in the pig intravesical ureter; as different antagonist profiles were obtained in inhibition of the phasic activity and tone, the present study suggests that different muscarinic receptor subtypes mediate the phasic and tonic contractile activity induced by a submaximal concentration of carbachol. This might be of importance for both the urine bolus transport during bladder filling and the parasympathetic constriction of the ureter preventing the vesicoureteral reflux during the emptying phase of the urinary bladder.

This work was supported by project PM 88-0035 (DGYCIT). The authors thank Dr Barlow, Bristol, U.K., for the gift of 4-DAMP, Dr Karl Thomae, GmbH, for the gift of AF-DX 116, and the Madrid Municipal Slaughterhouse for kindly donating the ureters.

References

BARAJAS, L. & WANG, P. (1983). Simultaneous ultrastructural visualization of acetylcholinesterase activity and tritiated norepinephrine uptake in renal nerves. *Anat. Rec.*, **205**, 185–195.

BARLOW, R.B., BERRY, K.J., GLENTON, P.A.M., NIKOLAOU, N.M. & SOH, K.S. (1976). A comparison of affinity constants for muscarine-sensitive acetylcholine receptors in guinea-pig atrial pacemaker cells at 29°C and 37°C. *Br. J. Pharmacol.*, **58**, 613–620.

BAROCELLI, E., CHIAVARINI, M., MORINI, G., BALLABENI, V., GENTILE, A. & IMPICCIATORE, M. (1990). Pharmacological evidence that muscarinic M_1-receptors are not involved in the contractile activity of guinea-pig oesophageal muscularis mucosae. XIth International Congress of Pharmacology. Amsterdam, The Netherlands. *Eur. J. Pharmacol.*, **183**, 1725–1732.

BONNER, T.I., BUCKLEY, N.J., YOUNG, A.C. & BRANN, M.R. (1987). Identification of a family of muscarinic acetylcholine receptor genes. *Science*, **237**, 527–532.

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469–476.

CHOO, L.K., MITCHELSON, F. & NAPIER, P. (1988). Difference in antagonist affinities at muscarinic receptors in chick and guinea-pig. *J. Auton. Pharmacol.*, **8**, 259–266.

CLAGUE, R.U., EGLEN, R.M., STRACHEN, A.C. & WHITING, R.L. (1985). Action of agonists and antagonists at muscarinic receptors present on ileum and atria *in vitro*. *Br. J. Pharmacol.*, **86**, 163–170.

DELMENDO, R.E., MICHEL, A.D. & WHITING, R.L. (1989). Affinity of muscarinic receptor antagonists for three putative muscarinic receptor binding sites. *Br. J. Pharmacol.*, **96**, 457–464.

DOODS, H.N., MATHY, M.J., DAVESKO, D., VAN CHARLDORP, K.J., DE JONGE, A. & VAN ZWEITEN, P.A. (1987). Selectivity of muscarinic agonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3 receptors. *J. Pharmacol. Exp. Ther.*, **242**, 257–262.

DUCKLES, S.P. (1990). p-Fluoro-hexahydro-sila-difenidol: affinity for vascular muscarinic receptors. *Eur. J. Pharmacol.*, **185**, 227–230.

EGLEN, R.M., MICHEL, A.D., MONTGOMERY, W.W., KUNYSZ, E.A., MACHADO, C.A. & WHITING, R.L. (1990). The interaction of parafluorohexahydrosiladiphenidol at muscarinic receptors *in vitro*. *Br. J. Pharmacol.*, **99**, 637–642.

EGLEN, R.M. & WHITING, R.L. (1986). Muscarinic receptor subtypes a critique of the current classification and a proposal for a working nomenclature. *J. Auton. Pharmacol.*, **5**, 323–346.

EGLEN, R.M. & WHITING, R.L. (1990). Heterogeneity of vascular muscarinic receptors. *J. Auton. Pharmacol.*, **19**, 233–245.

ELTZE, M. (1988). Muscarinic M_1- and M_2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.*, **151**, 205–221.

FUDER, H., KILBINGER, H. & MÜLLER, H. (1985). Organ selectivity of hexahydrosiladifenidol in blocking pre- and postjunctional muscarinic receptors studied in guinea-pig ileum and rat heart. *Eur. J. Pharmacol.*, **113**, 125–127.

GIACHETTI, A., MICHELETTI, R. & MONTAGNA, E. (1986). A cardioselective profile of AF-DX 116, a muscarine M_2 receptor antagonist. *Life Sci.*, **38**, 1663–1672.

GOYAL, R.K. (1988). Identification, localization and classification of muscarinic receptor subtypes in the gut. *Life Sci.*, **43**, 2209–2220.

GRANA, E., LUCCHELLI, A., ZONTA, F. & BOSELLI, C. (1987). Determination of dissociation constants and relative efficacies of some potent muscarinic agonists at postjunctional muscarinic receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 8–15.

GRIDER, J.R., BITAR, K.N. & MAKHLOUF, G.M. (1987). Identification of muscarinic M_2 receptors on single muscle cells of the human and guinea-pig intestine. *Gastroenterology*, **93**, 951–957.

HAMMER, R., BERRIE, C.P., BIRDSALL, N.J., BURGEN, A.S.V. & HULME, E.C. (1980). Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature*, **283**, 90–94.

HAMMER, R. & GIACHETTI, A. (1982). Muscarinic receptor subtypes M_1 and M_2. Biochemical and functional characterization. *Life Sci.*, **31**, 2991–2994.

HERNANDEZ, M., PRIETO, D., SIMONSEN, U., RIVERA, L., BARAHONA, M.V. & GARCIA-SACRISTAN, A. (1992). Noradrenaline modulates smooth muscle activity of the isolated intravesical ureter of the pig through different types of adrenoceptors. *Br. J. Pharmacol.*, **107**, 924–931.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

KARAHAN, F., ALICAN, I., OZKUTLU, U., ONAT, F., YEGEN, B.C., ULUSOY, B. & OKTAY, S. (1991). Muscarinic receptor subtypes of guinea-pig common bile duct. *Arch. Int. Pharmacodyn.*, **312**, 140–145.

KENAKIN, T.P. (1984). The classifications of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.*, **36**, 165–222.

KURTEL, H., DEDEOGLU, A., YEGEN, B.C., ULUSOY, N.B. & OKTAY, S. (1990). Muscarinic receptor subtypes of guinea-pig gallbladder smooth muscle. *Arch. Int. Pharmacodyn.*, **308**, 39–46.

LAMBRECHT, G., FEIFEL, R., FORTH, B., STROHMANN, C., TACKE, R. & MUTSCHLER, E. (1988). p-Fluoro-hexahydro-sila-difenidol: the first $M_{2\beta}$-selective muscarinic antagonist. *Eur. J. Pharmacol.*, **152**, 193–194.

LAZARENO, S., BUCKLEY, N.L. & ROBERTS, F.F. (1990). Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG 108-15 cells. *Mol. Pharmacol.*, **38**, 805–815.

LAZARENO, S., FARRIES, T. & BIRDSALL, N.J.M. (1993). Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors M_1–M_4. *Life Sci.*, **52**, 449–456.

LEFF, P. & DOUGALL, I.G. (1993). Further concerns over Cheng-Prusoff analysis. *Trends Pharmacol. Sci.*, **14** (Suppl.), 110–112.

MACLAGAN, J. & BARNES, P.J. (1990). Muscarinic pharmacology of the airways. *Trends Pharmacol. Sci.*, **10** (Suppl.), 88–92.

MELCHIORRE, C. (1988). Polymethylene tetramines: a new generation of selective muscarinic antagonists. *Trends Pharmacol. Sci.*, **9**, 216–220.

MICHEL, A.D., DELMENDO, E., STEFANICH & WHITING, R.L. (1989). Binding characteristics of the muscarinic receptor subtype of the NG108-15 cell line. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 62–71.

MITCHELSON, F., CHOO, L.K. & DARROCH, S. (1989). Muscarinic receptor differentiation. *Clin. Exp. Pharmacol. Physiol.*, **16**, 523–528.

MORITA, T., WADA, I., SAEKI, H., TSUCHIDA, S. & WEISS, R.M. (1987). Ureteral urine transport: changes in bolus volume, peristaltic frequency, intraluminal pressure and volume of flow resulting from autonomic drugs. *J. Urol.*, **137**, 132–135.

PERALTA, E.G., WINSLOW, J.W., PETERSON, G.L., SMITH, D.H., ASHKENAZI, A., RAMACHANDRAN, J., SCHIMERLIK, M.I. & CAPON, D.J. (1987). Primary structure and biochemical properties of an M_2 muscarinic receptor. *Science*, **236**, 600–605.

PRIETO, D., BENEDITO, S., RIVERA, L., HERNANDEZ, M. & GARCIA-SACRISTAN, A. (1990). Autonomic innervation of the equine urinary bladder. *Anat. Histol. Embryol.*, **19**, 276–285.

PRIETO, D., BENEDITO, S., RODRIGO, J., MARTINEZ-MURILLO, R. & GARCIA-SACRISTAN, A. (1989). Distribution and density of neuropeptide Y-immunoreactive nerve fibers and cells in the horse urinary bladder. *J. Auton. Nerv. Syst.*, **27**, 173–180.

RATTAN, S. & GOYAL, R.K. (1984). Identification of M_1 and M_2 muscarinic receptor subtypes in the control of the lower esophageal sphincter in the opossum. *Trends Pharmacol. Sci.*, **5** (Suppl.), 78–81.

RIVERA, L., HERNANDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992a). Mediation of contraction and relaxation by alpha and beta-adrenoceptors in the ureterovesical junction of the sheep. *Res. Vet. Sci.*, **52**, 57–61.

RIVERA, L., HERNANDEZ, M., BENEDITO, S., PRIETO, D. & GARCIA-SACRISTAN, A. (1992b). Mediation of contraction by cholinergic muscarinic receptors in the ureterovesical junction. *J. Auton. Pharmacol.*, **12**, 175–182.

RIVERA, L., PRIETO, D., HERNANDEZ, M., BENEDITO, S. & GARCIA-SACRISTAN, A. (1991). Distribution and function of cholinergic receptors in the sheep detrusor muscle. *J. Auton. Nerv. Syst.*, **34**, 95–102.

SCHULMAN, C.C. (1985). Innervation of the ureter: a histochemical and ultrastructural study. In *Urodynamics, Upper and Lower Urinary Tract*, II. ed. Lutzeyer, W. & Hannappel, ?. pp. 292–361. Berlin: Springer-Verlag.

SKARBY, T.V. & LARSSON, B. (1987). Theoretical and functional studies on $alpha_1$ and $alpha_2$-adrenoceptors: and examination using the Schild plot. *J. Auton. Pharmacol.*, **7**, 185–198.

WAELBROECK, M., TASTENOY, M., CAMUS, J. & CHRISTOPHE, J. (1990). Binding of selective antagonists to four muscarinic receptors (M_1–M_4) in rat forebrain. *Mol. Pharmacol.*, **38**, 267–274.

WALLESTEIN, S., ZUCKER, G.L. & FLEISS, J.L. (1980). Some statistical methods useful in circulation research. *Circ. Res.*, **47**, 1–9.

WATSON, M., ROESKE, N.R. & YAMAMURA, H.I. (1982). [^{3}H]-pirenzepine selectively identifies a high affinity population of muscarinic cholinergic receptors in the rat cerebral cortex. *Life Sci.*, **31**, 2019–2023.

(Received March 5, 1993
Revised August 4, 1993
Accepted August 13, 1993)

Br. J. Pharmacol. (1993), **110**, 1421–1428 © Macmillan Press Ltd, 1993

Dual contractile effects of ATP released by field stimulation revealed by effects of α,β-methylene ATP and suramin in rat tail artery

[1]Jian-Xin Bao & Lennart Stjärne

Department of Physiology I, Karolinska Institutet, S-171 77 Stockholm, Sweden

1 The field stimulation-induced release of endogenous ATP and noradrenaline (NA) and contractile response in rat isolated tail artery were examined. The release of ATP was studied by extracellular electrophysiological recording and that of NA by a novel voltammetrical technique. The effects of the P_2-purinceptor antagonist, suramin, on these parameters were compared with those of α,β-methylene ATP, a P_{2X}-purinoceptor desensitizing agent.

2 Neither α,β-methylene ATP (10 μM) nor suramin (100–500 μM) had significant effects on the extracellularly recorded nerve terminal action potential but both abolished the ATP-induced excitatory junction current caused by stimulation at 0.1 Hz. Neither agent affected significantly the voltammetrically measured release of NA induced by 10 or 100 pulses at 20 Hz.

3 Combined blockade of both postjunctional α_1- and α_2-adrenoceptors by prazosin and yohimbine (both 0.1 μM) profoundly depressed the contractile response to 10 pulses at 20 Hz. The small and fast residual contraction in the presence of these agents was abolished by α,β-methylene ATP (10 μM) and inhibited by suramin in a concentration-dependent manner (10–500 μM; IC_{50} 75 μM) and was hence probably caused by ATP or a related nucleotide.

4 When added first, α,β-methylene ATP (10 μM) or suramin (100–500 μM) delayed the onset and enhanced the amplitude of the neurogenic contraction. This enhanced response was abolished by further addition of prazosin and yohimbine (both 0.1 μM).

5 The K^+ channel blocker, tetraethylammonium (10 mM), dramatically enhanced the contractile response to 100 pulses at 1 Hz and caused it to become diphasic. Addition of α,β-methylene ATP (10 μM) or suramin (100–500 μM) abolished the large initial twitch component of this contraction and depressed the tonic phase.

6 Like α,β-methylene ATP, suramin (500 μM) had no effect on the contraction caused by exogenous NA (10 nM–100 μM) or KCl (60 mM); both agents almost abolished the contraction caused by ATP (100 μM).

7 In conclusion, (i) the contractile response of rat tail artery to electrical field stimulation is mediated by both ATP and NA, and is thus an expression of ATP-NA co-transmission, (ii) the released ATP exerts two opposite effects via 'P_{2X}-like' purinoceptors, triggering the initial rapid phase of the neurogenic contraction and restricting the NA-mediated component of the contraction; and (iii) the source and possible physiological role of the ATP which causes the inhibitory effect are unknown at present.

Keywords: Adenosine 5'-triphosphate; α,β-methylene ATP; noradrenaline; P_{2X}-purinoceptors; neurogenic contraction; rat tail artery; suramin; sympathetic neurotransmission

Introduction

Adenosine 5'-triphosphate (ATP) was proposed as a transmitter in non-adrenergic, non-cholinergic nerves in the gut and bladder in the 1970s and later as a co-transmitter with noradrenaline (NA) in sympathetic nerves (see Burnstock, 1990). Some doubts or controversies in this regard still exist (Hirst & Neild, 1980; Fredholm *et al.*, 1982), but the view that sympathetic nerves in addition to NA also release ATP (Burnstock, 1990; von Kügelgen & Starke, 1991a) as a co-transmitter is now increasingly accepted. ATP mediates the 'fast' electrical response to sympathetic nerve stimulation, the excitatory junctional current (e.j.c.) and potential (e.j.p.), and also often triggers the twitch component of the neurogenic contraction (Burnstock, 1990; von Kügelgen & Starke, 1991a). Recently, ATP has also been recognized as a fast ionotropic neurotransmitter both in the peripheral and central nervous systems (Silinksky *et al.*, 1992; Evans *et al.*, 1992; Edwards *et al.*, 1992).

In the tail artery of adult normotensive rats, the tissue examined in the present study, the e.j.cs and e.j.ps are caused by ATP via P_{2X}-purinoceptors (Sneddon & Burnstock, 1984; Neild & Kotecha, 1986; Åstrand & Stjärne, 1989). The neurogenic contraction has been thought to be mediated exclusively by NA (Cheung, 1984; Medgett & Langer, 1984; Vidal *et al.*, 1986), but we have found that the initial phase of this response was inhibited by α,β-methylene ATP (α,β-MeATP), a P_{2X}-purinoceptor desensitizing agent (Kasakov & Burnstock, 1983; Sneddon & Burnstock, 1984), and hence was mediated by ATP (Bao *et al.*, 1989; 1990; 1993a). We have also reported that α,β-MeATP strongly increased the amplitude of the NA-mediated neurogenic contraction. The potentiating effect was more marked on the α_1- than on the α_2-adrenoceptor-mediated component of this response; during stimulation at 20 Hz it was inversely related to the train length (Bao *et al.*, 1990; 1993a). A similar potentiating effect of α,β-MeATP on the neurogenic contraction has been reported to occur in other vascular tissues and in vas deferens (Bulloch *et al.*, 1990; MacDonald *et al.*, 1992). We have proposed the hypothesis that the two opposite effects of α,β-MeATP may unmask a dual, excitatory as well as inhibitory mechanical effect normally exerted by ATP via 'P_{2X}-like' purinoceptors (Bao *et al.*, 1990). In the present

[1] Author for correspondence.

paper we examined the validity of this hypothesis by comparing the effects of α,β-MeATP with those of suramin, a relatively selective P_2-purinoceptor antagonist (Dunn & Blakeley, 1988).

Methods

Recording of contractile responses

The method for recording the contractile responses has been described in detail elsewhere (Bao *et al.*, 1990). Briefly, ring segments (about 3 mm in length) of the proximal central caudal artery from male normotensive Sprague-Dawley rats (200–300 g) were mounted, under a resting tension of 5 mN, in a 2 ml perfusion chamber and perifused (at 1 ml min^{-1}, 37°C) by a Gilson Minipulse II pump with Tyrode solution of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and D-glucose 5.6. The solution in the reservoir was bubbled with 93.5% O_2 and 6.5% CO_2 to give a pH of 7.3–7.4. Electrical field stimulation (0.05 ms, 50–60 V) was applied via a pair of platinum electrodes (separation: about 10 mm) on either side of the preparation. The contraction of the circular muscle was measured isometrically by a Grass FTO3C force-displacement transducer and recorded by a BBC Goerz Metrawatt SE 120 potentiometric recorder. When necessary, the chart speed was increased and the stimulus artifacts were recorded for better visualization of the kinetics of the contractile responses. The contractile response to stimulation with the parameters used was abolished by tetrodotoxin (1 μM) or guanethidine (5 μM) and hence, caused by action potential-induced release of transmitters from sympathetic nerves.

The perivascular nerves of the preparations were stimulated, in most cases with 10 pulses at 20 Hz at 1 min intervals and in some cases with 100 pulses at 1 Hz at 5–8 min intervals. Drugs to be tested were added to the reservoir proximally to the pump to the final concentrations indicated, after the responses to stimulation had become stable. They were added at intervals of 10–30 min depending on the time required for the effects of each addition to become maximal; the response at the end of this period was expressed as a % of that before addition of the drug. When effects of suramin on contractions caused by exogenously applied NA, ATP and KCl were studied, these vasoconstrictors were added directly to the bath to the final concentrations indicated under stop-flow conditions with the solution in the bath bubbled.

Electrophysiological and electrochemical analysis of the release of ATP and NA

The release of ATP and NA from sympathetic nerve terminals was studied by electrophysiological (for details, see Brock & Cunnane, 1988; Åstrand & Stjärne, 1989; Stjärne & Stjärne, 1989) and electrochemical (see Mermet *et al.*, 1990; Gonon *et al.*, 1993; Bao *et al.*, 1993b) techniques, respectively. Separate chambers were used but the conditions were kept as similar as possible. Segments (about 2 cm) from the proximal region of the artery were mounted in a 2–3 ml Perspex organ chamber and pinned to the Sylgard layer. The preparations were perifused (at 2 ml min^{-1} and 37°C) with Tris-Tyrode solution (electrophysiology) or phosphate-buffered solution (electrochemistry). The Tris-Tyrode solution had the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.3, $MgCl_2$ 0.5, D-glucose 5.6, Tris 20. In the phosphate-buffered solution, Tris was replaced by Na_2HPO_4 4.06 and KH_2PO_4 0.73. Prazosin (0.1 μM) was routinely present in the phosphate-buffered solution to prevent or minimize contractions which might disturb the electrochemical measurements. The solution in the reservoir was bubbled with 100% O_2 (pH 7.3–7.4). The proximal end of the preparation was drawn into a stimulating suction electrode used to stimulate the perivascular sympathetic nerves with rectangular pulses (0.3 ms, 150–300 μA) at 0.1–20 Hz.

A glass micropipette (inner tip diameter 150–250 μm) was used as a recording electrode which was internally perfused at a constant rate (Tang *et al.*, 1990) with Tris-Tyrode solution of the same composition as that perfusing the bath. The recorded electrical responses, the nerve terminal spike (NTS) which reflects the nerve terminal action potential, and the excitatory junction current (e.j.c.) which reflects ATP release, were amplified by an a.c. amplifier, stored on magnetic tape, digitized and analysed off-line on an IBM AT personal computer. Addition of α,β-MeATP is known to abolish the ATP-induced, P_{2X}-purinoceptor-mediated intracellularly recorded excitatory junction potential (e.j.p.) (Sneddon & Burnstock, 1984; Neild & Kotecha, 1986). Similarly, addition of α,β-MeATP inside the recording electrode abolishes the e.j.c. (Åstrand & Stjärne, 1989; Stjärne & Stjärne, 1989). In the present experiments, α,β-MeATP (10 μM) was continuously present in the external perfusion medium to block the positive-going e.j.cs caused by ATP released from sites outside the recording electrode. Under control conditions, the e.j.cs declined somewhat during the first 10 min of stimulation at 0.1 Hz but then remained stable for at least 1 h (not shown). The agents to be tested were added to the medium perfusing the electrode to the final concentrations indicated.

An electrochemically pretreated carbon fibre electrode (active part: 8 μm in diameter, 50–80 μm in length) was used to monitor, by differential pulse amperometry (DPA) once per second, the oxidation current caused by NA released by nerve stimulation (see Gonon *et al.*, 1993; Bao *et al.*, 1993b). The signal recorded at + 120 mV, the oxidation potential of NA, reflects the rise in the local concentration of endogenous NA at the carbon fibre (CF) electrode and hence, is termed $\Delta[NA]_{CF}$. In these experiments, the preparations were stimulated with 10 or 100 pulses at 20 Hz every 2 min. After 2–3 control stimulations, the drugs to be tested were added to the reservoir proximally to the pump, to the final concentrations indicated, and their effects examined after they had been present for 20–30 min.

Analysis

The lag time of the contractile responses was measured from the onset of the stimulus artifact to the onset of contractions. Data are expressed as means ± s.e.mean; *n* refers to the number of preparations (= the number of animals). Means were compared by Student's unpaired *t* test, Mann-Whitney U-test or one way analysis of variance (ANOVA) followed by Fisher PLSD test, as appropriate. $P \leq 0.05$ was regarded as significant.

Drugs

α,β-Methylene adenosine 5'-triphosphate lithium salt, tetrodotoxin, yohimbine hydrochloride, and tetraethylammonium chloride (all from Sigma), guanethidine sulphate (Ciba-Geigy), prazosin hydrochloride (Pfizer) and suramin hexasodium salt (gift from Bayer) were used. Stock solutions of most drugs were prepared by dissolving them in distilled water but tetraethylammonium was dissolved directly in saline and prazosin in 50% ethanol. Control experiments showed that ethanol at its final concentration (0.0005%) did not *per se* affect the nerve stimulation-induced electrochemical and contractile responses.

Results

Effects of α,β-MeATP and suramin on e.j.cs

When present only in the external perfusion medium, α,β-MeATP (10 μM) for at least 1 h had no significant effect on the nerve terminal spike (not shown) or the e.j.c. (Figure 1a).

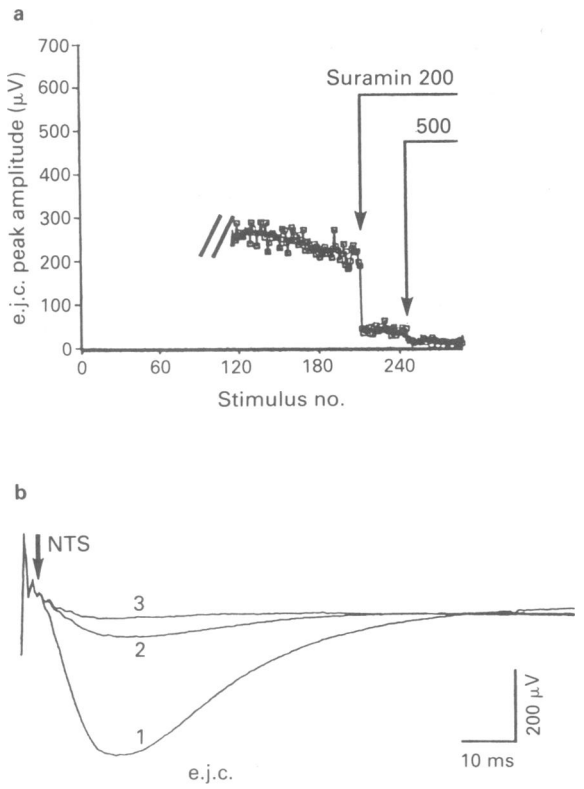


Figure 1 Effects of adding to the medium perfusing the recording electrode suramin (200 and 500 μM) on the nerve terminal spike (NTS) and the peak amplitude of the excitatory junction current (e.j.c.) evoked by stimulation at 0.1 Hz, in rat isolated tail artery. In (a), the e.j.c. peak amplitude is plotted against the stimulus number; initial parts of the e.j.c. responses are omitted. In (b) each trace is the average of 30 consecutive sweeps; traces 1–3 represent control, suramin 200 and 500 μM, respectively. (a) and (b) are from the same experiment. In all experiments, 10 μM α,β-methylene ATP was present in the external perfusion medium to block the positive-going e.j.cs caused by ATP released from sites outside the recording electrode. Four similar experiments were performed. Note that suramin did not affect the NTS but concentration-dependently inhibited the e.j.cs.

Nor did α,β-MeATP, when further added to the medium internally perfusing the electrode, affect the nerve terminal spike but now rapidly abolished the spontaneous and evoked e.j.cs (not shown). Similarly, addition of suramin (100–500 μM) to the medium perfusing the recording electrode did not significantly affect the nerve terminal spike but concentration-dependently inhibited the e.j.cs (Figures 1a,1b).

Effects of α,β-MeATP and suramin on $\Delta[NA]_{CF}$

The effects of α,β-MeATP and suramin on the nerve stimulation-induced rise in the concentration of endogenous NA at the carbon fibre electrode ($\Delta[NA]_{CF}$) was used to study their effects on NA release. The $\Delta[NA]_{CF}$ response to stimulation at 20 Hz with 10 or 100 pulse trains every 2 min was unaffected by 10 μM α,β-MeATP or 10–500 μM suramin (not shown).

Effects of α,β-MeATP and suramin on the contractile responses to electrical field stimulation, exogenous ATP, NA and KCl

Field stimulation with 10 pulses at 20 Hz at 1 min intervals produced reproducible, monophasic contractile responses (Figure 2). The contraction started after a delay of 374 ± 20 ms, continued to grow for 2.6 ± 0.2 s after the end of stimulation

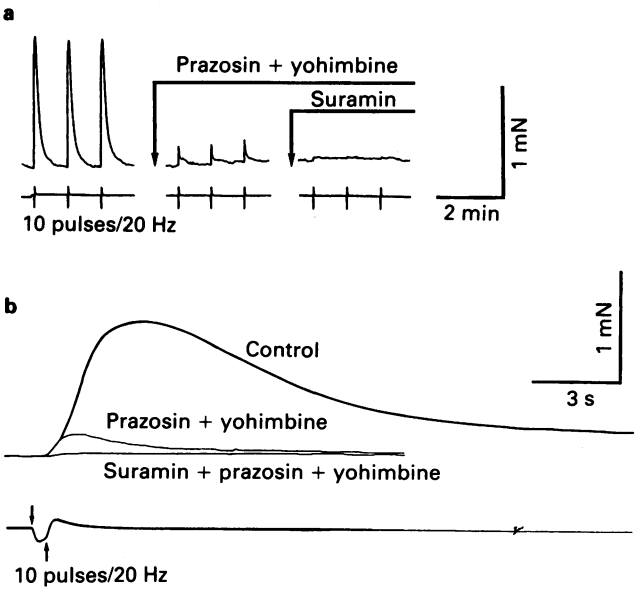


Figure 2 (a) Original recordings representative of 6 experiments in rat isolated tail artery, showing the effects of cumulative addition of prazosin, yohimbine (both 0.1 μM) and suramin (500 μM) on the contractile responses to electrical field stimulation with trains of 10 pulses at 20 Hz at 1 min intervals. The stimulation was discontinued for 20–30 min after addition of suramin; the intervals between addition of other drugs were 10–20 min. The bottom trace indicates the stimulus artifact. (b) Superimposed original recordings, same condition as in (a), on a faster time base to visualize the kinetics. Arrows in the bottom trace indicate the beginning and end of stimulation. Note that the contraction started after a delay (420 ms in this preparation) and continued to grow for several seconds (3.3 s) after the end of stimulation and that block of the NA-mediated contractions by prazosin and yohimbine in combination unmasked the small and fast, ATP-mediated component of the contraction.

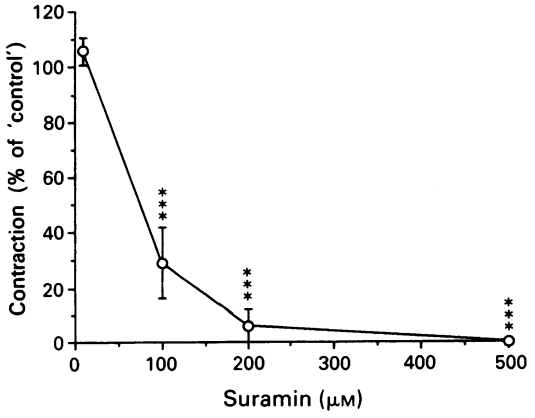


Figure 3 Concentration-dependence of the suramin-induced inhibition of the contractile response to 10 pulses at 20 Hz in the presence of prazosin and yohimbine (both 0.1 μM). Effects of suramin were evaluated after it had been present in the bath for 20–30 min. Contractions were expressed as a % of 'control' before addition of suramin (i.e. mean of 3–5 stable responses in the presence of prazosin and yohimbine). $n = 4–6$. ***$P < 0.001$ all *vs* control, one way ANOVA followed by Fisher PLSD test.

and had a peak amplitude of 0.9 ± 0.1 mN ($n = 14$). Combined addition of the α_1- and α_2-adrenoceptor antagonists, prazosin and yohimbine (both 0.1 μM), did not change the lag time but profoundly reduced the amplitude of the contraction (by 87.5 ± 2.9%, $n = 10$; $P < 0.001$) and shortened its duration (Figure 2b). Subsequent addition of α,β-MeATP

(10 μM) itself caused a contraction (peak value 6.5 ± 0.3 mN, $n = 51$) which subsided within 20–30 min. Now the small residual neurogenic contraction in the presence of prazosin and yohimbine was almost abolished (not shown, but see Figure 5 in Bao *et al.*, 1993a). Unlike α,β-MeATP, suramin (10–500 μM) did not change the resting tension, but like α,β-MeATP, concentration-dependently reduced the residual contractile response in the presence of prazosin and yohimbine (Figures 2,3); the IC_{50} for this effect was 75 μM (Figure 3).

When added alone (Figure 4), α,β-MeATP delayed the onset (from 374 ms to 673 ± 48 ms, $n = 4$; $P < 0.001$; Figure 4b) but increased the amplitude (by 244% on average, Figure 4c) of the neurogenic contraction. The enhanced contraction was abolished by further addition of prazosin and yohimbine (both 0.1 μM) (Figure 4). The effects of adding suramin alone varied somewhat with the experimental protocol. In one series of experiments in which the stimulation was discontinued for 20–30 min after each change in the concentration of suramin, to mimic the protocol in α,β-MeATP experiments (Figure 5), 10 μM suramin was without effect but 100 μM suramin delayed the onset (from 374 ms to 858 ± 18 ms, $n = 3$; $P < 0.001$) and tended to reduce slightly the amplitude of the neurogenic contraction (Figure 5). At a higher concentration (200–500 μM) suramin did not further change the lag time but concentration-dependently (by up to 207%) enhanced the amplitude of the contraction (Figure 5c). In another series in which stimulation was applied at fixed

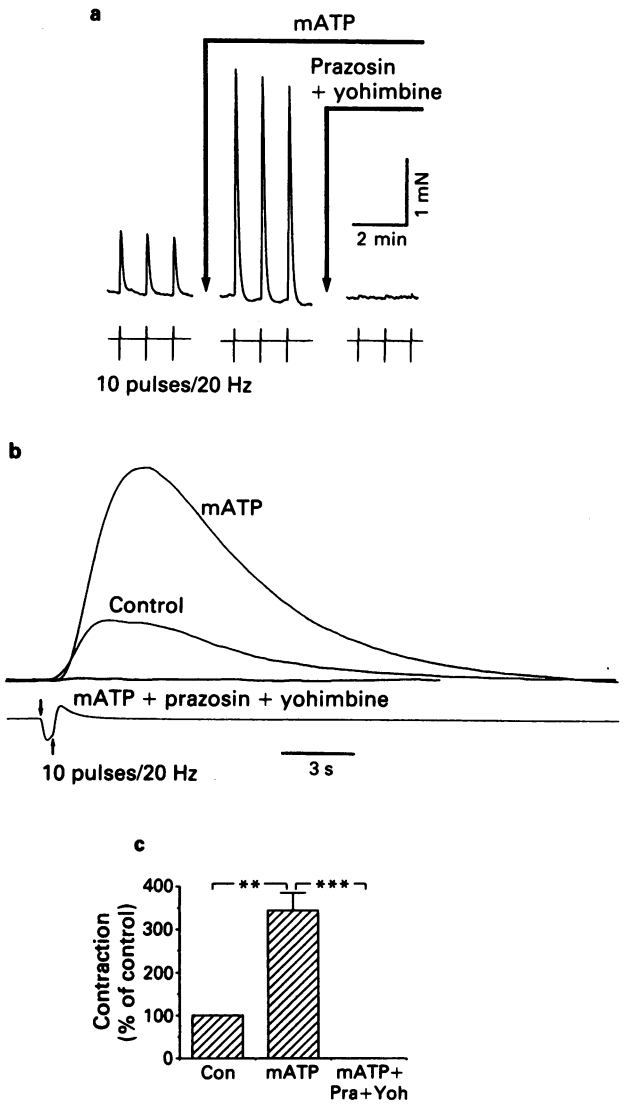


Figure 4 Effects of cumulative addition of α,β-methylene ATP (α,β-MeATP, mATP, 10 μM), prazosin (Pra) and yohimbine (Yoh) (both 0.1 μM) on the contractile responses to field stimulation with trains of 10 pulses at 20 Hz at 1 min intervals. Original recordings from one representative experiment shown at normal (a) and fast chart speed (b). The stimulation was discontinued for 20–30 min after addition of α,β-MeATP; other drugs were added at 10–20 min intervals. The bottom traces denote stimulus artifacts (the arrows indicate the beginning and end of the stimulation). Note that α,β-MeATP delayed the onset (from 430 to 770 ms in this experiment) but increased the amplitude of the contraction. (c) Summary of 4 experiments identical to that shown in (a). Contractions are expressed as a % of control (Con), i.e. the mean of 3–5 stable responses before addition of drugs. $^{**}P < 0.01$ and $^{***}P < 0.001$, one way ANOVA followed by Fisher PLSD test.

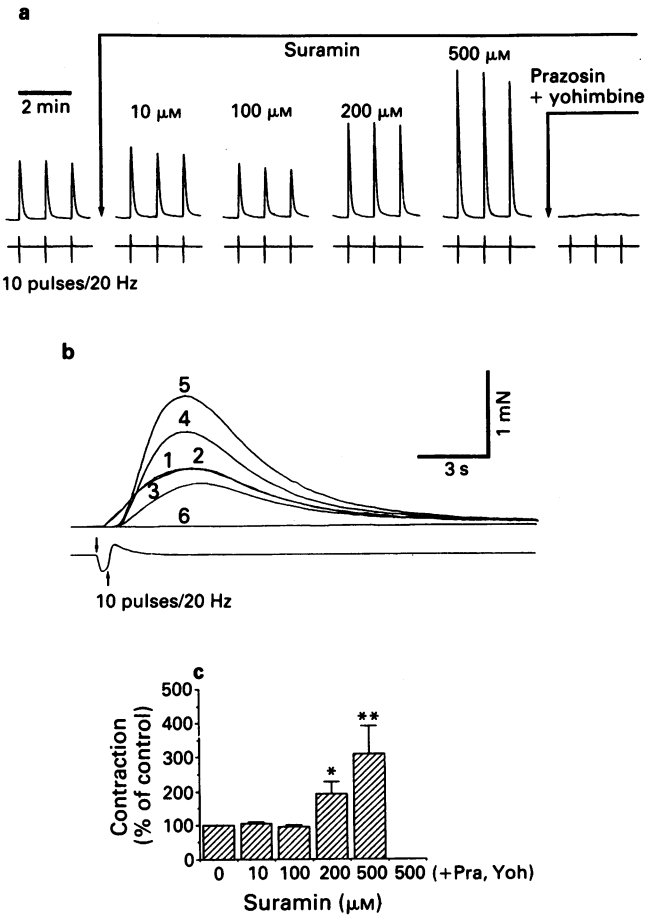


Figure 5 (a) Original recordings showing the effects of cumulative addition of suramin (10–500 μM), prazosin and yohimbine (both 0.1 μM) on the contractile responses to trains of 10 pulses at 20 Hz at 1 min intervals. The bottom traces denote the stimulus artifacts. The intervals between addition of drugs were 10–30 min. In this series of experiments, the stimulation was discontinued for 20–30 min after each addition of suramin before the next stimulation period was resumed. (b) Superimposed contractile responses, same condition as in (a), recorded at fast chart speed (the arrows indicate the beginning and end of the stimulation). Tracings 1–6 denote control, suramin 10 μM, 100 μM, 200 μM, 500 μM and further addition of prazosin and yohimbine (both 0.1 μM), respectively. Note (i) that 10 μM suramin was without effect, (ii) that 100 μM suramin delayed the onset (from 260 to 840 ms in this experiment) and slightly depressed the amplitude, and (iii) that 200–500 μM suramin increased the amplitude of the neurogenic contraction. (c) Summary of 3 experiments similar to that in (a) showing the concentration-dependent effects of suramin (10–500 μM) and the effects of further addition of prazosin (Pra) and yohimbine (Yoh) (both 0.1 μM). 'O' concentration indicates control, i.e. the mean of 3–5 stable responses before addition of drugs. Contractions were expressed as a % of control. $^{*}P < 0.05$ and $^{**}P < 0.01$ all *vs* control; one way ANOVA followed by Fisher PLSD test.

intervals, 10 μM suramin did not change the lag time or the amplitude of the contraction (the reduction by 37.5 ± 11.2%, $n = 6$, was not significant), 100 μM suramin delayed the onset (from 374 ms to 840 ± 26 ms, $n = 4$; $P < 0.001$) and further reduced the amplitude (by 43.0 ± 9.6%, $n = 6$; $P < 0.05$) while higher concentrations (200–500 μM) did not further delay the onset but increased the amplitude of the neurogenic contraction (by up to 168 ± 75%, $n = 6$; $P < 0.01$). The potentiating effect of suramin developed slowly and was maximal after about 20 min (not shown). The effect was slowly reversible by prolonged washing (for at least 30 min). Further addition of prazosin and yohimbine abolished the contraction in the presence of suramin (see Figure 5).

Addition of the K^+ channel blocker, tetraethylammonium (10 mM), accelerated the rate of rise and dramatically (by about 100 fold, $n = 14$) increased the peak amplitude of the contractile responses to 100 pulses at 1 Hz, and often caused the contraction to become diphasic (Figure 6). Spontaneous twitch contractions occurred frequently under the conditions (not shown). Further addition of prazosin (1 μM) had little effect on the rising phase of the contraction but substantially reduced the later phase (by 66.2 ± 4.9%, $n = 8$; $P < 0.01$) and always unmasked the diphasic nature of the contraction (Figure 6a). The initial twitch phase was abolished and the second phase strongly (by 78.0 ± 3.6%, $n = 8$; $P < 0.01$) depressed by 10 μM α,β-MeATP. The small residual response was profoundly reduced or abolished by further addition of 0.1–1 μM yohimbine (Figure 6a). Addition of 10 μM α,β-MeATP before prazosin abolished the initial rapid phase and slightly reduced the second phase; the latter was totally suppressed by further addition of prazosin and yohimbine (both 1 μM, Figure 6b). Similarly, suramin (100–500 μM) abolished the initial rapid phase of the contraction in the presence of tetraethylammonium but often caused the tension to oscillate (Figure 7). The oscillation was not studied in detail in the present paper.

Similarly to 10 μM α,β-MeATP (see Bao *et al.*, 1990), suramin did not even at the highest concentration used (500 μM) affect the contraction caused by KCl (60 mM, $n = 6$) or exogenous NA (10 nM–100 μM, $n = 6$), but profoundly inhibited the response to exogenous ATP (100 μM) ($n = 4$, not shown).

Discussion

The present results support our hypothesis that ATP released by field stimulation has a dual effect in the rat tail artery, i.e. triggers *per se* the early component of the contraction but restricts its later, NA-mediated component (Bao *et al.*, 1990). We will briefly discuss the usefulness of α,β-MeATP and suramin, then the main findings.

Usefulness of suramin

Although certain effects of α,β-MeATP have been claimed to be unrelated to P_{2X}-purinoceptors (Byrne & Large, 1986), this agent is generally considered to desensitize these receptors reasonably specifically (Bao *et al.*, 1990; Fedan & Lamport, 1990).

Suramin, a structurally different compound, has been shown to block specifically the contraction caused by ATP, but not by NA, KCl or acetylcholine (Dunn & Blakeley, 1988; Evans & Surprenant, 1992), and to block the inward currents induced by ATP or α,β-MeATP but not by 5-HT, acetylcholine or the nicotinic agonist, cytisine (Evans *et al.*, 1992; Edwards *et al.*, 1992; Silinsky *et al.*, 1992). Suramin has therefore been widely used for pharmacological analysis of the role of ATP in neurotransmission (von Kügelgen *et al.*, 1989a; Evans *et al.*, 1992; Edwards *et al.*, 1992; Silinsky *et al.*, 1992). Suramin is not selective for P_{2X}-purinoceptors, but also blocks P_{2Y}-purinoceptors in some smooth muscle preparations, e.g. mouse vas deferens (Blakeley *et al.*, 1991) and P_{2Z}-purinoceptors in platelets (Hourani *et al.*, 1992). These effects probably do not interfere with the neurogenic contraction in rat tail artery, however, because the P_{2Y}-purinoceptor antagonist reactive blue 2 (Burnstock & Warland, 1987) did not at 10–50 μM concentrations significantly affect the neurogenic contraction of rat tail artery caused by 10 pulses at 20 Hz, or the enhancing effect of α,β-MeATP on the contraction ($n = 3$, not shown), and because P_{2Z}-purinoceptors do not seem to exist on smooth muscle cells (see Gordon, 1986). The following findings in the present study indicate that the effects of suramin were due to block of the P_{2X}-purinoceptor-mediated ATP effects. (i) Like α,β-MeATP, suramin did not significantly change the nerve stimulation-induced release of

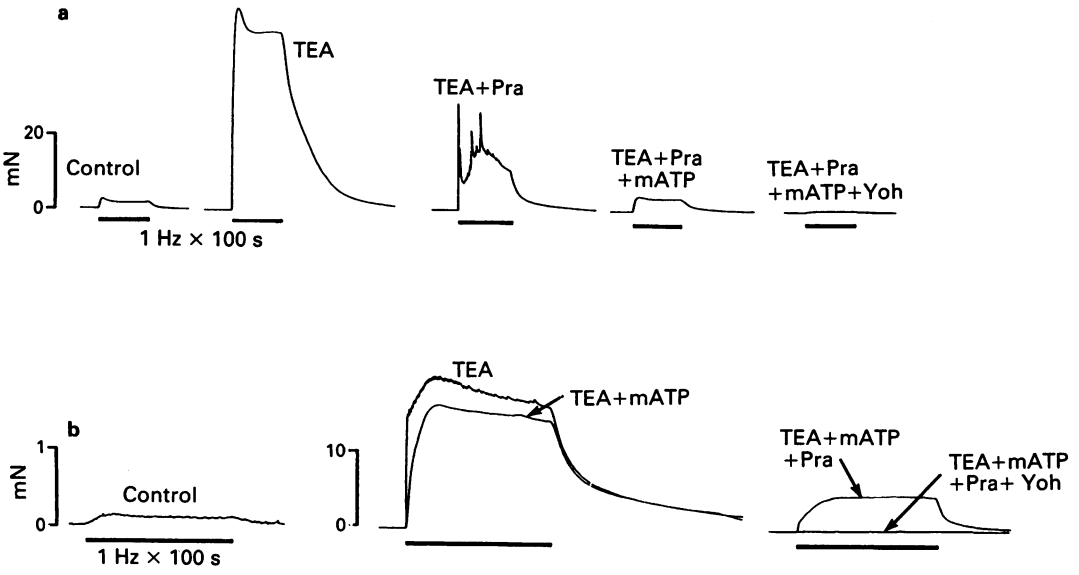


Figure 6 Original recordings representative of 8 (a) and 6 (b) experiments, showing the effects on the contractile responses to 100 pulses at 1 Hz (marked by bars) at 5–8 min intervals caused by cumulative addition of (a) tetraethylammonium (TEA, 10 mM), prazosin (Pra, 1 μM), α,β-methylene ATP (α,β-MeATP, mATP, 10 μM) and yohimbine (Yoh, 1 μM), or (b) TEA, α,β-MeATP, Pra and Yoh. The intervals between addition of drugs were 10–15 min. Note the different time bars in (a) and (b) and the different amplitude bars in (b). Note also that α,β-MeATP abolished the twitch phase or the initial rapid phase of the contraction in the presence of TEA and prazosin, and TEA alone, respectively.

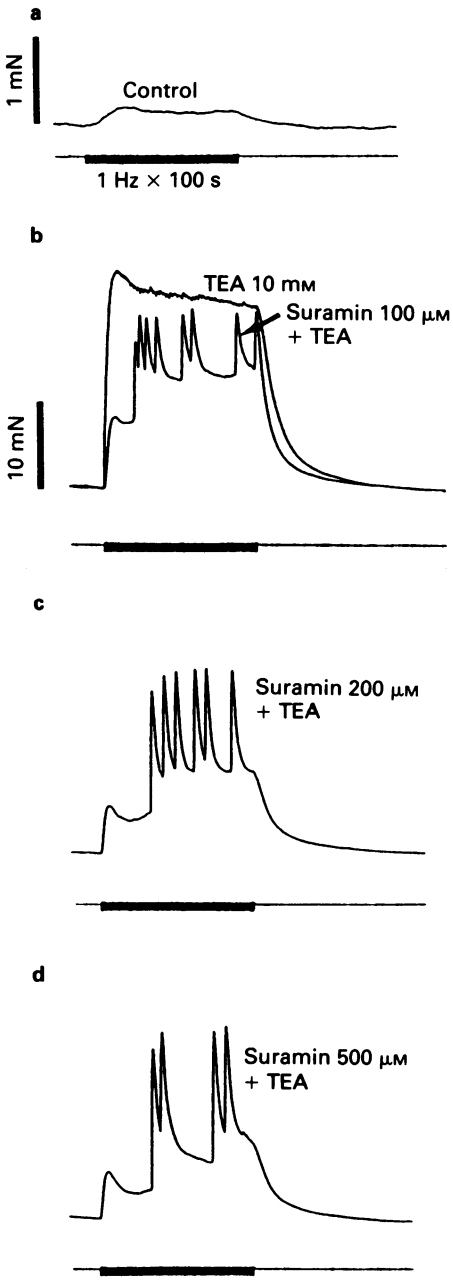


Figure 7 Original recordings representative of 4 experiments, showing the effects on the contractile responses to 100 pulses at 1 Hz (marked by bars) at 5–8 intervals caused by cumulative addition of tetraethylammonium (TEA, 10 mM) and suramin (100–500 μM). Similar results were obtained whether or not the stimulation was discontinued for 20–30 min after each addition of suramin. Note (i) the different amplitude bars in (a) and (b); amplitude bar in (b) also applies to (c) and (d), and (ii) that suramin abolished the initial rapid phase of the contraction in the presence of TEA and caused the contraction to oscillate.

endogenous NA, in line with the reported lack of effects of suramin on [^{3}H]-NA overflow in the mouse vas deferens (von Kügelgen *et al.*, 1989b). (ii) Like α,β-MeATP, suramin did not affect the nerve terminal spike but concentration-dependently blocked the e.j.cs. The effect was thus not due to blockade of nerve impulse conduction, in agreement with the finding of Evans & Surprenant (1992) in guinea-pig submucosal neurones, that suramin did not affect the threshold, amplitude or duration of the nerve action potential. (iii) Like α,β-MeATP, suramin almost abolished the contractile response to exogenous ATP but did not affect those to NA or KCl. It seems therefore reasonably safe to use suramin as a P_{2X}-purinoceptor antagonist under our experimental conditions.

Excitatory effect of ATP

We have previously reported that α,β-MeATP blocked the usually small, fast initial component of the neurogenic contractile response of the tail artery of normotensive rats (Bao *et al.*, 1989; 1993a). The demonstration in the present paper that suramin, like α,β-MeATP, delayed the onset of the neurogenic contraction (Figures 4b, 5b) and blocked (IC_{50} 75 μM) the small residual contractile response to nerve stimulation with short trains at 20 Hz in the presence of prazosin and yohimbine (Figures 2, 3), is further evidence that ATP indeed mediates a component of the neurogenic contraction in the tail artery of adult normotensive rats. Due to the relatively short incubation time used in the present study for suramin, especially at low concentrations (e.g. 10 μM), the calculated IC_{50} value for this effect may be overestimated (see Leff *et al.*, 1990).

Addition of the K^+-channel blocker, tetraethylammonium, greatly amplified the ATP- as well as NA-induced components of the neurogenic contraction, probably by dramatically increasing the release of both NA and ATP (Mermet *et al.*, 1990; Stjärne *et al.*, 1990) and the excitability of the smooth muscle (Haeusler & Thorens, 1980). In the presence of tetraethylammonium, α,β-MeATP or suramin not only abolished the early twitch phase but also depressed its tonic phase (Figures 6, 7) revealing a cooperative depolarizing effect of ATP-NA co-transmission, which helps lower the threshold of the cells for firing the muscle action potential. If all observed effects of α,β-MeATP and suramin were due to desensitization or block, respectively, of ATP receptors, these results then imply that ATP released by electrical field stimulation indeed triggers one component of the neurogenic contraction in rat tail artery. We have shown elsewhere that this neurogenic ATP-mediated contraction was abolished by nifedipine and thus probably caused by activation of L-type Ca^{2+} channels (Bao *et al.*, 1993b) and that the ATP-mediated contractile response faded rapidly during long high frequency trains (Bao *et al.*, 1993b), probably as a result of decline in per pulse release of ATP (Msghina & Stjärne, 1993).

Inhibitory effect of ATP

Our finding that α,β-MeATP (10 μM) substantially amplified the NA-mediated neurogenic contraction is not unique to the rat tail artery; a similar effect of α,β-MeATP has also been reported in other tissues (Bulloch & McGrath, 1988; Bulloch *et al.*, 1990; MacDonald *et al.*, 1992). Initially we considered the possibility that this effect of α,β-MeATP might be due to enhanced release of NA, as a result of block of prejunctional P_{2X}-purinoceptors (Bao *et al.*, 1989). This was clearly not the case, however, because α,β-MeATP did not significantly affect the [^{3}H]-NA overflow (Bao *et al.*, 1990) or $\Delta[NA]_{CF}$ (Bao *et al.*, 1993a and the present study) responses to nerve stimulation. We have therefore proposed a different explanation, namely that α,β-MeATP enhanced the NA-mediated contraction by blocking an inhibitory effect normally exerted by ATP released by field stimulation (Bao *et al.*, 1990). This hypothesis is strengthened by the present finding that suramin had a similar dual effect, i.e. blocked the ATP-mediated component of the neurogenic contraction but also amplified the NA-mediated contractions in rat tail artery. An alternative explanation, namely that α,β-MeATP may enhance the neurogenic contraction by depolarizing the smooth muscle, has been proposed, based on the finding that a similar degree of depolarization by KCl also amplified the neurogenic contraction (Neild & Kotecha, 1986). In our hands, however, 10–25 mM K^+ increased the amplitude of the contractile response to 10 pulses at 20 Hz by only 45% ($n = 2$), i.e. much less strongly than α,β-MeATP or suramin. The fact that the effects of α,β-MeATP were mimicked by suramin, which did not contract the rat tail artery and thus probably did not depolarize the smooth muscle, also argues against this explanation. It seems reasonable to assume, therefore,

that both the excitatory and inhibitory effects of α,β-MeATP and suramin were due to block of effects normally exerted by ATP. It should be kept in mind, however, that the effects of α,β-MeATP and suramin observed in this study were not identical. Suramin did not change the resting tension while α,β-MeATP *per se* caused a large transient contraction. Suramin (10–100 μM) tended to depress the amplitude of the neurogenic contraction under some experimental conditions while α,β-MeATP did not. Furthermore, suramin caused the tension in the presence of tetraethylammonium to oscillate, while α,β-MeATP did not.

Sources of ATP

The excitatory effect is almost certainly caused by ATP released exocytotically from the nerves as a sympathetic co-transmitter. After its release, ATP is rapidly (within 100 ms) metabolized by ecto-ATPase (Cunnane & Manchanda, 1988) and hence, probably exerts its effects locally, near the sites of release. However, the source of the ATP which inhibits the NA-mediated neurogenic contraction is less clear. It may be non-neuronal or neuronal. In favour of the former alternative, more than 90% of the ATP released by field stimulation has been claimed to be derived from non-neuronal sources (Sedaa *et al.*, 1990), and furthermore, α,β-MeATP-labelled P_{2X}-purinoceptors have been reported to be distributed throughout the media of blood vessel (Bo & Burnstock, 1989). In preliminary experiments we found that removal of the endothelium did not prevent the α,β-MeATP-induced enhancement of the neurogenic contraction ($n = 2$, not shown). An intriguing possibility is that the ATP restricting field stimulation-induced NA-mediated neurogenic contraction was released from smooth muscle and that the effect was exerted by preventing the electrotonic spread, perhaps via gap junctions, of the NA-mediated excitation. However, we have so far failed to find evidence supporting this possibility. Several gap junction inhibitors, e.g. endosulfan and (4β)phorbol 12-myristate 13-acetate (Fransson-Steen & Wärngård, 1992; Seseke *et al.*, 1992), failed to affect the neurogenic contraction in this vessel (unpublished data). Furthermore, neither α,β-MeATP nor suramin affected the contractile response to exogenous NA. Thus, the ATP inhibiting the neurogenic NA-mediated contraction was not released from smooth muscle by NA via postjunctional α-adrenoceptors (see Seddaa *et al.*, 1990; Katsuragi *et al.*, 1990; von Kügelgen & Starke, 1991b). The possibility remains, however, that ATP was released from smooth muscle by neurally released ATP (Katsuragi *et al.*, 1991; von Kügelgen & Starke, 1991b).

Alternatively, both the excitatory and inhibitory effect may be exerted by ATP released as a sympathetic co-transmitter. In support of this possibility, the enhancing effect of α,β-MeATP on the neurogenic contractile response at 20 Hz (Bao *et al.*, 1990) parallelled the decline in the per pulse release of ATP at this frequency (Msghina & Stjärne, 1993); both were inversely related to the length of the stimulus trains. As the excitatory effect probably involves activation of L-type Ca^{2+} channels and an increase in the intracellular Ca^{2+} concentration (Bao *et al.*, 1993b), the inhibitory effect of ATP may be due to hyperpolarization of smooth muscle secondary to activation of Ca^{2+}-activated K^+ channels. In direct support of this idea, ATP effects via P_2-purinoceptors have recently been reported to both increase the intracellular Ca^{2+} level and subsequently activate Ca^{2+}-activated K^+ channels in rat cultured aortic smooth muscle cells (von der Weid *et al.*, 1993). This would explain why α,β-MeATP and suramin did not enhance the neurogenic contraction in the presence of the K^+ channel blocker tetraethylammonium (Figures 6, 7).

In conclusion, the present results show that ATP released by electrical field stimulation exerts via 'P_{2X}-like' purinoceptors dual excitatory and inhibitory effects in rat isolated tail artery, triggering the initial component of the neurogenic contraction but also restricting its later, NA-mediated component. The source and possible physiological role of the ATP inducing the inhibitory effect remain to be established in future studies.

This work was supported by the Swedish Medical Research Council (project B93-14X-03027-24B), the Karolinska Institutes Fonder, the European Training Programme Twinning Grant (number 8912) and the Swedish Society for Medical Research. We thank Bayer (Germany) for supply of suramin.

References

ÅSTRAND, P. & STJÄRNE, L. (1989). On the secretory activity of single varicosities in the sympathetic nerves innervating the rat tail artery. *J. Physiol.*, **409**, 207–220.

BAO, J.X., ERIKSSON, I.E. & STJÄRNE, L. (1989). On pre- and/or post-junctional roles of ATP and an unknown 'substance X' as sympathetic co-transmitters in rat tail artery. *Acta Physiol. Scand.*, **135**, 65–66.

BAO, J.X., ERIKSSON, I.E. & STJÄRNE, L. (1990). Neurotransmitters and pre- and postjunctional receptors involved in the vasoconstrictor response to sympathetic nerve stimulation in rat tail artery. *Acta Physiol. Scand.*, **140**, 467–479.

BAO, J.X., GONON, F. & STJÄRNE, L. (1993a). Frequency- and train length-dependent variation in the role of postjunctional α_1- and α_2-adrenoceptors for the field stimulation-induced neurogenic contraction of rat tail artery. *Naunyn-Schmied Arch. Pharmacol.*, **347**, 601–616.

BAO, J.X., GONON, F. & STJÄRNE, L. (1993b). Kinetics of ATP- and noradrenaline-mediated sympathetic neurotransmission in rat tail artery. *Acta Physiol. Scand.*, **149** (in press).

BLAKELEY, A.G.H., BROCKBANK, J.E., KELLY, S.S. & PETERSEN, S.A. (1991). Effects of suramin on the concentration-response relationship of α,β-methylene ATP on the mouse vas deferens. *J. Auton. Pharmacol.*, **11**, 45–49.

BO, X. & BURNSTOCK, G. (1989). [^{3}H]-α,β-methylene ATP, a radioligand labelling P_2-purinoceptors. *J. Auton. Nerv. Syst.*, **28**, 85–88.

BROCK, J.A. & CUNNANE, T.C. (1988). Electrical activity at the sympathetic neuroeffector junction in the guinea-pig vas deferens. *J. Physiol.*, **399**, 607–632.

BULLOCH, J.M. & MCGRATH, J.C. (1988). Blockade of vasopressor and vas deferens responses by α,β-methylene ATP in the pithed rat. *Br. J. Pharmacol.*, **94**, 103–108.

BULLOCH, J.M., DALY, C.J., DUNN, W.R., MACDONALD, A. & MCGRATH, J.C. (1990). α,β-Methylene ATP can potentiate as well as inhibit nerve mediated responses of rabbit blood vessels and guinea-pig vas deferens. *Eur. J. Pharmacol.*, **183**, 543–544.

BURNSTOCK, G. (1990). Noradrenaline and ATP as cotransmitters in sympathetic nerves. *Neurochem. Int.*, **17**, 357–368.

BURNSTOCK, G. & WARLAND, J.J.I. (1987). P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P_{2Y}- but not the P_{2X}-purinoceptors. *Br. J. Pharmacol.*, **90**, 383–391.

BYRNE, N.G. & LARGE, W.A. (1986). The effect of α,β-methylene ATP on the depolarization evoked by noradrenaline (γ-adrenoceptor response) and ATP in the immature rat basilar artery. *Br. J. Pharmacol.*, **88**, 6–8.

CHEUNG, D.W. (1984). Neural regulation of electrical and mechanical activities in the rat tail artery. *Pflügers Arch.*, **400**, 335–337.

CUNNANE, T.C. & MANCHANDA, R. (1988). Electrophysiological analysis of the inactivation of sympathetic transmitter in the guinea-pig vas deferens. *J. Physiol.*, **404**, 349–364.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

EDWARDS, F.A., GIBB, A.J. & COLQUHOUN, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature*, **359**, 144–147.

EVANS, R.J., DERKACH, V. & SURPRENANT, A. (1992). ATP mediates fast synaptic transmission in mammalian neurones. *Nature*, **357**, 503–505.

EVANS, R.J. & SURPRENANT, A. (1992). Vasoconstriction of guinea-pig submucosal arterioles following sympathetic nerve stimulation is mediated by the release of ATP. *Br. J. Pharmacol.*, **106**, 242–249.

FEDAN, J.S. & LAMPORT, S.J. (1990). P_2-purinoceptor antagonists. *Ann. New York Acad. Sci.*, **603**, 182–197.

FRANSSON-STEEN, R. & WÄRNGÅRD, L. (1992). Inhibitory effects of endosulfan on gap junctional intercellular communication in WB-F344 rat liver cells and primary rat hepatocytes. *Carcinogenesis*, **13**, 657–662.

FREDHOLM, B.B., FRIED, G. & HEDQVIST, P. (1982). Origin of adenosine released from rat vas deferens by nerve stimulation. *Eur. J. Pharmacol.*, **79**, 233–243.

GONON, F., BAO, J.X., MSGHINA, M., SUAUD-CHAGNY, M.F. & STJÄRNE, L. (1993). Fast and local electrochemical monitoring of noradrenaline release from sympathetic terminals in isolated rat tail artery. *J. Neurochem.*, **60**, 1251–1257.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

HAEUSLER, G. & THORENS, S. (1980). Effects of tetraethylammonium chloride on contractile, membrane and cable properties of rabbit artery muscle. *J. Physiol.*, **303**, 203–224.

HIRST, G.D.S. & NEILD, T.O. (1980). Evidence for two populations of excitatory receptors for noradrenaline on arteriolar smooth muscle. *Nature*, **283**, 767–768.

HOURANI, S.M.O., HALL, D.A. & NIEMAN, C.J. (1992). Effects of the P_2-purinoceptor antagonist, suramin, on human platelet aggregation induced by adenosine 5'-diphosphate. *Br. J. Pharmacol.*, **105**, 453–457.

KASAKOV, L. & BURNSTOCK, G. (1983). The use of the slowly degradable analog, α,β-methylene ATP, to produce desensitization of the P_2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. *Eur. J. Pharmacol.*, **86**, 291–294.

KATSURAGI, T., TOKUNAGA, T., OGAWA, S., SOEJIMA, O., SATO, C. & FURUKAWA, T. (1991). Existence of ATP-evoked ATP release system in smooth muscles. *J. Pharmacol. Exp. Ther.*, **259**, 513–518.

KATSURAGI, T., TOKUNAGA, T., USUNE, S. & FURUKAWA, T. (1990). A possible coupling of postjunctional ATP release and transmitters' receptor stimulation in smooth muscle. *Life Sci.*, **46**, 1301–1307.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly-equilibrating but competitive antagonist at P_{2X}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645–649.

MACDONALD, A., DALY, C.J., BULLOCH, J.M. & MCGRATH, J.C. (1992). Contributions of α_1-adrenoceptors, α_2-adrenoceptors and P_{2X}-purinoceptors to neurotransmission in several rabbit isolated blood vessels: role of neuronal uptake and autofeedback. *Br. J. Pharmacol.*, **105**, 347–354.

MEDGETT, I.C. & LANGER, S.Z. (1984). Heterogeneity of smooth muscle alpha adrenoceptors in rat tail artery *in vitro*. *J. Pharmacol. Exp. Ther.*, **229**, 823–830.

MERMET, C., GONON, F.G. & STJÄRNE, L. (1990). Online electrochemical monitoring of the local noradrenaline release evoked by electrical stimulation of the sympathetic nerves in isolated rat tail artery. *Acta Physiol. Scand.*, **140**, 323–329.

MSGHINA, M. & STJÄRNE, L. (1993). Sympathetic transmitter release in rat tail artery and mouse vas deferens: facilitation and depression during high frequency stimulation *Neurosci. Lett.*, **155**, 37–41.

NEILD, T.O. & KOTECHA, N. (1986). Effects of alpha, beta-methylene ATP on membrane potential, neuromuscular transmission and smooth muscle contraction in the rat tail artery. *Gen. Pharmacol.*, **17**, 461–464.

SEDAA, K.O., BJUR, R.A., SHINOZUKA, K. & WESTFALL, D.P. (1990). Nerve and drug-induced release of adenine nucleosides and nucleotides from rabbit aorta. *J. Pharmacol. Exp. Ther.*, **252**, 1060–1067.

SESEKE, F.G., GARDEMANN, A. & JUNGERMANN, K. (1992). Signal propagation via gap junctions, a key step in the regulation of liver metabolism by the sympathetic hepatic nerves. *FEBS*, **301**, 265–270.

SILINSKY, E.M., GERZANICH, V. & VANNER, S.M. (1992). ATP mediates excitatory synaptic transmission in mammalian neurones. *Br. J. Pharmacol.*, **106**, 762–763.

SNEDDON, P. & BURNSTOCK, G. (1984). ATP as a co-transmitter in rat tail artery. *Eur. J. Pharmacol.*, **106**, 149–152.

STJÄRNE, L. & STJÄRNE, E. (1989). Basic features of an extracellular recording method to study secretion of a sympathetic co-transmitter, presumably ATP. *Acta Physiol. Scand.*, **135**, 217–226.

STJÄRNE, L., BAO, J.-X., GONON, F.G., MERMET, C., MSGHINA, M., STJÄRNE, E. & ÅSTRAND, P. (1990). Presynaptic receptors and modulation of noradrenaline and ATP secretion from sympathetic nerve varicosities. *Ann. New York Acad. Sci.*, **604**, 250–265.

TANG, J.-M., WANG, J., QUANDT, F.N. & EISENBERG, R.S. (1990). Perfusing pipettes. *Pflügers Arch.*, **416**, 347–350.

VIDAL, M., HICKS, P.E. & LANGER, S.Z. (1986). Differential effects of α-β-methylene ATP on responses to nerve stimulation in SHR and WKY tail arteries. *Naunyn-Schmied Arch. Pharmacol.*, **332**, 384–390.

VON DER WEID, P.-Y., SEREBRYAKOV, V.N., ORALLO, F., SNETKOV, V.A. & TAKEDA, K. (1993). Effects of ATP on cultured smooth muscle cells from rat aorta. *Br. J. Pharmacol.*, **108**, 638–645.

VON KÜGELGEN, I., BÜLTMANN, R. & STARKE, K. (1989a). Effects of suramin and α,β-methylene ATP indicate noradrenaline-ATP co-transmission in the response of the mouse vas deferens to single and low frequency pulses. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 760–763.

VON KÜGELGEN, I., SCHÖFFEL, E. & STARKE, K. (1989b). Inhibition by nucleotides acting at presynaptic P_2-receptors of sympathetic neuro-effector transmission in the mouse isolated vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 522–532.

VON KÜGELGEN, I. & STARKE, K. (1991a). Noradrenaline-ATP co-transmission in the sympathetic nervous system. *Trend Pharmacol. Sci.*, **12**, 319–324.

VON KÜGELGEN, I. & STARKE, K. (1991b). Release of noradrenaline and ATP by electrical stimulation and nicotine in guinea-pig vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 419–429.

(Received March 1, 1993
Revised August 12, 1993
Accepted August 16, 1993)

Br. J. Pharmacol. (1993), **110**, 1429–1436 © Macmillan Press Ltd, 1993

Binding and structure-activity-relation of benzo[*f*]isoquinoline- and norcodeinone-derivatives at μ-opioid receptors in the rat cerebral cortex

[1]Michael Freissmuth, *Wolfgang Beindl & *Martin Kratzel

Institute of Pharmacology, University of Vienna; Währinger Straße 13a and *Institute of Pharmaceutical Chemistry, University of Vienna; Währinger Straße 12; A-1090 Vienna, Austria

1 We have probed the ligand binding site of the μ-opioid receptor using a series of isoquinoline- and norcodeinone-derivatives; in these morphine- and codeine-analogues, the position of the piperidine-nitrogen as well as its mobility is altered relative to that found in morphine.

2 The μ-receptor in rat cortical membranes was labelled with [^{3}H]-naloxone and competition experiments were carried out in the absence and presence of Gpp(NH)p and NaCl: conditions, which are associated with affinity shifts for agonists whilst antagonist affinity remains unaffected. Moving the piperidine-nitrogen closer to the phenolic ring or reducing its mobility by incorporation into an additional ring drastically decreases the affinity.

3 In contrast, we find that the piperidine-nitrogen in a distal position is well tolerated provided that additional structural criteria, in particular a phenolic hydroxyl-group and a 6 carbon ring corresponding to ring C in morphine, are met. This assumption was verified by the synthesis of WB4/PH (4a**R**, 10b**S**, 11**R**)-10, 11-epoxy-1, 2, 3, 4, 5, 6-hexahydro-9-hydroxy-3-methyl-4a,10b-butano-benzo[*f*]isochinolin-12-on(10). This compound is an agonist with an affinity comparable to that of morphine.

4 We therefore conclude that both the mobility of the piperidine nitrogen of the ligand and of its counterpart anionic site in the ligand binding pocket of the μ-opioid receptor (presumably aspartic acid) are important determinants for fruitful interaction. The mobility of the anionic site is restricted in one direction but is sufficient to bridge the 2Å distance that exists between the position of the nitrogen in morphine and WB4/PH.

Keywords: μ-Opioid receptor; ligand binding site; anionic centre; isoquinoline; norcodeinone; G protein

Introduction

Endogenous opioid peptides and exogenous opiates interact with several types of receptors which based on pharmacological criteria are classified as μ-, δ-, and κ-subtypes (Höllt, 1986; Haynes, 1988); these receptors belong to the family of G protein-coupled receptors and are therefore presumed to contain the characteristic hydrophobic core of seven α-helices which contains the ligand binding pocket (Strosberg, 1991). The structure of the ligand binding domain has been probed with chimaeric receptors, random or site-directed mutagenesis thereby identifying critical amino acids, which line the binding pocket (see Kobilka *et al.*, 1988; Marullo *et al.*, 1990; Breyer *et al.*, 1990; Strader *et al.*, 1991; Olah *et al.*, 1992). However, primary sequence information for the opioid receptors has become available only very recently (Evans *et al.*, 1992; Kieffer *et al.*, 1992). Hence, models for the ligand binding site on the opioid receptor depend on the analysis of structure-activity relation of natural and synthetic compounds (Porthoghese, 1988; Goldblum & Loew, 1991).

The minimal structural requirement (Schaumann-principle) for interaction with opioid receptors is a nitrogen 3 to 4 carbon atoms removed from a phenolic ring, where the carbon-1-atom of this intervening side chain is quarternary. The phenylpiperidine, pethidine (meperidine), met these criteria and was the first synthetic opioid. In morphine the piperidine ring (ring D) is rigid since the carbon atoms 9,13,14 are shared by ring D and ring B. In contrast, the mobility of the nitrogen is higher in meperidine and its congeners as well as in methadone and other diphenylpropylamin-derivatives. In the present study, we have focussed on the steric position of the nitrogen by using compounds, in which the mobility of the nitrogen and the configuration of the nitrogen-containing ring has been altered. The biological activity of these compounds was assessed by determining their affinity for the μ-opioid receptor.

[1] Author for correspondence.

Methods

Materials

[^{3}H]-naloxone (specific activity 60 Ci mmol^{-1}) was from NEN (Boston, MA, U.S.A.); guanine nucleotides were from Boehringer Mannheim (Germany); solvents and common reagents (analytical or reagent grade) were obtained commercially and used as received except chloroform, which was distilled under nitrogen from phosphorus pentoxide.

Chemical synthesis

The synthesis of compounds WB1 and WB2 (Figure 1a) was performed as previously described (Kratzel & Fleischhacker, 1987; Kratzel & Völlenkle, 1991). The compounds R1-R7 (Figure 1b) were kindly provided by B. Richter (Fleischhacker *et al.*, 1991; Fleischhacker & Richter, 1992).

The synthesis of WB2/PH, WB3, WB4, and WB4/PH is outlined in Figure 2 and described in detail below. The preparation of WB4 can be achieved by use of divergent ring expansion methods, starting with WB2. The simpliest way is to react WB2 with diazomethane yielding WB4 in a single reaction step. The disadvantage of this method is the necessity of an additional purification step by preparative thin layer or column chromatography separating the epoxide WB6 and higher ketone homologues such as WB5. The phenolic compounds WB2/PH and WB4/PH were obtained by conventional ether hydrolysis (boron tribromide or hy-

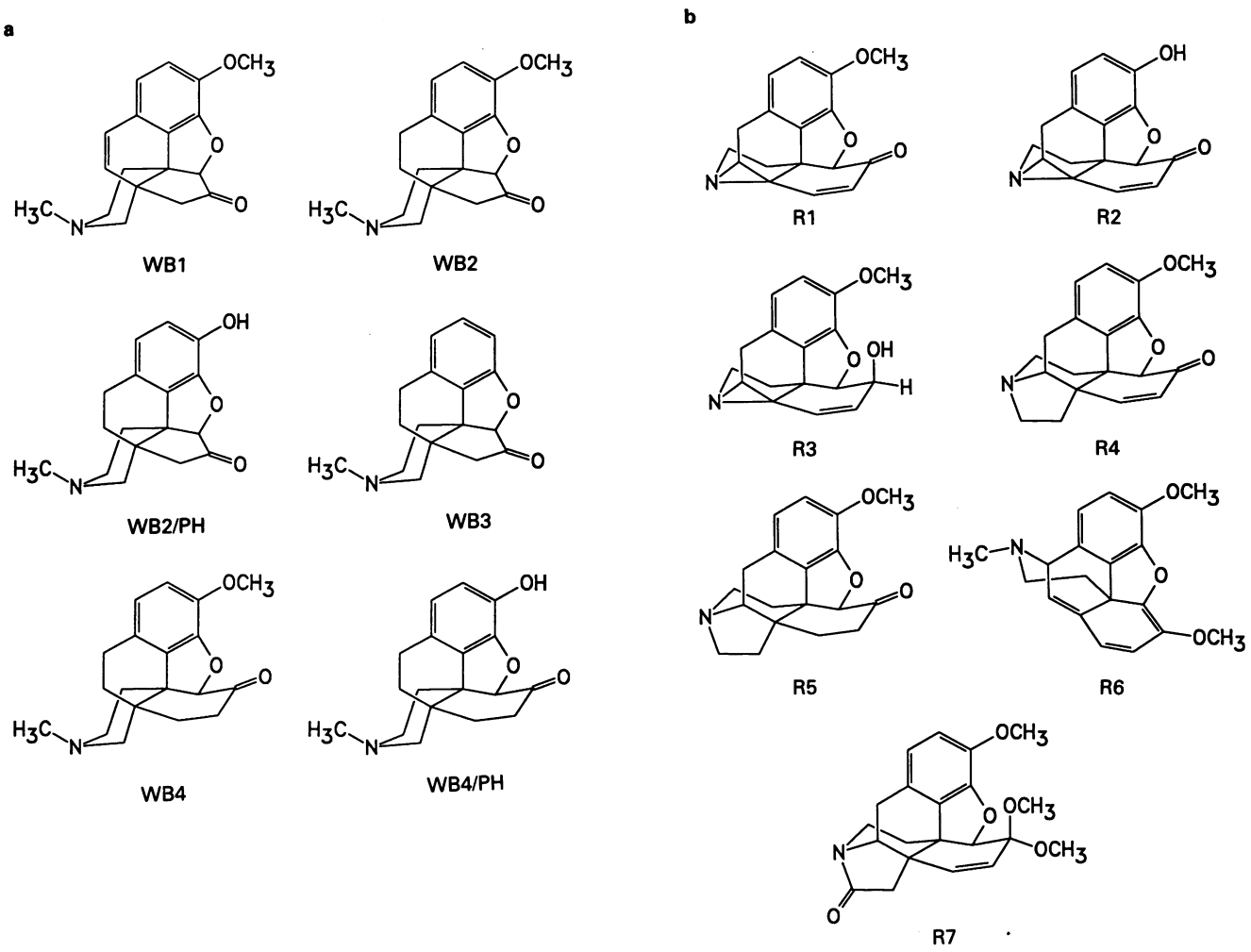


Figure 1 Compounds of the benzo[*f*]isoquinoline (a) and of the norcodeinone (b) series. The chemical names are as follows: WB1, (4a**R**, 10b**S**, 11**R**)-10,11-epoxy-1,2,3,4-tetrahydro-9-methoxy-3-methyl- 4a,10b-propano-benzo[*f*]isoquinolin-12-one; WB2, (4a**R**, 10b**S**, 11**R**)-10, 11-epoxy-1,2,3,4,5,6-hexahydro-9-methoxy-3-methyl-4a, 10b-propano-benzo[*f*]isoquinolin-12-one; WB2/PH, (4a**R**), 10b**S**, 11**R**)-10,11-epoxy-1,2,3,4,5,6 -hexahdyro-9-hydroxy-3-methyl- 4a, 10b-propano-benzo[*f*]isoquinolin-12-one; WB3, (4a**R**, 10b**S**, 11**R**)-10,11-epoxy-1,2, 3,4,5,6 -hexahyro-3-methyl-4a, 10b-propano-benzo[*f*]isoquinoline-12-one; WB4, (4a**R**, 10b**S**, 11**R**)-10,11-epoxy-1,2,3,4,5,6-hexahydro-9-methoxy-3-methyl-4a, 10b-butano-benzo[*f*]isoquinolin-12-one; WB4/PH, (4a**R**, 10b**S**, 11**R**)-10,11-epoxy-1,2,3,4,5,6-hexahydro-9-hydroxy-3-methyl-4a,10b-butano-benzo[*f*]isoquinolin-12-one. R1, 14,17-Cyclo-norcodeinone; R2, 14,17-normorphinone; R3, 14,17-norcodeine; R4, 14,17-Ethano-norcodeinone; R5, 14,17-nordihydrocodeinone; R6, (10*H*)-10,13-iminoethano-3,6, dimethoxy-*N*-methyl-phenanthro[4,5-b, c,d]furan; R7, 14,17-β-Oxo-thano-norcodeinone-dimethylacetal.

drobromic acid, respectively) of the corresponding methyl ethers WB2 and WB4. Removal of the hydroxy group of WB2/PH was carried out by treatment with 5-chloro-1-phenyl-tetrazole/K_2CO_3 in acetone to get the corresponding tetrazolyl ether which is cleaved to WB3 by catalytic hydrogenolysis (Pd/C, methanol).

Melting points were taken on a Kofler hot stage apparatus and are uncorrected. I.r. spectra were recorded as thin films on salt discs on a Perkin Elmer model 298 spectrometer. N.m.r. spectra were determined by using the spectrometer Bruker AC 80. All substances were measured in $CDCl_3$ as solvent. ^1H n.m.r. spectra (80 MHz) were recorded with $(CH_3)_4Si$ as the internal reference, the chemical shifts of the ^{13}C spectra (20.1 MHz) were given in p.p.m. related to the resonance of $CDCl_3$ (77.0 p.p.m.). Mass spectra were recorded on a Varian MAT 111A. Preparative thin layer chromatography (t.l.c.) was conducted on Merck aluminium oxide F_{254} plates (type T, 1.5 mm).

*(4a**R**, 10b**S**, 11**R**)-10,11-epoxy-1,2,3,4,5,6-hexahydro-9-hydroxy-3-methyl-4a,10b-propano-benzo[f]isoquinolin-12-one (WB2/PH)* (A) 300 mg (1 mmol) of WB2 were dissolved in hydrobromic acid (10 ml, 37%) and refluxed for 2 h. The solution was diluted with water, basified with 2 N NaOH to pH 8, and extracted with dichloromethane (3 × 20 ml). The combined organic layers were dried over Na_2SO_4. Evaporation of the solvent afforded 175 mg (61%) of WB2/PH. (B) To a solution of 300 mg (1 mmol) of WB2 in abs. chloroform (50 ml) 2.0 g (8 mmol) of boron tribromide were added at 15°C under nitrogen. After 45 min the solution was basified with 2 N NH_4OH to pH 8 and the organic phase was separated. After drying over Na_2SO_4 the solution was evaporated to yield 185 mg (65%) of WB2/PH. Brownish oil. ^1H n.m.r.: d (p.p.m.) 6.71, 6.62 (AB-system, 2H, J = 8 Hz, 7-H, 8-H), 4.67 (s, 1H, 11-H), 2.31 (s, 3H, N-CH_3); i.r.: n (cm^{-1}) 3400 (OH), 1750 (C = O); m.s. (m/z): 285 (M^+).

*(4a**R**, 10b**S**, 11**R**)-10,11-epoxy-1,2,3,4,5,6-hexahydro-3-methyl-4a,10b-propano-benzo[f]isoquinolin-12-one (WB3)* A mixture of WB2/PH (300 mg, 1.05 mmol), 5-chloro-1-phenyl-tetrazole (190 mg, 1.05 mmol) and K_2CO_3 (290 mg, 1 mmol) was heated under reflux for 24 h. The reaction mixture was filtered, evaporated, and redissolved in 2 N Na_2CO_3 solution. Extraction with dichloromethane (3 × 20 ml) afforded after drying (Na_2SO_4) and evaporating 405 mg (90%) of the corresponding tetrazolyl either as a brownish oil. ^1H n.m.r.: d

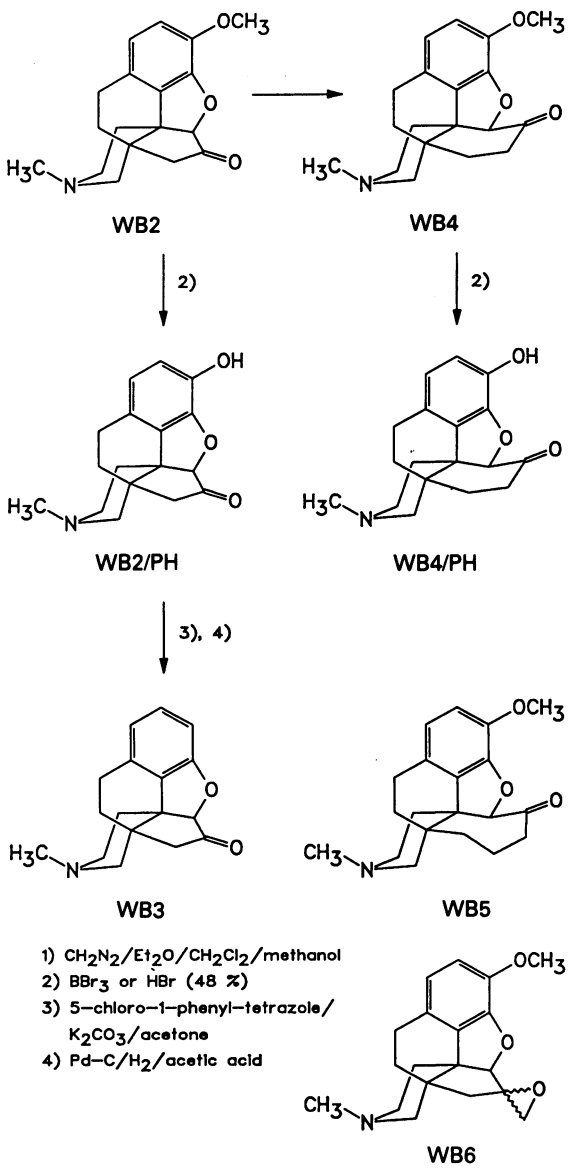


Figure 2 Synthesis of benzo[*f*]isoquinoline derivatives. The chemical synthesis is described in detail under Methods.

(p.p.m.) 7.9–7.3 (m, 5H, 2'-H-6'-H), 7.14, 6.74 (AB-system, 2H, $J = 8$ Hz, 7-H, 8-H), 4.70 (s, 1H, 11-H), 2.26 (s, 3H, N-CH_3); i.r.: n (cm^{-1}) 3400 (OH), 1760 (C = O); m.s. (m/z): 429 (M^+). 300 mg (0.7 mmol) of the tetrazolyl ether derivative, dissolved in 20 ml of acetic acid, was hydrogenated at 80°C for 24 h. The reaction mixture was filtered and evaporated to dryness. The oily residue was dissolved in water, made alkaline with solid Na_2CO_3, and extracted with dichloromethane (3 × 20 ml). The combined organic layers were dried over Na_2SO_4 and concentrated, leaving 140 mg (73%) of WB3. Colourless oil. ^{1}H n.m.r.: d (p.p.m.) 7.2–6.5 (3 spin system, 3H, 7-H, 8-H, 9-H), 4.64 (s, 1H, 11-H), 2.31 (s, 3H, N-CH_3); i.r.: n (cm^{-1}) 1740 (C = O); m.s. (m/z): 269 (M^+).

(4aR, 10bS, 11R)-10,11-epoxy-1,2,3,4,5,6-hexahydro-9-methoxy-3-methyl-4a,10b-butano-benzo[f]isoquinolin-12-one (WB 4) 300 mg (1 mmol) of WB2 was dissolved in dichloromethane (10 ml) and methanol (5 ml). An ethereal solution of diazomethane was added in small portions (1 ml) until the completed reaction was indicated by t.l.c. The solution was evaporated *in vacuo* and purified by preparative t.l.c. (petroleum ether/ethyl acetate/triethylamine = 90 + 8 + 4). The first zone afforded 185 mg (59%) of WB4 as colourless crystals. m.p. 167–168°C (methanol); ^{1}H n.m.r.: d (p.p.m.) 6.69, 6.60 (AB-system, 2H, 7-H, 8-H), 4.82 (s, 1H, 11-H), 3.89 (s, 3H, OCH_3), 2.34 (s, 3H, N-CH_3); ^{13}C-n.m.r.: d (p.p.m.) 208.0 (12-C), 145.7 (10-C), 142.7 (9-C), 130.4 (10a-C), 124.2 (6a-C), 121.2 (7-C), 114.7 (8-C), 88.9 (11-C), 63.2, 56.8 (2-C and 4-C), 56.8 ($O\underline{C}H_3$), 46.7 ($N\underline{C}H_3$), 49.0, 36.2, 35.0, 32.5, 30.5, 29.5, 22.1 (1-C, 4a-C, 5-C, 6-C, 10b-C, 13-C, 14-C); i.r.: n (cm^{-1}) 1725 (C = O); ms (m/z): 313 (M^+). Anal. Calcd. C 72.81, H 7.39, N 4.46; Found C 72.72, H 7.32, N 4.48.

(4aR, 10bS, 11R)-10,11-epoxy-1,2,3,4,5,6-hexahydro-9-hydroxy-3-methyl-4a,10b-butano-benzo[f]isoquinolin-12-one (WB4/PH) Prepared similarly to WB2/PH. Yellowish oil. ^{1}H-n.m.r.: d (p.p.m.) 6.71, 6.61 (AB-system, 2H, $J = 8$ Hz, 7-H, 8-H), 4.85 (s, 1H, 11-H), 2.37 (s, 3H, N-CH_3); ir: n (cm^{-1}) 3400 (OH), 1725 (C = O); ms (m/z): 299 (M^+).

Preparation of rat cortical membranes

Male Sprague-Dawley rats were killed by cervical dislocation; the brains were removed and the cerebral cortices were dissected free of white matter. Cortices from ten animals were pooled and homogenized by means of an Ultra-Turrax (2 × 15 s at half-maximal, 1 × 2 s at maximal setting) in ice-cold buffer containing (mM composition): Tris.HCl, pH 8.0, 20, EDTA 1, sucrose 250; all subsequent steps were carried out at 2°C. The homogenate was centrifuged at 12,000 *g* for 10 min, the resulting pellet was rehomogenized in sucrose-containing buffer and recentrifuged at 12,000 *g*. The supernantants from the first and second spin were combined and centrifuged at 50,000 *g* for 15 min. The resulting pellet was washed free of sucrose, resuspended at a protein concentration of ~5 mg ml^{-1}, quickly frozen in liquid nitrogen and stored at −80°C.

Receptor binding assays

In saturation experiments, brain membranes (~0.2 mg/assay) were incubated with increasing concentrations of [^{3}H]-naloxone ranging from 0.125 to 6 nM in a final volume of 0.5 ml containing (mM composition): Tris.HCl, pH 7.5, 50, EDTA 1, $MgCl_2$ at 20°C for 60 min. Preliminary time course experiments showed that binding equilibrium was reached after 50 min at 0.3 nM [^{3}H]-naloxone; binding remained stable for up to 90 min. The incubation was terminated by rapid filtration using a Skatron cell harvester; the glassfibre filters were washed with ice-cold buffer containing (mM composition): Tris-HCl, pH 7.5, 10, NaCl 100, $MgCl_2$ 5. Radioactivity retained on the filters was quantified by liquid scintillation counting at an efficiency of 50%. Where indicated in the legends of Figures 2 and 3 and Table 2, GppNHp and NaCl were present at 100 μM and 100 mM, respectively. Nonspecific binding was defined in the presence of 10 μM naloxone or morphine, which gave equivalent results, and amounted to 10% of total binding in the K_D-concentration range.

Competition experiments were carried out in an analogous way in the absence and presence of Gpp(NH)p and NaCl; the concentration of [^{3}H]-naloxone and the membrane suspension were ~4 nM and ~0.4 mg/assay, respectively; the concentration of the competing compounds is indicated in the legends of the respective figures and tables.

All experiments were carried out in duplicate and performed at least three times with different membrane preparations. The amount of protein was determined by dye-binding (Coomassie-Blue) with bovine serum albumin as the standard using the assay kit provided by Biorad (Richmond, CA, U.S.A.).

Data analysis

Data obtained in saturation binding experiments were subjected to non-linear least-squares curve-fitting using the equation describing a saturation isotherm; since [^{3}H]-naloxone

was found to bind to a homogeneous class of binding sites irrespective of whether NaCl and Gpp(NH)p were present or not, the data from competition experiments were fitted to equations describing the interaction of the competitor with one or two binding sites yielding estimates for the IC_{50} (concentration that caused half-maximal inhibition of radioligand binding) at the low- and high-affinity binding site as well as the relative proportion thereof (Schütz *et al.*, 1988). The appropriate model was chosen by comparing the goodness of fit using an F-test based on the extra-sum of squares principle. IC_{50} values were converted by the Cheng-Prusoff-approximation into estimates for the dissociation of the competitors, K_i ($K_i = IC_{50}/[1 + L/K_D]$). The slope from competition curves were calculated from a three-parameter logistic equation (Hill-equation).

Results

Characterization of [^{3}H]-naloxone binding

Binding of [^{3}H]-naloxone to the rat cortical membrane preparation was saturable and of high-affinity; in the absence of Gpp(NH)p and NaCl, the binding isotherms was adequately described assuming the interaction of the radioligand with a single class of binding sites yielding a K_D value of 2.8 ± 1.3 nM and a B_{max} of 154 ± 41 fmol mg^{-1} membrane protein. Addition of the Gpp(NH)p and NaCl enhanced binding of [^{3}H]-naloxone by an increase in the amount of detectable receptors (B_{max} = 268 ± 28 fmol mg^{-1}) whereas the K_D remained virtually unchanged (2.5 ± 0.5 nM) (Figure 3a). Originally, the binding of antagonist to G protein-coupled receptors was believed to be unaffected by guanine nucleotides; however, several reports have indicated that the hydrolysis-resistant analogues of GTP are capable of promoting antagonist binding (reviewed in Schütz & Freissmuth, 1992); a negative intrinsic activity of antagonists at the δ-opioid has been demonstrated in membranes from NG108 cells (Costa & Herz, 1989) and a direct reciprocal modulation of agonist and antagonist binding has been verified in reconstituted systems using purified components (Freissmuth *et al.*, 1991a,b). In competition binding experiments, both unlabelled naloxone and morphine potently inhibited specific [^{3}H]-naloxone binding (Figure 3b); the competition curve for naloxone was monophasic irrespective of whether Gpp(NH)p and NaCl were absent or present. Conversely, in the absence of guanine nucleotide and NaCl, the curve for morphine was shallow and could be adequately described by a two-site model; addition of Gpp(NH)p and NaCl resulted in a steep competitive curve which was shifted to the right. These data verify that the binding site labelled by [^{3}H]-naloxone corresponds to a μ-opioid receptor; based on the observed affinities of naloxone and morphine (summarized in Table 2) this receptor can be tentatively assigned to the μ_2-opioid subtype (Pasternak, 1988), which is the predominant subtype in the rat cerebral cortex (Goodman & Pasternak, 1985).

Competition experiments with the benzo[f]isoquinoline- and norcodeinone-derivatives

The results of an initial screening experiment with WB1-3 and R1-R7 are given in Figure 4a. Compounds which were not capable of inhibiting specific [^{3}H]-naloxone binding by more than 30% at a concentration of 100 μM were considered inactive (R3-R7) and excluded from further characterization. Competition curves were obtained for the 6 active compounds (Figure 4b, Table 1) and the following rank order of (increasing) potency was obtained WB1 ≤ R1 < WB3 < WB2 < WB2/PH = R2. Analysis of the structure-activity-relationship and comparison with published data (compiled by Casy & Partfitt, 1986) led to the following hypothetical conclusion; compounds in which the phenolic ring A was methoxylated (WB1, WB2, R1) or the hydroxyl-

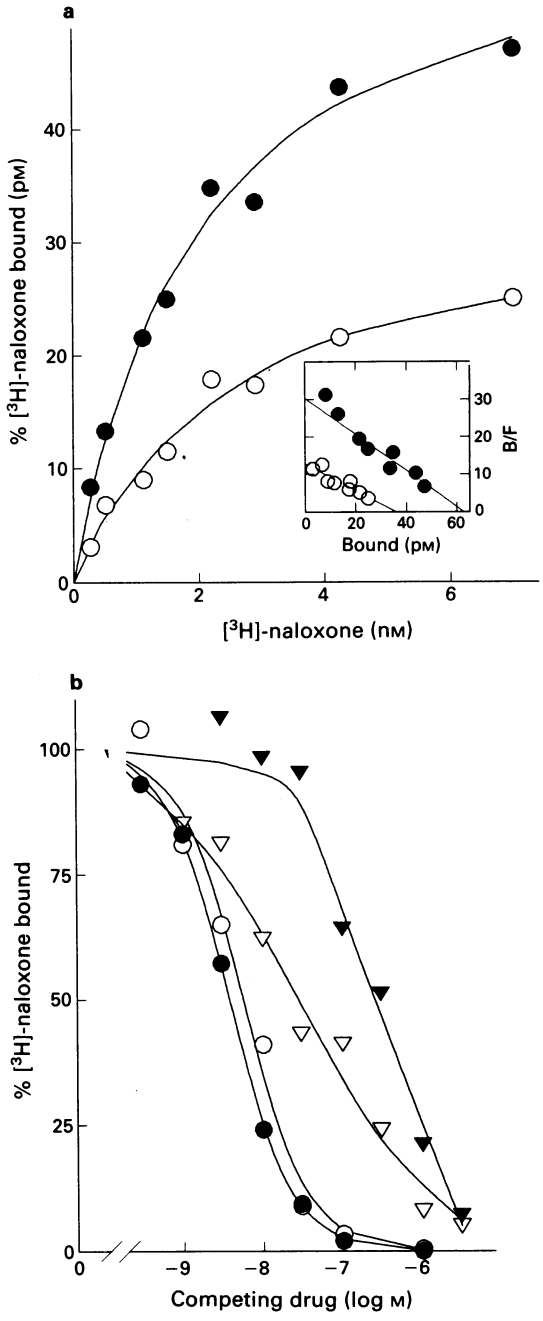


Figure 3 Characterization of specific [^{3}H]-naloxone binding to rat cerebral cortical membranes. (a) Saturation isotherms; rat cortical membranes (0.13 mg) were incubated with [^{3}H]-naloxone in the absence (○) and presence of 0.1 mM Gpp(NH)p + 100 mM NaCl (●) for 60 min as described under Methods. The inset shows a Scatchard transformation. (b) Competition of [^{3}H]-naloxone binding by unlabelled naloxone (○,●) and morphine (▽,▼) in the absence (○,▽) and presence of 0.1 mM Gpp(NH)p + 100 mM NaCl (●,▼). The concentration of [^{3}H]-naloxone was 4 nM, the protein level was 0.4 mg, specific binding in the absence of a competitor amounted to 52 pM in the absence and 93 pM in the presence Gpp(NH)p + NaCl, respectively and was taken as 100%. Data represent means from duplicate determinations in a single experiment which is representative of two additional experiments.

group was missing had a reduced affinity when compared to their homologues (WB2/PH and R2). R2 and WB2/PH displayed comparable affinities although the relative position of the nitrogen atom as well as ring C (5 carbon atoms vs. 6 carbon atoms) differed. Hence, compound WB4 was synthesized (see Figure 2 and Methods), the ring C was enlarged

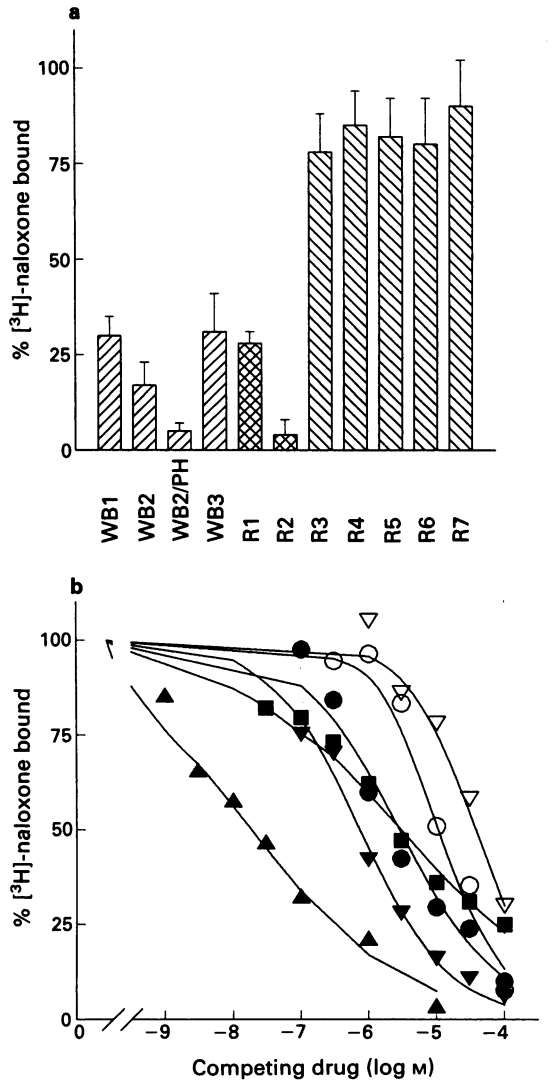


Figure 4 Inhibition of specific [^{3}H]-naloxone binding by isoquinoline- and norcodeinone-derivatives. (a) Screening of WB- and R-compounds (100 μM) for inhibition of specific [^{3}H]-naloxone (4 nM) binding, which amounted to 60 pM in the absence of a competitor. Data are means ± s.d. from 3 experiments carried out in duplicate. (b) Competition curves for WB2 (○), WB3 (▽), WB2/PH (●), WB4 (■), WB4/PH (▲), R2 (▼). Specific binding in the absence of a competitor was 58 pM and was taken as 100%; protein amounted to 0.38 mg. Data are means from duplicate determinations in a single experiment which was repeated twice with similar results; intrassay and interassay variation (s.d.) were 5% and 25%, respectively.

Table 1 Affinity estimates (IC_{50} = concentration giving half-maximal inhibition of specific [^{3}H]-naloxone binding to cerebral cortical μ-opioid receptors) and slope factors (n_H) for benzo[*f*]isoquinoline- (WB-series) and norcodeinone- (R-series) derivatives

Compound	IC_{50} (μM)	n_H
WB1	82 ± 22	0.98 ± 0.12
WB2	18 ± 4	0.97 ± 0.08
WB2/PH	1.6 ± 0.6	0.63 ± 0.03
WB3	50 ± 12	0.80 ± 0.05
WB4	2.3 ± 0.5	0.53 ± 0.17
WB4/PH	0.07 ± 0.04	0.49 ± 0.11
R1	75 ± 22	1.00 ± 0.10
R2	1.5 ± 0.5	0.62 ± 0.05
Morphine	0.02 ± 0.006	0.53 ± 0.06
Naloxone	0.005 ± 0.001	0.97 ± 0.11

Data represent means ± s.d. from 3 to 4 determinations; IC_{50} and slope factors were calculated by fitting the inhibition curves to a three parameter logistic equation.

by one C-atom. In spite of its methoxylated hydroxyl-group in ring A, the affinity of WB4 was only slightly lower than that of R2 and WB2/PH and was clearly superior to that of its 5-ring homologue WB2 (Table 1). This analysis resulted in the synthesis of WB4/PH which combines the favourable characteristics, i.e. a hydroxyl-group in ring A and a 6 carbon-ring C, whilst the relative position of the nitrogen remained similar to its homologues. This compound clearly surpassed the affinity all other substances with a potency approaching that determined for the reference drug morphine (Figure 4b, Table 1).

Analysis of agonist versus antagonist properties

The slope of the individual competition curves in Figure 4b and Table 1 differs with steep inhibition curves being observed for R1, WB1, WB2, WB3; the shallow curves (slopes <1 for R2, WB4/PH, WB2/PH and WB4) suggests the presence of heterogeneous binding sites for these compounds. We have verified the possibility that this binding heterogeneity arises from the agonist activity of these compounds by carrying out competition experiments in the presence and absence of Gpp(NH)p. As can be seen from Figure 5, addition of GppNHp and NaCl shifted the shallow competition curve for WB4/PH to the right, with a slope approaching unity (Table 2); similar results were obtained for R2, WB2/PH and WB4 (Table 2). In contrast, the competition curves for WB2 (Figure 5) and R1, WB1, WB3 were similar in the presence and absence of Gpp(NH)p and NaCl (Table 2).

Molecular modelling

The crystal structure of compounds of the WB series has been determined by X-ray diffraction (Kratzel & Völlenke, 1991); we have used these coordinates to model the structure of WB4/PH and to compare it with the structure of its homologue, hydromorphone (Figure 6). This analysis highlights the difference in the relative position of the nitrogen atom.

Discussion

Recently, a three-component molecular model for the ligand binding site of the μ-opioid receptor has been evaluated which considers three critical regions on the morphine molecule, namely the nitrogen, the phenolic hydroxylgroup (ring A), and the hydrophilic region (furan oxygen and hydroxyl-group at carbon 6 (ring C)) (Goldblum & Loew, 1991). The corresponding functional groups lining the binding site of the receptor are an anionic residue interacting with the nitrogen; this carboxyl-group is provided by either glutamate or aspartate, the latter being the more likely candidate; an aspartate residue in the third transmembrane spanning α-helix (Asp^{113} in the β_2-adrenoceptor) is conserved in all receptors that bind protonated amines as agonist (Strosberg, 1991) and site-directed mutagenesis has confirmed that this residue interacts with the protonated amine of the catecholamine side-chain (Strader *et al.*, 1991). In addition, the structural model encompasses a hydroxyl-group in a serine- or threonine-side chain and a lysine-derived protonated nitrogen, which form hydrogen bonds to the hydroxyl-groups and to the furan-oxygen.

One of the major structural variations within the compounds used in the present study is the different position of the nitrogen atom relative to the aromatic ring A; the following pertinent conclusions can be drawn from our results: (i) the mobility of the nitrogen is important; if the nitrogen is incorporated into an additional ring thereby reducing its mobility (R1, R2), the affinity is reduced; this is best illustrated with R2, which is a structural homologue to morphine, but displays a 100 fold higher K_i in spite of a comparable molecular volume. The difference in the oxidation-state at

Table 2 Classification of benzo[*f*]isoquinoline- (WB-series) and norcodeinone- (R-series) derivatives as agonists and antagonists based on affinity shifts induced by the combination of Gpp(NH)p and NaCl

Agonists:		− [Gpp(NH)p + NaCl]			+ [Gpp(NH)p + NaCl]	
Compound	*% H*	K_{iH} (nM)	K_{iL} (μM)	n_H	K_i (μM)	n_H
WB2/PH	26 ± 3	111 ± 55	6 ± 3	0.63 ± 0.03	2 ± 0.4	0.95 ± 0.01
WB4	50 ± 8	160 ± 30	10 ± 5	0.53 ± 0.17	11 ± 1	0.97 ± 0.01
WB4/PH	40 ± 7	1 ± 0.4	0.2 ± 0.05	0.49 ± 0.11	0.2 ± 0.04	0.92 ± 0.12
R2	65 ± 5	90 ± 30	8 ± 4	0.62 ± 0.05	6 ± 0.8	1.07 ± 0.05
Morphine	60 ± 3	1.5 ± 0.5	0.1 ± 0.03	0.53 ± 0.06	0.3 ± 0.1	0.85 ± 0.10

Antagonists:	− [Gpp(NH)p + NaCl]		+ [Gpp(NH)p + NaCl]	
Compound	K_i (μM)	n_H	K_i (μM)	n_H
WB1	29 ± 8	0.98 ± 0.12	21 ± 1	0.85 ± 0.05
WB2	7 ± 1	0.97 ± 0.08	8 ± 2	1.07 ± 0.10
WB3	18 ± 4	0.80 ± 0.05	26 ± 5	0.82 ± 0.09
R1	27 ± 8	1.00 ± 0.10	24 ± 4	1.15 ± 0.15
Naloxone	0.002 ± 0.001	0.97 ± 0.11	0.002 ± 0.001	0.93 ± 0.09

Data represent means ± s.d. from 3 to 4 determinations; K_{iH} and K_{iL}, (the dissociation constant for the competing ligand at the high- and low-affinity site, respectively) and %H (the percentage of high-affinity binding sites) were determined by fitting the competition curves to an equation describing the interaction of the competitor with two binding sites and converting the thus obtained IC_{50H} and IC_{50L} to K_i-values using the Cheng-Prusoff relationship; slope factors (n_H) were calculated using a three parameter logistic equation.

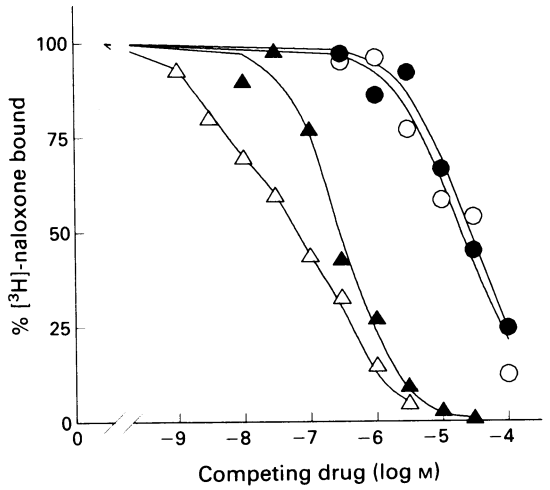


Figure 5 Inhibition of specific [^{3}H]-naloxone binding by WB2 and WB4/PH in the absence and presence of Gpp(NH)p + NaCl. WB2 (○,●) and WB4/PH (△,▲) were incubated in the absence (○,△) and presence of Gpp(NH)p + NaCl (●,▲) at a protein level of 0.4 mg and [^{3}H]-naloxone (4 nM); specific binding in the absence of competitors amounted to 50 and 90 pM, respectively and was taken as 100%. Data are means from duplicate determinations in a single experiment, which is representative for 3 separate experiments; intr-assay and interassay variation (s.d.) were 5% and 25%, respectively.

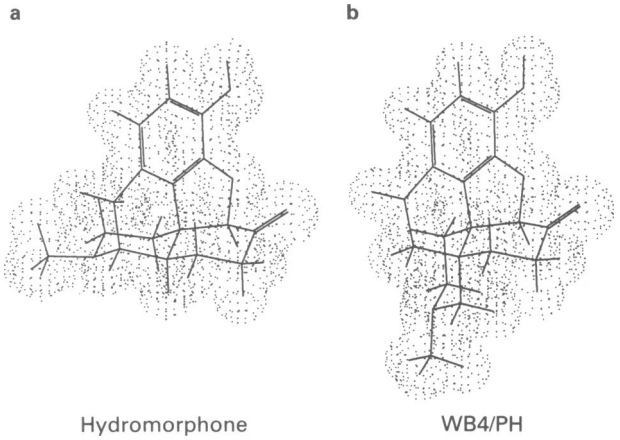


Figure 6 Structural comparison of hydromorphone (a) and WB4/PH (b). The molecular models were built by using the X-ray coordinates of hydromorphone and a WB-precursor (Kratzel & Völlenke, 1991) and simultaneous geometric optimisation for the two compounds with Hyperchem-Software (Autodesk Inc).

carbon 6 (keto-vs. hydroxyl-group) can be disregarded in this context, since this produces only modest effects on binding affinity; the higher oxidation state of carbon 6 is actually associated with an increased affinity (Chang *et al.*, 1979; Magnan *et al.*, 1982); similarly, the lack of the methyl-group on the nitrogen cannot account for the difference as normorphine and morphine are essentially equipotent at μ-receptors (Magnan *et al.*, 1982). (ii) Increasing the size of the hetrocyclic ring (R3-R5,R7) leads to further decline in activity; this effect probably arises from steric hindrance and can best be seen by comparing the inactive compound R4 and its direct homologue R1. (iii) In R6, the nitrogen is moved closer to the phenolic ring when compared to its position in morphine; this change is not tolerated, since R6 is completely inactive. The fact that the hydroxyl-group (attached to the carbon corresponding to carbon 6 in morphine) is methylated in R6 does not explain its low affinity; buprenorphine, which is also methoxylated at carbon-6, is 20 fold more potent than morphine at μ-receptors (Toll, 1992). (iv) In contrast, moving the nitrogen distal to its position in morphine is well tolerated as documented by the compounds of the WB-series; this holds true for WB4/PH, in particular, which is almost as potent as morphine. A comparison of the crystal structure of WB2 with that of morphine shows that the nitrogen in the WB-series is 2Å removed from the position where it is found in morphine (Kratzel & Völlenke, 1991). Molecular modelling indicates that this different steric position is maintained in WB4/PH. We therefore conclude that the mobility of the anionic site in the receptor (presumably aspartate) is sufficient to bridge this gap in the distal direction but is restricted in the opposite direction.

Sodium ions and guanine nucleotides act synergistically to reduce the affinity of the opioid receptor for agonists (Blume, 1978); we have used the affinity shifts induced by this combination to classify our test compounds as agonists or antagonists. Compounds with a phenolic hydroxyl-group

behaved as agonists whilst the methoxylated derivatives and then non-hydroxylated WB3 behaved as low-affinity antagonists, WB4 being the one notable exception. We are currently at a loss to explain this inconsistency. In addition, it is well known that methylation of the phenolic hydroxyl-group is associated with a reduced potency; codeine, the methoxylated analogue of morphine is about 3000 fold less potent in displacing radiolabelled naloxone from μ-opiate receptors (Pert & Snyder, 1973). Our results are in line with this observation; WB2/PH, WB4/PH, and R2 are more potent than their methoxylated counterparts WB2, WB4, R1; likewise, lack of the hydroxylgroup in WB3 results in a lower affinity. We also find that the configuration of ring C is important for binding to the μ-opioid receptor; apart from the position of the nitrogen atom, the only feature that distinguishes WB2/PH from hydromorphone is its 5-carbon-ring C (as opposed to a reduced 6-carbon ring in hydromorphone). Nevertheless, the potency of WB2/PH is 2–3 orders of magnitude lower than that of hydromorphone. Affinity is restored by enlarging the ring C as in WB4/PH. Hence, the size of ring C is clearly critical, implying that there is a functionally important hydrophobic domain in the ligand binding site of the μ-opioid receptor; this assumption is also substantiated by the observation that, in comparison with morphine, the phenylpiperidine pethidine (meperidine), which lacks a structure analogous to ring C, displays a modest affinity (K_i in the μmolar range) for μ-opioid receptors (Pert *et al.*, 1973; Magnan *et al.*, 1982).

The substitutions at the nitrogen are also important as they alter the affinity of the compound for individual receptor subtypes; substitutions with unsaturated and rigid side chains such as an allyl and cyclopropylmethyl reduce the agonistic activity at μ-receptors while enhancing the affinity for κ-receptors and δ-opioid receptors (Magnan *et al.*, 1982). In addition, recent studies on a series of *trans*-isoquinolinylphenols suggest that modifications of the equivalent to ring C differentially affect the affinity of and selectivity for μ- and κ-receptors (Judd *et al.*, 1992). It will therefore be interesting to use the test compounds presented in this work to probe the binding site of these receptor types.

We are grateful to B. Richter for providing compounds R1-R7, and to Dr Zak, Institute of Physical Chemistry, University of Vienna, for performing elemental analyses. We thank Drs W. Fleischhacker and W. Schütz for support and discussion. This work was supported by a grant from the Austrian Science foundation (P8875-MOB) to MF.

References

BLUME, A.J. (1978). Interactions of ligands with the opiate receptors of brain membranes: regulation by ions and nucleotides. *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 1713–1717.

BREYER, R.M., STROSBERG, A.D. & GUILLET, J.-G. (1990). Mutational analysis of ligand binding activity of β_2-adrenergic receptor expressed in *Escherichia coli*. *EMBO J.*, **9**, 2679–2684.

CASY, A.F. & PARTFITT, R.T. (1986). *Opioid Analgesics, Chemistry and Receptors*. (ed.) New York: Plenum Press.

CHANG, K.-J., COOPER, B.R., HAZUM, E. & CUATRECASAS, P. (1979). Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. *Mol. Pharmacol.*, **16**, 91–104.

COSTA, T. & HERZ, A. (1989). Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 7321–7325.

EVANS, C.J., KEITH, D.E., MORRISON, H., MAGENDZO, K. & EDWARDS, R.H. (1992). Cloning of a delta opioid receptor by functional expression. *Science*, **258**, 1952–1955.

FLEISCHHACKER, W. & RICHTER, B. (1992). 14,17-Ethano-norcodeinone. *Monatsh. Chem.*, **123**, 837–848.

FLEISCHHACKER, W., RICHTER, B. & VÖLLENKLE, H. (1991). 14, 17-Cyclonorcodeinondimethyl-acetal: Ein neues, reaktionsfreudiges Codeinonderivat, II. *Monatsh. Chem.*, **122**, 399–411.

FREISSMUTH, M., SELZER, E., MARULLO, S., SCHÜTZ, W. & STROSBERG, A.D. (1991a). Expression of two human β-adrenergic receptors in *Escherichia coli:* functional interaction with two forms of the stimulatory G protein. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 8548–8552.

FREISSMUTH, M., SELZER, E. & SCHÜTZ, W. (1991b). Interactions of the purified bovine brain A_1-adenosine receptor with G-proteins: Reciprocal modulation of agonist and antagonist binding. *Biochem. J.*, **276**, 651–656.

GOLDBLUM, A. & LOEW, G.H. (1991). A molecular model for an anionic μ-receptor; affinity and activation of morphine conformers. *Eur. J. Pharmacol.*, **206**, 119–131.

GOODMAN, R.R. & PASTERNAK, G.W. (1985). Visualisation of mu 1 opiate receptors in rat brain using a computerized autoradiographic subtraction technique. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 6667–6671.

HAYNES, L. (1988). Opioid receptor and signal transduction. *Trends Pharmacol. Sci.*, **9**, 309–311.

HÖLLT, V. (1986). Opioid peptide processing and receptor selectivity. *Annu. Rev. Pharmacol. Toxicol.*, **26**, 59–90.

JUDD, D.B., BROWN, D.S., LLOYD, J.E., MCELROY, A.B., SCOPES, D.I.C., BIRCH, P.J., HAYES, A.G. & SHEEHAN, M.J. (1992). Synthesis, antinociceptive activity and opioid receptor profiles of substituted trans-3-(decahydro- and octahydro-4a-isoquinyl)phenols. *J. Med. Chem.*, **35**, 48–56.

KIEFFER, B.L., BEFORT, K., GAVERIAUX-RUFF, C. & HIRTL, C.G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterisation. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 12048–12052.

KOBILKA, B.K., KOBILKA, T.S., DANIEL, K., REGAN, J.W., CARON, M.G. & LEFKOWITZ, R.J. (1988). Chimeric α_2-, β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science*, **240**, 1310–1316.

KRATZEL, M. & FLEISCHHACKER, W. (1987). An efficient synthesis of 14-halogenomethyl-substituted C-normorphinans. *Heterocycles*, **26**, 2703–3710.

KRATZEL, M. & VÖLLENKLE, H. (1991). 10,11-Epoxy-3-methyl-1,2,3,4-tetrahydro-4a, 10b-pro-pano-benzo[*f*] isoquinolin-12-ones: a novel type of opioid analgesics – synthetic approach and structure elucidation. *Heterocycles*, **32**, 2381–2388.

MAGNAN, J., PATERSON, S.J., TAVANI, A. & KOSTERLITZ, H.W. (1982). The binding spectrum of narcotic analgesic drugs. *Naunyn Schmied. Arch. Pharmacol.*, **319**, 197–205.

MARULLO, S., EMORINE, L.J., STROSBERG, A.D. & DELAVIER-KLUTCHKO, C. (1990). Selective binding of ligands to β_1, β_2 or chimeric β_1/β_2-adrenergic receptors involves multiple subsites. *EMBO J.*, **9**, 1471–1476.

OLAH, M., REN, H., OSTROWSKI, J., JACOBSON, K.A. & STILES, G.L. (1992). Cloning, expression and characterization of the unique bovine A_1-adenosine receptor: studies on the ligand binding site by site-directed mutagenesis. *J. Biol. Chem.*, **267**, 10764–10770.

PASTERNAK, G.W. (1988). Multiple mu opiate receptors. *ISI Atlas of Pharmacology 1988*, 148–154.

PERT, C. & SNYDER, S.H. (1973). Opiate receptor: demonstration in nervous tissue. *Science*, **179**, 1011–1014.

PERT, C., PASTERNAK, G.W. & SNYDER, S.H. (1973). Opiate agonists and antagonists discriminated by receptor binding in brain. *Science*, **182**, 1359–1361.

PORTOGHESE, P.S. (1989). Bivalent ligands and the message-address concept in the design of selective opioid receptor antagonists. *Trends Pharmacol. Sci.*, **10**, 230–235.

SCHÜTZ, W. & FREISSMUTH, M. (1992). Reverse intrinsic activity of antagonists at G protein-coupled receptors. *Trends Pharmacol. Sci.*, **13**, 376–380.

SCHÜTZ, W., NANOFF, C. & FREISSMUTH, M. (1988). The fallacy of non-selectivity of radioactive ligands. *Trends Pharmacol. Sci.*, **9**, 261–264.

STRADER, C.D., GAFFNEY, T., SUGG, E.E., RIOS CANDELORE, M., KEYS, R., PATCHETT, A.A. & DIXON, R.A.F. (1991). Allele-specific activation of genetically engineered receptors. *J. Biol. Chem.*, **266**, 5–8.

STROSBERG, A.D. (1991). Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. *Eur. J. Biochem.*, **196**, 1–10.

TOLL, L. (1992). Comparison of mu opioid receptor binding on intact neuroblastoma cells with guinea pig brain and neuroblastoma cell membranes. *J. Pharmacol. Exp. Ther.*, **260**, 9–15.

(Received March 12, 1993
Revised July 22, 1993
Accepted August 18, 1993)

Br. J. Pharmacol. (1993), **110**, 1437–1440 © Macmillan Press Ltd, 1993

Mechanism of rat uterine smooth muscle contraction induced by endothelin-1

[1]Hajime Tsunoda, *Takashi Miyauchi, Kayo Fujita, Takeshi Kubo & *Katsutoshi Goto

Department of Obstetrics and Gynecology, Institute of Clinical Medicine, and *Department of Pharmacology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

1 Endothelin (ET)-1 has been demonstrated to cause contraction of uterine smooth muscle. We investigated the role of ET receptor subtypes (ET_A and ET_B receptors) in ET-1-induced contraction of rat uterine smooth muscle by using the ET_A receptor antagonist BQ-123 and the ET_B receptor agonist BQ-3020.

2 ET-1 caused a contraction with superimposed oscillations of the rat isolated uterus suspended in Krebs-Ringer solution; both the amplitude of contraction as well as the oscillation frequency increased in a dose-dependent manner (10^{-11}–10^{-7} M).

3 BQ-123 (10^{-6} M) markedly shifted the dose-response curve of ET-1 for both contractile effects and oscillation frequency to the right.

4 BQ-3020 (10^{-11}–3×10^{-7} M) did not cause uterine contraction; neither did it affect the dose-response curve of ET-1 for either the contractile effect or the increase in oscillation frequency. Thus, stimulation of ET_B receptors is not involved in these responses.

5 The present findings suggest that ET-1-induced contractile responses and the increase in oscillation frequency in rat uterine smooth muscle is mediated through ET_A receptors, and that ET_B receptors play no role in these responses.

Keywords: Endothelin-1; ET_A receptor; ET_B receptor; BQ-123; BQ-3020; uterus; contraction

Introduction

Endothelin (ET)-1, a potent vasoconstrictor peptide, was first isolated from culture supernatant of endothelial cells (Yanagisawa *et al.*, 1988). Further studies revealed the existence of two related peptides, ET-2 and ET-3 (Yanagisawa & Masaki, 1989), and of two distinct ET receptor subtypes termed the ET_A receptor (ET-1 selective) and the ET_B receptor (equally sensitive to isopeptides of the endothelin family) (Arai *et al.*, 1990; Sakurai *et al.*, 1990; 1992). These peptides produce contractions of smooth muscle from various vascular and nonvascular tissues, such as the uterus, trachea, and stomach (Yanagisawa & Masaki, 1989; Sakurai *et al.*, 1992). It has been reported that ET-1-induced contraction of the porcine coronary artery is mediated mainly through ET_A receptors (Ihara *et al.*, 1992a), whereas ET-1-induced contraction of the guinea-pig trachea has been shown to be mediated mainly through ET_B receptors (Takai *et al.*, 1992).

Although it has been demonstrated that ET-1 causes contraction of the rat uterus (Bousso-Mittler *et al.*, 1989), the roles of the endothelin receptor subtypes (ET_A and ET_B receptors) in this response have not been established. In the present study, contraction of the rat uterus in response to ET-1 was analyzed by using the ET_A receptor antagonist BQ-123 (Ihara *et al.*, 1992a) and the ET_B receptor agonist BQ-3020 (Ihara *et al.*, 1992b).

Methods

Preparation and solutions

Virgin female Sprague-Dawley rats (200–250 g) were injected subcutaneously with 0.2 mg of β-oestradiol 24 h before the experiments. After anaesthetization with sodium pentobarbitone (50 mg kg^{-1}, i.p.), the uterine horns were removed and mounted in an organ bath containing 5 ml of Krebs-Ringer solution of the following composition (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5. The Krebs-Ringer solution was maintained at 37°C and aerated with a mixture of 95% O_2–5% CO_2.

Mechanical activity

The isometric contraction of the uterus was measured with a force-displacement transducer (TB-612T, Nihon Kohden, Tokyo, Japan) connected to a polygraph system (amplifier, AP-601G, Nihon Kohden; thermal-pen recorder, WT-687G, Nihon Kohden). A resting tension of 1 g was applied to the tissue, and an equilibration period of 2 h was allowed during which the tissue was washed with fresh solution every 15 min. After equilibration, a maximum response to KCl (50 mM) was established repeatedly at 30 min intervals until a steady response was obtained (usually, three to four times). Thereafter, the dose-response relationship for ET-1 or BQ-3020 was determined by means of cumulative application. To examine the effects of BQ-123 on the dose-response curve of ET-1, BQ-123 (10^{-6} M) was applied 15 min before the cumulative application of ET-1. For normalization, the responses to ET-1 or BQ-3020 were expressed as % of the maximum response to KCl. Since ET-1 also induced oscillation-like contractions, we measured both the frequency of the oscillation and the increase in peak tension. To study the effect of BQ-3020 on the ET-1-induced uterine contraction, the dose-response curve of ET-1 in the presence of BQ-3020 was also determined.

Statistical analysis

Values are expressed as mean ± s.e.mean. Statistical analyses were performed by an unpaired Student's *t* test and a *P* value of less than 0.05 was considered significant.

Drugs

ET-1 was purchased from Peptide Institute Inc. (Osaka. Japan). The selective ET_A receptor antagonist BQ-123 (cyclo

[1] Author for correspondence.

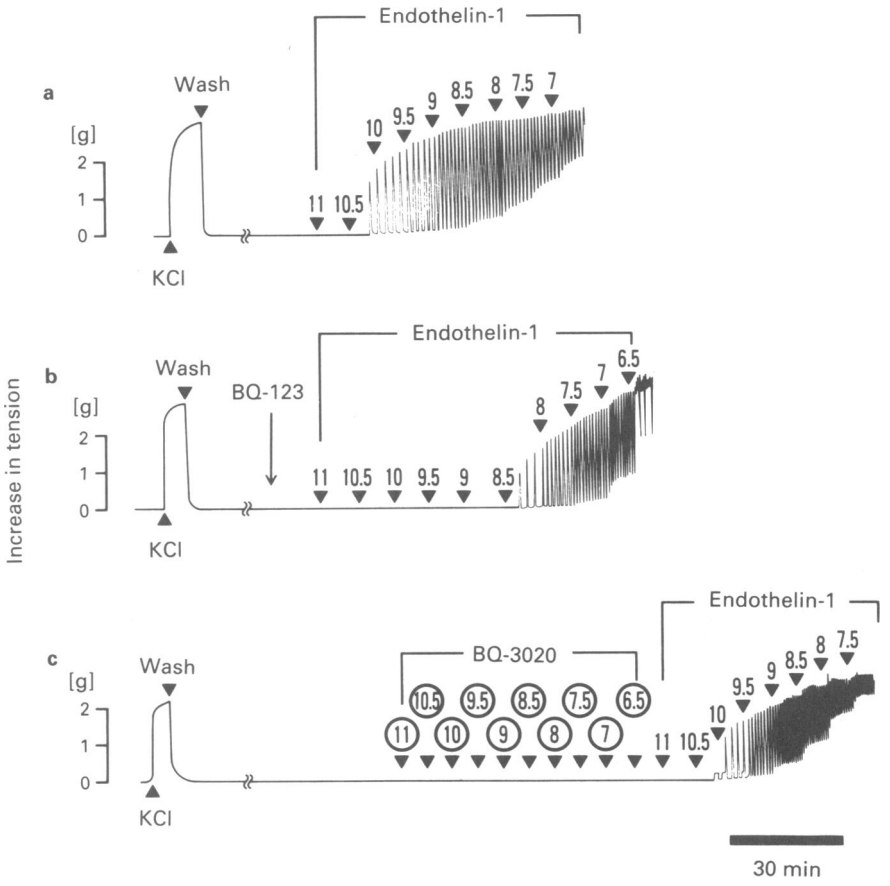


Figure 1 Representative traces of the responses to increasing concentrations of endothelin-1 of the rat uterus in the absence (a) or presence (b) of the ET_A receptor antagonist BQ-123 (10^{-6} M). As shown in (c), although the ET_B receptor agonist BQ-3020 caused no contraction of the uterus, ET-1 caused a contraction of the same tissue. Numbers, which are not encircled, in the figure indicate the − log molar concentration of endothelin-1. Encircled numbers in (c) indicate the − log molar concentration of BQ-3020. The transverse bar indicates time.

(-D-Trp-D-Asp-Pro-D-Val-Leu)), and the selective ET_B receptor agonist BQ-3020 (N-acetyl-LeuMetAspLysGluAlaValTyrPheAlaHisLeuAspIleIleTry) were synthesized at Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan).

Results

ET-1 produced dose-dependent contraction of the rat uterus (Figures 1a and 2a). This response to ET-1 included oscillation-like contractions superimposed on an increase in baseline tension. Since this oscillation-like response sometimes lacked the relaxation phase and became a tetanic contraction, we measured the contractile tension only at the peak of the systolic phase for the analysis in Figures 2a, 3 and 4. The dose-response relationship for the contractile effects of ET-1 on the rat uterus are shown in Figure 2a; the pD_2 value was 9.99 ± 0.26, $n = 7$. In the presence of BQ-123 (10^{-6} M), an ET_A receptor antagonist, the dose-response curve for the contractile effects of ET-1 was shifted significantly to the right (Figures 1b and 2a).

The calculated pK_B value of BQ-123 for antagonizing ET-1-induced contractile responses was 7.51 ± 0.04. ET-1 also caused an increase in the oscillation frequency in a dose-dependent manner (Figures 1a and 2b). Since ET-1-induced uterine contraction sometimes lacked the relaxation phase when ET-1 concentrations were high, we quantified oscillation frequency only up to a concentration of 10^{-8} M (Figure 2b). In the presence of BQ-123 (10^{-6} M), the dose-oscillation-frequency curve for ET-1 was shifted significantly to the right (Figure 2b). The calculated pK_B value of BQ-123 for blockade of the ET-1-induced increase in oscillation frequency was 7.39 ± 0.11.

BQ-3020 ($10^{-11} - 3 \times 10^{-7}$ M), a selective ET_B receptor agonist, produced no contraction of the uterine smooth muscle (Figures 1c and 3). Furthermore, BQ-3020 (3×10^{-7} M) did not modify the dose-response curve of ET-1 for either the contractile effect or the increase in oscillation frequency (Figure 4).

Discussion

We have shown that the ET_A receptor antagonist, BQ-123, effectively antagonized ET-1-induced contraction of the uterine smooth muscles, and that the ET_B receptor agonist BQ-3020 produced no contractile response on the tissues. Since it has been demonstrated that the potency of BQ-3020 with respect to stimulation of ET_B receptors is almost the same as that of ET-1 (Ihara *et al.*, 1992b), the dose of BQ-3020 used in the present study ($10^{-11} - 3 \times 10^{-7}$ M) was considered to be appropriate for the stimulation of ET_B receptors. We have also shown that BQ-123 effectively antagonizes the ET-1-induced increase in the oscillation frequency of the rat uterus. These data suggest that only ET_A receptors play a role in uterine contraction induced by ET-1 in rats, and that ET_B receptors are not involved in this response. The pK_B value (7.51) of BQ-123 for blockade of the ET-1-induced increase in the contraction was similar to that for antagonism of the ET-1-induced increase in the oscillation frequency (7.39). These pK_B values are similar to the pA_2 value (7.4) which has been determined for blockade of ET-1 by BQ-123 on the porcine isolated coronary artery (Ihara *et al.*, 1992a). Thus, it

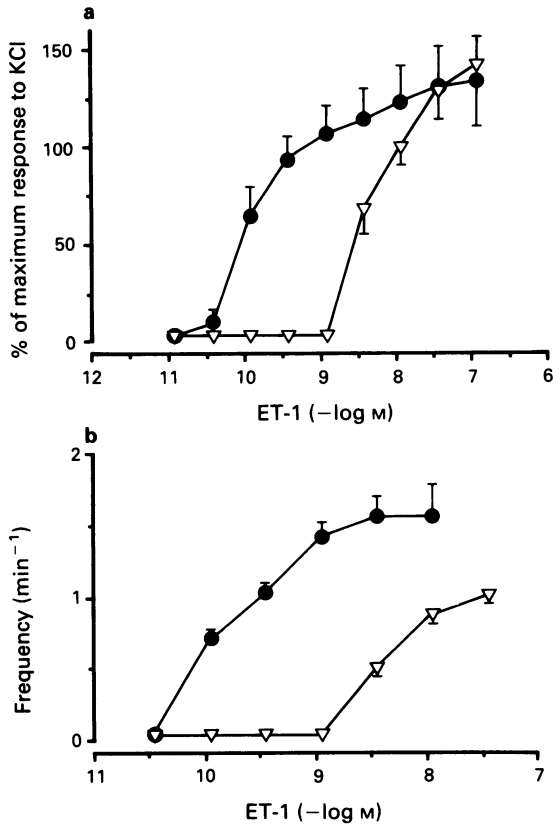


Figure 2 Dose-response curves for the contractile effects (a) and increase in oscillation frequency (b) induced by ET-1 on the rat uterus in the absence (●; $n = 7$) or presence of the ET_A receptor antagonist BQ-123 (10^{-6} M; ▽; $n = 7$). In (a), the contractile effects on the rat uterine smooth muscle are expressed as % of maximum contraction to 50 mM KCl. In (b), oscillation frequency was calculated per min for each dose until the contraction of some tissues became tetanic (up to 10^{-8} M). Points and vertical lines represent means ± s.e.mean, respectively.

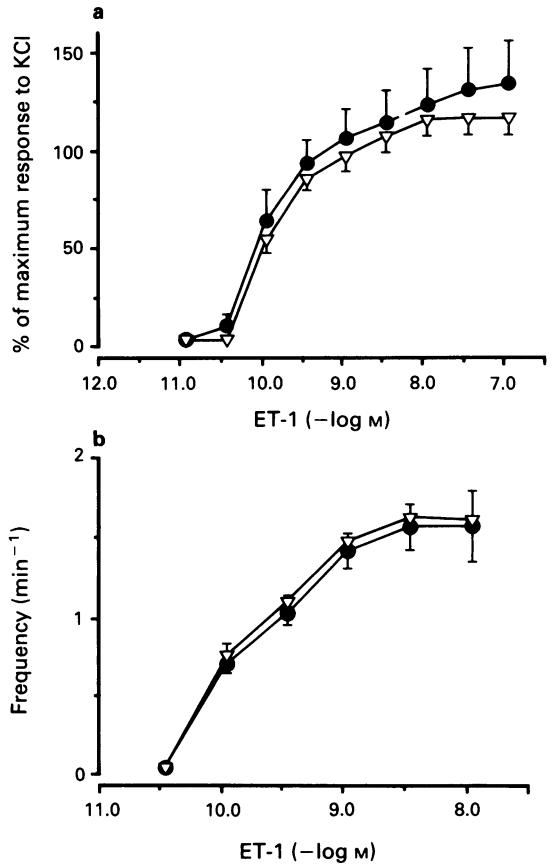


Figure 4 Dose-response curves for the contractile effects (a) and oscillation frequency (b) of ET-1 on the rat uterus in the absence (●; $n = 7$) or presence (▽; $n = 7$) of the ET_B receptor agonist BQ-3020 (3×10^{-7} M). In (b), oscillation frequency was calculated per min for each concentration until the contraction of some tissues became tetanic (up to 10^{-8} M). Points and bars represent means ± s.e.mean, respectively.

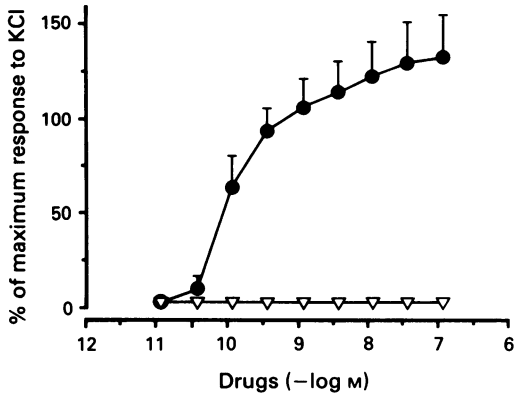


Figure 3 Effects of increasing doses of the ET_B receptor agonist BQ-3020 (▽; $n = 7$) or ET-1 (●; $n = 7$) on the rat uterus. Points show means and vertical lines s.e.mean.

is suggested that the ET-1-induced increase in both the contraction and the oscillation frequency is mediated through ET_A receptors.

The ET_A receptor shows far higher affinity for ET-1 than ET-3, whereas the ET_B receptor exhibits a high affinity for both ET-1 and ET-3 (Arai *et al.*, 1990; Sakurai *et al.*, 1990; 1992). Bousso-Mittler *et al.* (1989) and Sakata & Kuraki (1992) demonstrated that the potency of ET-1 for causing contraction of the rat uterus is markedly higher than that of ET-3. Based on these results, they speculated that the ET-1-induced contraction of the rat uterus is mediated mainly through ET_A receptors. The present data obtained using an ET_A receptor antagonist support this conclusion.

An important finding in the present study is that BQ-3020, a selective ET_B receptor agonist, had no effects *per se* on rat uterus. Further, neither the increases in the contractile tension nor the increases in oscillation frequency induced by ET-1 were affected by BQ-3020 in the rat uterus. Therefore, it can be concluded that activation of ET_B receptors neither stimulates the rat uterus nor affects the ET_A receptor-mediated uterine contraction. However, since the expression of ET_B receptor mRNA has been demonstrated in the rat uterus (Sakurai *et al.*, 1990), it is possible that ET_B receptors may play a role other than inducing contraction of the smooth muscle in this tissue.

It has been shown that ET-1 is produced by human endometrial tissues (Economos *et al.*, 1992). Furthermore, Usuki *et al.* (1990) reported that the maternal circulating ET-1 level increases at the end of pregnancy and during labour, and that a large amount of immunoreactive ET-1 exists in human amniotic fluid. These findings suggest that ET-1 may have a role in the modulation of the uterine function. Since ET-1 showed extremely potent contractile effects on estrogen-dominated uterine smooth muscles in rats through activation of ET_A receptors, possible alteration in the function and expression of ET receptor subtypes during the later stages of pregnancy may be relevant to the marked increase in drug sensitivity in this period. This possibility is now under investigation in our laboratory.

This work was supported by a grant of the Special Research Project on the Circulation Biosystem in the University of Tsukuba and by the University of Tsukuba Project Research. We thank Drs Masaru Nishikibe and Mitsuo Yano, at Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba, Japan for useful discussions and for the generous gifts of BQ-123 and BQ-3020. We also thank Mrs Yumiko Kawakura for her excellent technical assistance.

References

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

BOUSSO-MITTLER, D., KLOOG, Y., WOLLBERG, Z., BDOLAH, A., KOCHVA, E. & SOKOLOVSKY, M. (1989). Functional endothelin/sarafotoxin receptors in the rat uterus. *Biochem. Biophys. Res. Commun.*, **162**, 952–957.

ECONOMOS, K., MACDONALD, P.C. & CASEY, M.L. (1992). Endothelin-1 gene expression and protein biosynthesis in human endometrium: potential modulator of endometrial blood flow. *J. Clin. Endocrinol. Metab.*, **74**, 14–19.

IHARA, M., NOGUCHI, K., SAEKI, T., FUKURODA, T., TSUCHIDA, S., KIMURA, S., FUKAMI, T., ISHIKAWA, K., NISHIKIBE, M. & YANO, M. (1992a). Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci.*, **50**, 247–255.

IHARA, M., SAEKI, T., FUKURODA, T., KIMURA, S., OZAKI, S., PATEL, A.C. & YANO, M. (1992b). A novel radioligand [^{125}I]BQ-3020 selective for endothelin (ET_B) receptors. *Life Sci.*, **51**, 47–52.

SAKATA, K. & KARAKI, H. (1992). Effects of endothelin on cytosolic Ca^{2+} level and mechanical activity in rat uterine smooth muscle. *Eur. J. Pharmacol.*, **221**, 9–15.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of endothelin receptor. *Nature*, **348**, 732–735.

SAKURAI, T., YANAGISAWA, M. & MASAKI, T. (1992). Molecular characterization of endothelin receptors. *Trends Pharmacol. Sci.*, **13**, 103–108.

TAKAI, M., UMEMURA, I., YAMASAKI, K., WATANABE, T., FUJITANI, Y., ODA, K., URADE, Y,. INUI, T., YAMAMURA, T. & OKADA, T. (1992). A potent and specific agonist, Suc-[Glu^9, Ala^{11,15}]-endothelin-1(8–21), IRL 1620, for the ET_B receptor. *Biochem. Biophys. Res. Commun.*, **184**, 953–959.

USUKI, S., SAITOH, T., SAWAMURA, T., SUZUKI, N., SHIGEMITU, S., YANAGISAWA, M., GOTO, K., ONDA, H., FUJINO, M. & MASAKI, T. (1990). Increased maternal plasma concentration of endothelin-1 during labor pain or on delivery and the existence of a large amount of endothelin-1 in amniotic fluid. *Gynecol. Endocrinol.*, **4**, 85–97.

YANAGISAWA, M., KURIHARA, H,. KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 441–445.

YANAGISAWA, M. & MASAKI, T. (1989). Molecular biology and biochemistry of the endothelins. *Trends Pharmacol. Sci.*, **10**, 374–378.

(Received July 13, 1993
Revised August 9, 1993
Accepted August 18, 1993)

Br. J. Pharmacol. (1993), **110**, 1441-1444 © Macmillan Press Ltd, 1993

Induction of bradykinin B_1 receptors *in vivo* in a model of ultra-violet irradiation-induced thermal hyperalgesia in the rat

[1]M.N. Perkins & D. Kelly

Sandoz Institute for Medical Research, Gower Place, London EC1E 6BN

1 The role of bradykinin B_1 receptors in the thermal hyperalgesia following unilateral ultra-violet (u.v.) irradiation of the hindpaw of rats has been investigated.

2 In non-irradiated (naive) animals the B_1 receptor agonist des-Arg^9-bradykinin and bradykinin (BK) (up to 1 μmol kg^{-1} i.v.) had no effect on withdrawal latency to a noxious heat stimulus when administered 60 min before testing.

3 Following exposure of one hindpaw to strong u.v. irradiation the withdrawal latency of the u.v.-treated paw to radiant noxious heat fell by a maximum of 50% after 48 h. There was no reduction in latency in the contralateral paw.

4 des-Arg^9-BK (1-100 nmol kg^{-1} i.v.) administered 24 h after u.v. exposure caused a further dose-dependent fall (50 $\pm$ 4% reduction from saline injected animals at 100 nmol kg^{-1} i.v.) in withdrawal latency in the u.v.-treated paw when measured 60 min after injection. The withdrawal latency of the contralateral paw was also reduced but to a lesser extent following des-Arg^9-BK (100 nmol kg^{-1} i.v.) with a maximum reduction of 19 $\pm$ 3%.

5 Bradykinin also induced a further reduction in withdrawal latency (33 $\pm$ 5% reduction at 1 μmol kg^{-1}) although it was not as effective as des-Arg^9-BK. Bradykinin did not reduce the withdrawal latency in the contralateral paw.

6 The hyperalgesic action of both des-Arg^9-BK (10 nmol kg^{-1} i.v.) and bradykinin (100 nmol kg^{-1} i.v.) were antagonized by the B_1 receptor antagonist, des-Arg^9,Leu^8-BK (200 nmol kg^{-1} i.v.) but not by the B_2 receptor antagonist, HOE 140 (0.5 μmol kg^{-1} i.v.).

7 The results suggest that in conditions of inflammatory hyperalgesia bradykinin B_1 receptors are induced both locally and distant to the inflamed area, activation of which leads to further thermal hyperalgesia. In addition, in these conditions bradykinin appears to act predominantly via B_1 receptors, presumably after degradation to des-Arg^9-BK.

Keywords: Bradykinin; B_1 receptors; inflammation; nociception

Introduction

The acute activation of sensory neurones by kinins in normal animals has been shown to be mediated through the bradykinin B_2 receptor (Dray *et al.*, 1988; Steranka *et al.*, 1989; Rang *et al.*, 1991).

Recently we have shown that in two persistent inflammatory models in rats the accompanying thermal and mechanical hyperalgesia is reversed by the bradykinin (BK) B_1 receptor antagonist des-Arg^9,Leu^8-BK (Perkins *et al.*, 1992; 1993). In contrast, the potent B_2 antagonist HOE 140 (D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK) (Hock *et al.*, 1991) was only weakly active in these models (Perkins *et al.*, 1992; 1993) suggesting a different pharmacology for bradykinin under these pathophysiological conditions. In support of this was the finding that des-Arg^{9}BK could induce a small exacerbation of the hyperalgesia particularly in the model of ultra-violet (u.v.) irradiation-induced thermal hyperalgesia (Perkins *et al.*, 1992; 1993). This study was the first demonstration *in vivo* that in inflammatory hyperalgesia there was the induction of a bradykinin B_1 receptor and further suggested that the accompanying kinin contribution to the maintenance of this hyperalgesia was predominantly via the activation of B_1 and not B_2 receptors.

That study, however, relied mainly on the use of bradykinin B_1 and B_2 antagonists rather than agonists. We have extended that previous study in order to establish the pharmacology of the hyperalgesic actions of des-Arg^9-BK and bradykinin as well as the time course of the induction of the B_1 receptor following u.v. irradiation. In addition, in view of recent findings suggesting that there can be expression of bradykinin B_1 receptors systemically following a localised inflammatory insult (Farmer *et al.*, 1991), we have explored the possibility of induction of bradykinin B_1 receptors mediating hyperalgesia distant to the u.v.-treated area.

Methods

The thermal hyperalgesia induced by the u.v. irradiation protocol previously described (Perkins *et al.*, 1992; 1993) approached the maximum that could be obtained and was not, therefore suitable for studying the pharmacology of agonists which might be expected to produce further hyperalgesia.

Therefore, in these experiments rats (female Sprague Dawley 100 g) received one instead of two unilateral exposures to u.v.A light (90 s, intensity maximum 365 nm, 69 mwatt cm^{-2}). On each day for the next four days, different groups of u.v.-treated rats (typically four) were placed in a transparent perspex box with a thin glass floor. The time taken to withdraw each hind paw following a heat stimulus, applied to the underside of the paw was measured. The heat stimulus was a focused radiant heat beam provided by a light bulb. The hind paws were exposed to the heat stimulus in a random manner so that there was no consistent order of testing between ipsilateral and contralateral paws and no rat had both paws exposed to heat immediately after each other. The withdrawal latencies of both paws were then measured at various times after the u.v. exposure. Drugs were administered intravenously, typically 60 min prior to

[1] Author for correspondence.

measurement of withdrawal latency and antagonists were co-administered with agonists.

All drugs except the B_1 and B_2 agonists and antagonists were obtained from Sigma. Bradykinin was synthesized at Sandoz, Basel. des-Arg^9-BK and des-Arg^9,Leu^8-BK were obtained from Bachem A.G. (D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK) (HOE 140) was synthesized by Dr A. Hallett, The Sandoz Institute for Medical Research, London.

Statistical analysis

Results are expressed as mean latency (s) ± s.e.mean in the case of the determination of time courses. Drugs effects were expressed as the % reduction in withdrawal latency following drug administration, compared with the pre-drug measurement in the same animal. For the time course measurement data were analysed by Dunnett's test for multiple comparison, otherwise one way analysis of variance followed by paired Student's *t* test (for comparison of post- and pre-drug treatment values) or unpaired Student's *t* test (for comparison between drug and vehicle treated groups).

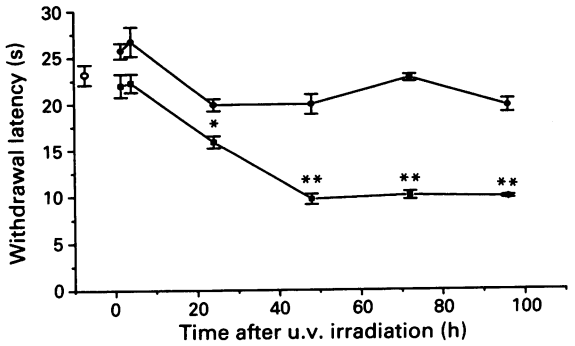


Figure 1 Time course (h) of foot withdrawal latency (s, means ± s.e. mean, $n = 4$) to a thermal stimulus in the ipsilateral (solid squares) and contralateral (solid circles) hindpaws of rats following u.v. irradiation of the ipsilateral paw. The open circle shows the withdrawal latency of naive rats. There was a significant reduction in paw withdrawal latency by 24 h, which persisted throughout the period of measurement to 96 h. There was no hyperalgesia in the contralateral paw. $^{**}P<0.01$ and $^{*}P<0.05$ denote significant differences compared with naive animals.

Results

Naive animals

In non-u.v. treated (naive) rats neither des-Arg^9-BK nor bradykinin had any effect when injected 60 min before testing at up to 1 μmol kg^{-1} i.v. Additionally, as has been shown previously, neither HOE 140 nor des-Arg^9,Leu^8-BK had any effect on withdrawal latency when administered to naive animals (data not shown and Perkins *et al.*, 1992).

Time course of hyperalgesia

On the day after the u.v. exposure there was reddening of the treated hind paw but no evidence of skin damage. Mild inflammation was evident and was characterized by erythema but there was no evidence of swelling of the digits or foot pad. There was some mild blistering of the glabrous skin without signs of weeping or abrasions, the blistering lasting for 2–3 days. Animals appeared to groom normally and did not exhibit any obvious signs of distress while being observed or when being handled. There was no weight loss at any stage following u.v. treatment.

Four hours after u.v. treatment the withdrawal latency of the ipsilateral paw remained unchanged from that of naive animals, but at 24 h, the latency fell significantly from 22 ± 1 s (mean ± s.e.mean, $n = 4$) to 15.9 ± 0.7 s ($n = 8$, see Figure 1). There was then a further fall in latency to 10.8 ± 0.7 s ($n = 4$) by 48 h after which the latency remained fairly constant up to 96 h (see Figure 1). In the contra-lateral paw there was no significant reduction in latency at any time point.

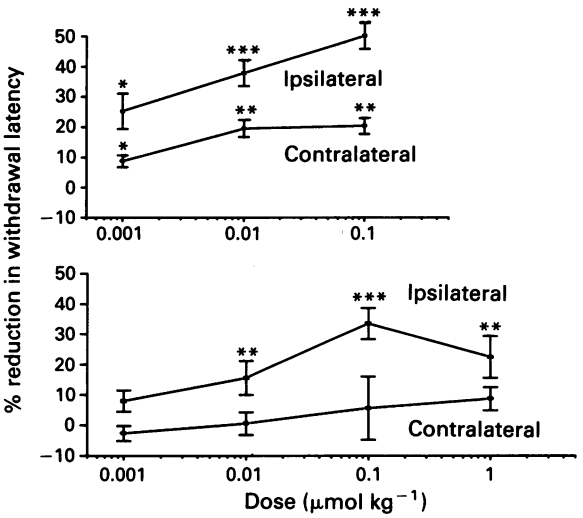


Figure 2 Withdrawal latencies (% reduction from vehicle controls, mean ± s.e.mean, $n = 4$) for both the ipsilateral (u.v.-treated paw, solid squares) and contralateral (solid circles) hindpaws of rats following intravenous injections (μmol kg^{-1}) of des-Arg^9-bradykinin (BK) (a) and bradykinin (b). There was a significant reduction in withdrawal latency in both paws after all doses of des-Arg^9-BK (a). Bradykinin (b) was less effective in reducing latency and only in the ipsilateral paw. $^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$ denote significant differences compared with vehicle injected animals.

Effect of bradykinin receptor agonists and antagonists

Ipsilateral paw 24 h after u.v.-irradiation, des-Arg^9-BK, injected 60 min before testing, caused a dose-dependent reduction in withdrawal latency with a 25 ± 6% ($P<0.05$, $n = 4$) reduction in withdrawal latency at 1 nmol kg^{-1} i.v. rising to 50 ± 4% at 100 nmol kg^{-1} i.v. (see Figure 2). Bradykinin also produced a reduction in latency although it was less efficacious than des-Arg^9-BK producing a 33 ± 5% ($P<0.001$, $n = 4$) reduction at 100 nmol kg^{-1} with no further reduction at 1 μmol kg^{-1} i.v. (see Figure 2).

Co-administration of the B_1 antagonist, des-Arg^9,Leu^8-BK (200 nmol kg^{-1} i.v.), antagonized the hyperalgesic action of both des-Arg^9-BK (10 nmol kg^{-1} i.v.) and bradykinin (100 nmol kg^{-1} i.v.) (see Figure 3). Co-administration of the B_2 antagonist, HOE 140 (0.5 μmol kg^{-1} i.v.) neither prevented the hyperalgesic action of des-Arg^9-BK nor bradykinin in the ipsilateral paw (see Figure 3).

Contralateral paw Bradykinin had no significant effect on the withdrawal latency of the contralateral paw at doses from 1 nmol kg^{-1} i.v. to 1 μmol kg^{-1} i.v. In contrast, des-Arg^9-BK produced a small but significant reduction in withdrawal latency of 9 ± 2% ($P<0.05$, $n = 4$) in the contra-lateral paw at 1 nmol kg^{-1} i.v. increasing to a reduction of 19.5 ± 3% ($P<0.01$, $n = 4$) at 10 nmol kg^{-1} i.v. with no further increase at 100 nmol kg^{-1} i.v. (see Figure 2).

This hyperalgesic action of des-Arg^9-BK was antagonized by des-Arg^9,Leu^8-BK (200 nmol kg^{-1} i.v., $n = 4$) but not by HOE 140 (0.5 μmol kg^{-1} i.v.) (see Figure 3).

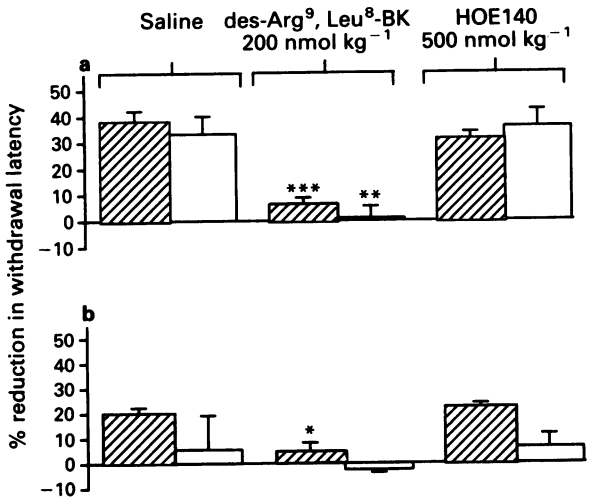


Figure 3 Percentage reduction in withdrawal latency (mean ± s.e. mean, $n = 4$) for the ipsilateral (a) and contralateral (b) hindpaws following administration of des-Arg^9-bradykinin (10 nmol kg^{-1} i.v., shaded columns) and bradykinin (BK, 100 nmol kg^{-1} i.v., open columns). The B_1 receptor antagonist, des-Arg^9,Leu^8-BK co-administered (200 nmol kg^{-1} i.v.) with the agonist significantly reduced the response to both des-Arg^9-BK and bradykinin. The B_2 antagonist, HOE 140 (500 nmol kg^{-1} i.v.) co-administered in the same way did not reduce the response to either agonist. $***P<0.001$, $**P<0.01$ and $*P<0.05$ denote significant differences compared with respective saline injected animals.

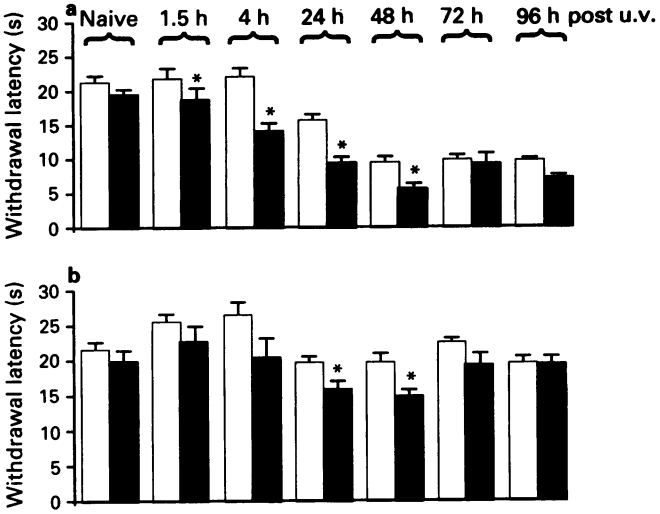


Figure 4 Withdrawal latencies (s, mean ± s.e.mean, $n = 4$) of ipsilateral (a) and contralateral (b) hindpaws of rats showing the time course of induction of des-Arg^9-bradykinin (BK)-induced hyperalgesia. In both (a) and (b) the open columns represent the withdrawal latency preceding administration of des-Arg^9-BK (10 nmol kg^{-1} i.v.) and the black columns the withdrawal latency 60 min after des-Arg^9-BK injection. In naive animals des-Arg^9-BK had no effect. Following u.v. exposure to the ipsilateral paw there was a reduction in withdrawal latency signifying hyperalgesia only in the ipsilateral paw. des-Arg^9-BK was able to produce a further reduction in withdrawal latency 1.5–4 h after u.v. exposure in the ipsilateral paw (a) and 24 h later in the contralateral paw (b). $*P<0.05$ denote significant differences compared with respective saline injected animals.

Time course of induction of B_1 response

The time course of the induction of the response to des-Arg^9-BK following u.v. irradition was determined for both the ipsi- and contra-lateral paws; 1.5 h after u.v. exposure des-Arg^9-BK (10 nmol kg^{-1} i.v.) caused a small but significant reduction in withdrawal latency from 22 ± 1 s to 19 ± 1 s ($P<0.05$, $n = 4$) in the ipsi-lateral paw (see Figure 4). By 4 h there was a much more marked hyperalgesic effect of des-Arg^9-BK the withdrawal latency falling to 14 ± 0.8 s ($n = 4$) (see Figure 4). The hyperalgesic action of des-Arg^9-BK was clearly present for up to 48 h post-u.v. and then became erratic in that there was no significant effect at 72 h but a small, though significant, reduction in withdrawal latency at 96 h post u.v. exposure (see Figure 4).

In the contralateral paw des-Arg^9-BK produced significant hyperalgesia (20-22% reduction in latency) only at the 24 and 48 h points (Figure 4).

Discussion

The data presented here show that following an inflammatory insult des-Arg^9-BK is able to induce thermal hyperalgesia both in the inflamed area and in the contralateral paw. A previous study, with B_1 and B_2 receptor antagonists, had provided evidence that B_1 receptor-mediated effects were a predominant factor in the hyperalgesia observed following u.v.-irradiation (Perkins *et al.*, 1993).

The reduction of des-Arg^9-BK-induced hyperalgesia by a B_1, but not a B_2, receptor antagonist suggests that this action of des-Arg^9-BK is due to the induction of B_1 receptors which are not normally present, as des-Arg^9-BK is inactive in naive animals. In addition, it appears that the action of bradykinin in inducing thermal hyperalgesia is also via a B_1 receptor. Bradykinin is rapidly broken down after systemic administration with over 90% being degraded to des-Arg^9-BK and other metabolites after a single passage through the lungs (see Regoli & Barabe, 1980). It is likely, therefore, that the bradykinin-induced hyperalgesia was due to the production of des-Arg^9-BK.

The presence of contralateral hyperalgesia induced by des-Arg^9-BK may imply that u.v. irradiation of one paw leads to a systemic induction of B_1 receptors. This is consistent with a recent study by Farmer *et al.* (1991) showing that following antigen-induced arthritis in a knee joint of the rabbit there was induction of B_1 receptors on the aorta. In view of the evidence *in vitro* linking the induction of B_1 receptors with inflammatory mediators (Toda *et al.*, 1987; Bouthillier *et al.*, 1987; Cahill *et al.*, 1988; deBlois *et al.*, 1988; 1989; D'Orleans-Juste *et al.*, 1989; Drapeau *et al.*, 1991), one explanation for this contralateral hyperalgesia is that the circulating cytokines produced during inflammation are sufficient to induce the formation of the B_1 receptor.

The time course of the induction of the B_1 response is consistent with that observed in studies *in vitro* where responsiveness to des-Arg^9-BK increases over a 6 h period (Regolie & Barabe 1980; Bouthillier *et al.*, 1987; deBlois *et al.*, 1989). With respect to the contralateral side it is not possible to determine whether the apparent delay in induction of the des-Arg^9-BK-induced hyperalgesia is a real phenomenon or is a consequence of the smaller hyperalgesic response. In either case, if the B_1 receptor induction is dependent on inflammatory mediators such as cytokines then the reduced degree of des-Arg^9-BK-induced hyperalgesia in this paw may reflect lower levels of these mediators in areas distant to the u.v.-inflamed paw.

Previous studies, in which B_2 and B_1 agonists have been used with respect of activation of nociceptors, have all been performed in animals with no persistent inflammatory condition. BK but not des-Arg^9-BK has been shown to activate polymodal nociceptors in a number of circumstances. These range from direct recording *in vitro* of C-fibre nociceptor activity and bradykinin-evoked nociceptive reflexes (Griesbacher & Lembeck 1987; Lang *et al.*, 1990), to recording *in vivo* of bradykinin mediated excitation of nociceptors in skin, skeletal muscle, joints and visceral organs (Fock & Mense, 1976; Berkely *et al.*, 1988; 1990; Mizumura *et al.*, 1990). In

man it has been shown that bradykinin but not des-Arg^9-BK produces pain when applied to an acutely produced blister base and this pain was inhibited by a B_2 but not a B_1 antagonist (Whalley *et al.*, 1987).

It is not possible, from these experiments, to determine whether the B_1 receptor-mediated hyperalgesia is due to an induction of B_1 receptors on the nociceptive terminal itself with consequent direct activation by des-Arg^9-BK or whether there is an indirect action of des-Arg^9-BK via the release of sensitizing agents from other cell types. des-Arg^9-BK has been shown to increase the synthesis and release *in vitro* of inflammatory mediators such as prostacyclin (Toda *et al.*, 1987; Cahill *et al.*, 1988). and interleukin-1 (IL-1) (Tiffany & Burch 1989; Burch *et al.*, 1989). In the case of IL-1 release this has been shown to occur from macrophage cell lines (Tiffany & Burch 1989; Burch *et al.*, 1989), a cell type which would be expected to increase in numbers in inflamed areas. In addition, there may be further interactions between des-Arg^9-BK and inflammatory mediators such as the synergism that has been demonstrated between des-Arg^9-BK and IL-1 in increasing prostaglandin E_2 formation in human fibroblasts (Lerner & Modeer, 1991).

In summary, the data presented here provide further evidence that in persistent inflammatory conditions in the rat bradykinin B_1 receptors are expressed, activation of which produces significant hyperalgesia. This expression of B_1 receptors is not restricted to the area of inflammation occuring in the contralateral paw. Whether the B_1 receptor-induced hyperalgesia is a direct or indirect action on the nociceptive neurone remains to be elucidated.

References

BERKELY, K.J., HOTTA, H., ROBBINS, A. & SATO, Y. (1990). Functional properties of afferent fibres supply reproductive and other organs in pelvic area of female rat. *J. Neurophysiol.*, **63**, 256–272.

BERKELY, K.J., ROBBINS, A. & SATO, Y. (1988). Afferent fibres supplying the uterus in the rat. *J. Neurophysiol.*, **59**, 142–163.

BOUTHILLIER, J., DEBLOIS, D. & MARCEAU, F. (1987). Studies on the induction of pharmacological responses to des-Arg^9-bradykinin *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **92**, 257–264.

BURCH, R.M., CONNOR, J.R. & TIFFANY, C.W. (1989). The kallikrein-kininogen-kinin system in chronic inflammation. *Agents Actions*, **27**, 258–260.

CAHILL, M., FISHMAN, J.B. & POLGAR, P. (1988). Effect of des arginine^9-bradykinin and other bradykinin fragments on the synthesis of prostacyclin and the binding of bradykinin by vascular cells in culture. *Agents Actions*, **24**, 224–231.

DEBLOIS, D., BOUTHILLIER, J. & MARCEAU, F. (1988). Effect of glucocorticoids, monomines and growth factors on the spontaneously developing responses of the rabbit isolated aorta to des-Arg^9-bradykinin. *Br. J. Pharmacol.*, **93**, 969–977.

DEBLOIS, D., BOUTHILLIER, J. & MARCEAU, F. (1989). Pharmacological modulation of the up-regulated responses to des-Arg^9-bradykinin *in vivo* and *in vitro*. *Immunopharmacology*, **17**, 187–198.

D'ORLEANS-JUSTE, P., DE NUCCI, G. & VANE, J.R. (1989). Kinins act on B_1 or B_2 receptors to release conjointly endothelium-derived factor and prostacyclin from bovine aortic endothelial cells. *Br. J. Pharmacol.*, **96**, 920–926.

DRAPEAU, G., BEBLOIS, D. & MARCEAU, F. (1991). Hypotensive effects of Lys-des-Arg^9-bradykinin and metabolically protected agonists of B_1 receptors for kinins. *J. Pharmacol. Exp. Ther.*, **259**, 997–1003.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988). Activation of a bradykinin receptor in peripheral nerve and spinal cord in the neonatal rat *in vitro*. *Br. J. Pharmacol.*, **95**, 1008–1010.

FARMER, S.G., MCMILLAN, B.A., MEEKER, S.N. & BURCH, R.M. (1991). Induction of vascular smooth muscle bradykinin B_1 receptors *in vivo* during antigen arthritis. *Agents Actions*, **34**, 191–193.

FOCK, S. & MENSE, S. (1976). Excitatory effects of 5-hydroxytryptamine, histamine and potassium ions on muscular group IV afferent units: a comparison with bradykinin. *Brain Res.*, **105**, 459–467.

GRIESBACHER, T. & LEMBECK, F. (1987). Effect of bradykinin antagonists on bradykinin-induced plasma extravasation, venoconstriction, prostaglandin E_2 release, nociceptor stimulation and contraction of the iris sphincter muscle in the rabbit. *Br. J. Pharmacol.*, **92**, 333–340.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., ST. HENKE, G., BREIPOHL, G., KONIG, W., KNOLLE, J. & SCHOLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–774.

LANG, E., NOVAK, A., REEH, P.W. & HANDWERKER, H.O. (1990). Chemosensitivity of fibre afferents from rat skin *in vitro*. *J. Neurophysiol.*, **63**, 887–901.

LERNER, U.H. & MODEER, T. (1991). Bradykinin B_1 and B_2 receptor agonists synergistically potentiate interleukin-1-induced prostaglandin biosynthesis in human gingival fibroblasts. *Inflammation*, **15**, 427–436.

MIZUMURA, K., MINAGAWA, M., TSUJII, Y. & KUMAZAWA, T. (1990). The effects of bradykinin agonists and antagonists on visceral polymodal receptor activities. *Pain*, **40**, 221–227.

PERKINS, M.N., CAMPBELL, E.A., DAVIS, A. & DRAY, A. (1992). Anti-nociceptive activity of bradykinin B_1 and B_2 antagonists in two models of persistent hyperalgesia in the rat. *Br. J. Pharmacol.*, **107**, 237P.

PERKINS, M.N., CAMPBELL, E.A. & DRAY, A. (1993). Anti-nociceptive activity of the bradykinin B_1 and B_2 receptor antagonists, desArg^{9}Leu^{8}Bk and HOE 140, in two models of persistent hyperalgesia in the rat. *Pain*, **53**, 191–197.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

RANG, H.P., BEVAN, S. & DRAY, A. (1991). Chemical activation of nociceptive peripheral neurones. *Br. Med. Bull.*, **47**, 534–548.

STERANKA, L.R., FARMER, S.G. & BURCH, R.M. (1989). Antagonists of B_2 bradykinin receptors. *FASEB. J.*, **3**, 2019–2025.

TIFFANY, C.W. & BURCH, R.M. (1989). Bradykinin stimulates tumor necrosis factor and interleukin 1 release from macrophages. *FEBS Lett.*, **247**, 189–192.

TODA, N., BIAN, K., AKIBA, T. & OKAMURA, T. (1987). Heterogeneity in mechanisms of bradykinin action in canine isolated blood vessels. *Eur. J. Pharmacol.*, **135**, 321–329.

WHALLEY, E.T., CLEGG, S., STEWART, J. & VAVREK, R.J. (1987). The effect of kinin agonists and antagonists on the pain response of the human blister base. *Arch. Pharmacol.*, **336**, 652–655.

(Received March 5, 1993
Revised July 23, 1993
Accepted August 20, 1993)

Br. J. Pharmacol. (1993), **110**, 1445–1448 © Macmillan Press Ltd, 1993

Effects of sumatriptan on the cerebral intraparenchymal microcirculation in the cat

[1]Masahiro Kobari, Yasuo Fukuuchi, Minoru Tomita, Norio Tanahashi, Shizuko Konno & Hidetaka Takeda

Department of Neurology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo, Japan

1 Sumatriptan, a 5-hydroxytryptamine $(5\text{-HT})_1$-like receptor agonist, is effective against the headache of migraine. The effects of sumatriptan injected via the carotid artery on the cerebral microcirculation were studied in 10 anaesthetized cats.

2 The local cerebral blood volume (CBV), mean transit time of blood (MTT) and cerebral blood flow (CBF) in the parieto-temporal cortex were measured by a photoelectric method. CBV represents the cumulative dimensions of the cerebral microvessels.

3 Sumatriptan at 5 and 50 $\mu g\,kg^{-1}$ had no significant effects on the CBV, MTT, CBF, and mean arterial blood pressure (MABP); 500 $\mu g\,kg^{-1}$ of sumatriptan reduced the CBV, prolonged the MTT, and decreased the CBF (approximately -20%) without affecting the MABP. Sumatriptan, 5 $mg\,kg^{-1}$, elicited transient reductions in CBV and CBF, which were attributable to the rapid and marked falls of MABP seen with this dose.

4 Thus, while a high dose of sumatriptan (500 $\mu g\,kg^{-1}$) exhibits direct vasoconstrictor actions on the cerebral vessels, low doses of sumatriptan, within the therapeutic range, elicit no vasoconstriction. The data do not support a vasoconstrictor action of sumatriptan playing a primary role in reversing the headache of migraine.

Keywords: Cerebral blood flow; cerebral vessel; 5-hydroxytryptamine receptor; migraine; photoelectric method; sumatriptan

Introduction

Sumatriptan is a novel 5-hydroxytryptamine (5-HT) receptor agonist selective for the 5-HT_1-like receptors (Feniuk *et al.*, 1989; Humphrey *et al.*, 1990; Humphrey & Feniuk, 1991). When given orally (Goadsby *et al.*, 1991) or subcutaneously (Cady *et al.*, 1991; The Subcutaneous Sumatriptan International Study Group, 1991), it aborts the headache of migraine. The mechanisms involved, however, remain a matter of dispute in part because the pathogenesis of migraine itself is not yet fully understood. According to the widely held vascular hypothesis, the head pain of migraine arises from dilatation of previously contracted intra- and extracranial vessels, which are sensitive to pain (Kobari *et al.*, 1990). Sumatriptan may exert its therapeutic action by preventing the dilatation of, or by constricting, these vessels through the vascular 5-HT_1-like receptors (Humphrey *et al.*, 1990; Humphrey & Feniuk, 1991; Caekebeke *et al.*, 1992). *In vitro* studies have consistently demonstrated that sumatriptan constricts basilar arteries of dogs, primates (Connor *et al.*, 1989), and man (Parsons *et al.*, 1989). It also causes contraction of human dural arteries (Humphrey *et al.*, 1990; Jansen *et al.*, 1992), which are more likely to be involved in the generation of headache (Blau, 1978).

An alternative explanation has been put forward by Moskowitz (1992), who proposed that the headache of migraine is caused by activation of the 'trigeminovascular system', consisting of the cranial vessels and their trigeminal innervation. The antidromic transmission of impulses induces the release of vasoactive neuropeptides (Goadsby & Edvinsson, 1993), which may in turn elicit cerebral vasodilatation and plasma extravasation (Buzzi & Moskowitz, 1990), while the orthodromic conduction of potentials conveys the pain sensation to the central nervous system. Sumatriptan appears to suppress these mechanisms through activating neuronal inhibitory 5-HT_1-like receptors (Moskowitz, 1992).

The reported effects of sumatriptan on cerebral perfusion *in vivo* are far from conclusive. Sumatriptan reduced carotid blood flow measured with an electromagnetic flow probe in dogs and pigs (Feniuk *et al.*, 1989; den Boer *et al.*, 1991). However, sumatriptan did not affect the cerebral circulation measured by single photon emission computed tomography (SPECT) in healthy volunteers (Scott *et al.*, 1992) or in patients with migraine during headache (Friberg *et al.*, 1991). Friberg *et al.* (1991), employing transcranial Doppler flowmetry, found that sumatriptan improved the reduced blood flow velocity in the middle cerebral artery on the headache side, however, Diener *et al.* (1991) failed to detect similar changes.

One reason for such discrepancies may be the lack of appropriate methods for continuously monitoring the cerebral perfusion. Carotid blood flow measurements do not represent the cerebral circulation *per se*, and transcranial Doppler flowmetry provides only the blood flow velocity in the major cerebral arteries. SPECT does not permit quantitative determination of the cerebral blood flow (CBF). In addition, there have so far been no investigations examining the effects of sumatriptan on the cerebral intraparenchymal microvessels and circulation. Our photoelectric method (Tomita *et al.*, 1978; 1983) permits continuous, quantitative, and on-line measurements of the local cerebral blood volume (CBV), and intermittent estimations of the mean transit time of blood (MTT) and the CBF. The purpose of the present study was to elucidate the basic actions of sumatriptan injected into the carotid artery on the cerebral microcirculation by use of this new technique.

Methods

Ten adult cats of either sex weighing 2.1–2.7 kg were employed. The animals were anaesthetized by intraperitoneal injection of 50 $mg\,kg^{-1}$ α-chloralose and 500 $mg\,kg^{-1}$ urethane. The body temperature of the animals was maintained

[1] Author for correspondence.

at 37–37.5°C by a heated blanket. Endotracheal intubation was performed, and respiration was kept constant with a volume-controlled respirator for experimental animals (Harvard, model 662). A catheter was inserted into the left lingual artery, and used for injecting saline and drugs into the carotid artery. Another catheter was inserted into the left femoral artery, and used for monitoring the arterial blood pressure (BP). The mean arterial blood pressure (MABP) was calculated from the formula: MABP = (systolic pressure − diastolic pressure)/3 + diastolic pressure.

The head of each animal was fixed in a stereotaxic headholder. The local CBV was measured continuously with a photoelectric apparatus consisting of a micro-lamp, 1 mm in outer diameter (Hamai Electric), and a silicon photodiode (Sharp Electric) covered with a band pass filter (Tomita *et al.*, 1978; 1983). Through a small cranial hole made in the left parieto-temporal region, the micro-lamp was inserted into the brain tissue to approximately 5 mm below the surface. The photodiode was attached to the skull just above the inserted lamp. The photodiode continuously detected the intensity of transmitted light passing through a thin layer of cerebral cortex. Since the intensity of light depends largely on the content of haemoglobin, it can be calibrated to the amount of CBV if the tissue haematocrit is constant (Tomita *et al.*, 1978). To obtain tissue dilution curves from the same region, 0.2 ml of 0.9% saline was injected as a bolus into the left carotid artery via the lingual artery. The tissue dilution curves were drawn on the CBV recording. The MTT of blood through the brain tissue was estimated from the dilution curve employing the area-over-height method (Grodins, 1962; Zierler, 1962). The local CBF was calculated according to the Stewart-Hamilton equation, i.e. CBF = CBV/MTT (Grodins, 1962; Zierler, 1962; Tomita *et al.*, 1978).

In 10 animals each, 5 μg, 50 μg, 500 μg or 5 mg kg^{-1} of sumatriptan (Glaxo, UK) dissolved in 0.5 ml of saline was injected sequentially into the carotid artery via the lingual artery in approximately 10 s. The changes in CBV, MTT, CBF, and SABP were recorded for 15 min following the injection of sumatriptan. There was a 20 min interval between each dose.

The experimental protocols conformed to the guiding principles of the American Physiological Society. The data are presented as the mean ± s.e.mean. Statistical analysis was performed by Student's *t* test and Wilcoxon's signed-rank test.

Results

Figure 1 shows an example of recordings of CBV, tissue dilution curves (present on the CBV recording), and BP before and after intracarotid injection of either 500 μg kg^{-1} or 5 mg kg^{-1} of sumatriptan. After the injection of 500 μg kg^{-1}, both CBV and BP fell slightly. The MTT calculated from the dilution curves was slightly prolonged. Following the 5 mg kg^{-1} injection, the BP initially showed a marked reduction, but subsequently tended to recover. The CBV also exhibited a rapid decline, but then gradually increased to exceed the initial level.

There were no significant differences in the absolute values of MABP, CBV, MTT, and CBF prior to the injection of each dose of sumatriptan. Figure 2a summarizes the time course of MABP following intracarotid injection of 5 μg, 50 μg, 500 μg, or 5 mg kg^{-1} of sumatriptan. Following the administration of either 5 or 50 μg kg^{-1}, there were no significant changes in MABP. A small MABP reduction ($P<0.01$) was observed at 1 min after the injection of 500 μg kg^{-1}. Immediately after the injection of 5 mg kg^{-1}, there was a marked decline in MABP ($P<0.01$), which had returned to the initial level at 15 min.

The effects of sumatriptan on CBV are shown in Figure 2b. There were no significant alterations in CBV following the injection of either 5 or 50 μg kg^{-1}. Following the injection of 500 μg kg^{-1}, the CBV was decreased by approximately 10% ($P<0.05$) during the observation period. After the infusion of 5 mg kg^{-1}, the CBV initially exhibited a 12% reduction ($P<0.05$), but then showed an increase exceeding the initial level.

Figure 2c summarizes the effects of sumatriptan on MTT. There were no significant changes in MTT after the injection of either 5 or 50 μg kg^{-1}. Following the injection of 500 μg kg^{-1}, MTT was significantly prolonged ($P<0.05$) at 1 min. Immediately after the injection of 5 mg kg^{-1}, MTT was markedly prolonged ($P<0.05$), but returned to the initial level thereafter.

The time course of CBF following the intracarotid injection of sumatriptan is shown in Figure 2d. There were no significant changes in CBF after the injection of either 5 or 50 μg kg^{-1}. Following the injection of 500 μg kg^{-1}, the CBF was significantly decreased by approximately 20% ($P<0.05$). Immediately after the injection of 5 mg kg^{-1}, the CBF was markedly reduced ($P<0.05$), but then returned to the control level.

The Pa_{O_2}, Pa_{CO_2}, and pH values of the animals in the steady-state before the administration of the first dose of sumatriptan were 96.4 ± 4.9 mmHg, 29.6 ± 2.2 mmHg, and 7.336 ± 0.027, respectively. There were no statistically significant changes in Pa_{O_2}, Pa_{CO_2}, or pH after the injection of sumatriptan in any of the groups.

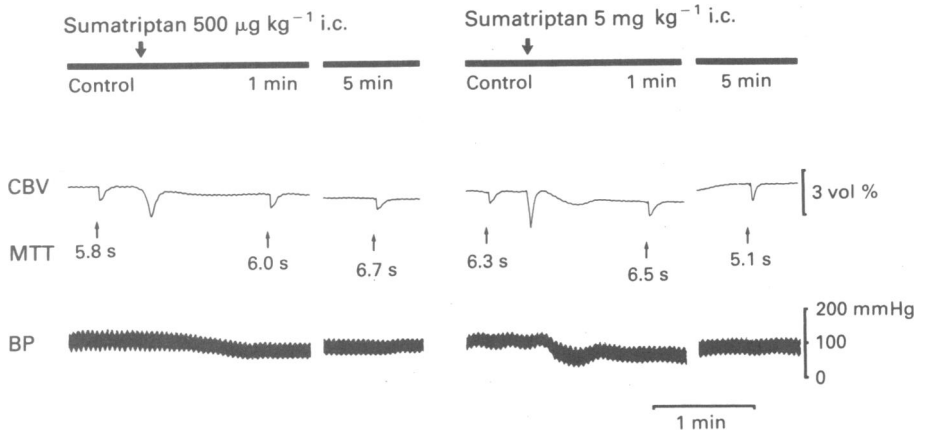


Figure 1 An example of recordings of cerebral blood volume (CBV) and blood pressure (BP) before and after intracarotid (i.c.) injection of sumatriptan. The small upward arrows, which indicate injections of 0.2 ml of saline, are followed by tissue dilution curves. The mean transit time of blood (MTT) estimated from the individual dilution curves is shown under the arrows.

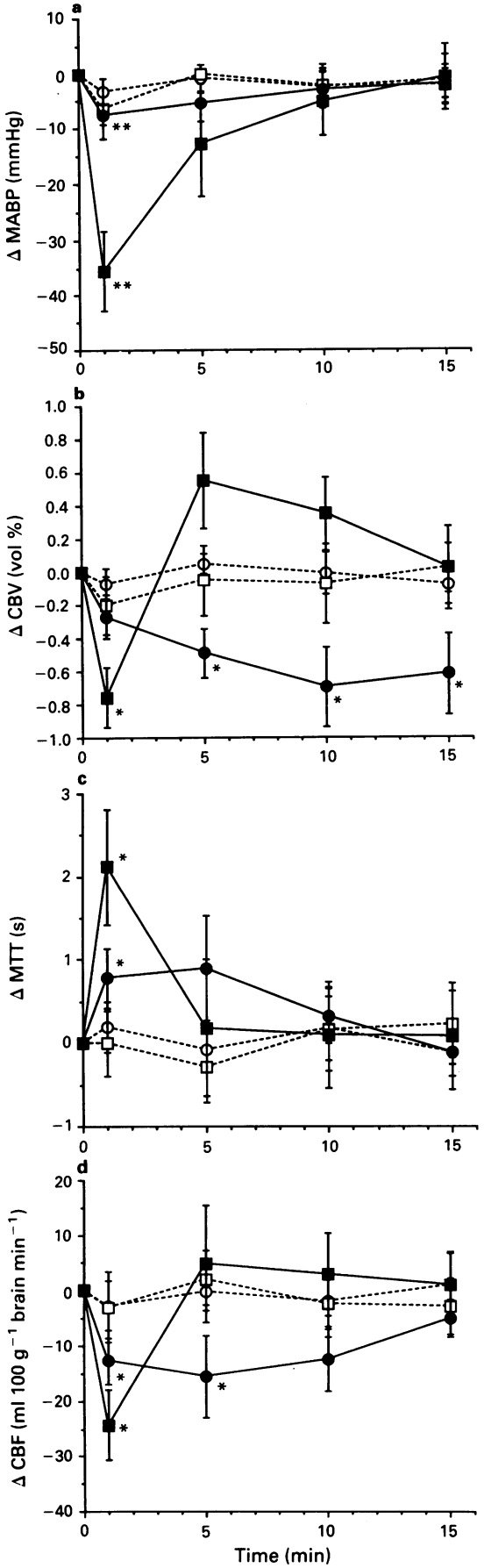


Figure 2 The changes in (a) mean arterial blood pressure (MABP), (b) cerebral blood volume (CBV), (c) mean transit time of blood (MTT) and (d) cerebral blood flow (CBF) following intracarotid injection of 5 μg (○), 50 μg (□), 500 μg (●), or 5 mg (■) kg^{-1} of sumatriptan. Values are mean ± s.e.mean ($n = 10$). $^{*}P<0.05$ and $^{**}P<0.01$ compared to the values at 0 min.

Discussion

Full details of the photoelectric method have been described elsewhere (Tomita *et al.*, 1978; 1983). CBV reflects the cumulative dimensions of the cortical microvessels. Insertion of the present micro-lamp into the brain tissue does not affect the cerebrovascular responses, such as the autoregulation or CO_2 reactivity of the CBF (Tomita *et al.*, 1978). The CBF values measured by the photoelectric and the hydrogen gas clearance methods were found to correlate well (Tomita *et al.*, 1988).

In the present study, low doses of sumatriptan (5 and 50 μg kg^{-1}) exerted no significant effects on the intraparenchymal circulation of the cortex, as evaluated from the CBV, MTT, and CBF. These results are consistent with those of Connor *et al.* (1992) who observed no significant changes in the diameter of feline pial arteries following intravenous infusion of 64 μg kg^{-1} of sumatriptan. The results are also in line with those of Scott *et al.* (1992) and Friberg *et al.* (1991) who found no changes in the regional CBF following intravenous injection of 2 mg (or approximately 30 μg kg^{-1} for an average person) of sumatriptan.

Sumatriptan, 500 μg kg^{-1}, was found to reduce the CBV, to prolong the MTT, and to decrease the CBF (by approximately 20%) without an associated change in MABP. A high dose of sumatriptan thus exerts a vasoconstrictor action on the intraparenchymal microvessels. Sumatriptan has been considered unlikely to cross the blood-brain barrier, since it induces contraction of the pial arteries when applied from the abluminal side, but not when applied from the luminal side (Connor *et al.*, 1992). However, our present results suggest that intravascular sumatriptan, albeit at a high concentration, may reach the smooth muscles of the microvessels, or that receptors are located in the intima. In either event, the 5-HT_1-like receptors appear to mediate the contractile responses to sumatriptan (Parsons, 1991; Humphrey & Feniuk, 1991).

A very high dose of sumatriptan (5 mg kg^{-1}) elicited transient reductions in CBV and CBF in the present study, which may be attributable to rapid and marked declines of MABP (Barzó *et al.*, 1993). It is possible that the marked falls in MABP may be due to cardiac depression elicited by a toxic dose of sumatriptan.

According to a dose-finding study on migraine patients (Perrin *et al.*, 1989), intravenous infusion of 64 μg kg^{-1} of sumatriptan was sufficient to relieve the headache in over 90% of the patients. In the present study, a vasoconstrictor action of sumatriptan was observed only after the administration of 500 μg kg^{-1}, which amounted to approximately eight times the above-cited therapeutic dosage. In contrast, a clinically effective dose of sumatriptan (50 μg kg^{-1}) did not influence the intraparenchymal circulation of the brain despite being given by the carotid route.

Our data suggest that vasoconstriction by sumatriptan may not be involved in aborting the pain of migraine. There is a possibility that sumatriptan may constrict the large intra- or extracranial vessels without affecting the intraparenchymal small vessels. However, from our experience, it seems very unlikely that the downstream intraparenchymal circulation is totally unaffected by the constriction of the large cerebral vessels. Further, the distribution, density, and subtypes of the 5-HT receptors in the cerebral vasculature differ among different species (Parsons, 1991). A problem thus remains as to whether or not the cerebrovascular responses observed in cats can be extrapolated to man. The present result does not exclude a possibility that sumatriptan may constrict the pathologically dilated vessels of migraine at doses lower than 500 μg kg^{-1}.

In conclusion, a high dose of sumatriptan given into the carotid artery decreased the local CBV and CBF through constriction of the intraparenchymal vessels. However, lower doses of sumatriptan, equivalent to those used in clinical practice, did not affect the cerebral vessels or perfusion. The

data do not support the vasoconstrictor action of sumatriptan playing a primary role in ameliorating the headache of migraine.

The authors are grateful to Glaxo Pharmaceuticals Inc., Tokyo, Japan, for providing sumatriptan.

References

BARZÓ, P., BARI, F., DÓCZI, T., JANCSÓ, G. & BODOSI, M. (1993). Significance of the rate of systemic changes in blood pressure on the short-term autoregulatory response in normotensive and spontaneously hypertensive rats. *Neurosurgery*, **32**, 611–618.

BLAU, J.N. (1978). Migraine: a vasomotor instability of the meningeal circulation. *Lancet*, **ii**, 1136–1139.

BUZZI, M.G. & MOSKOWITZ, M.A. (1990). The antimigraine drug, sumatriptan (GR43175), selectively blocks neurogenic plasma extravasation from blood vessels in dura mater. *Br. J. Pharmacol.*, **99**, 202–206.

CADY, R.K., WENDT, J.K., KIRCHNER, J.R., SARGENT, J.D., ROTHROCK, J.F. & SKAGGS, H. Jr. (1991). Treatment of acute migraine with subcutaneous sumatriptan. *J. Am. Med. Ass.*, **265**, 2831–2835.

CAEKEBEKE, J.F.V., FERRARI, M.D., ZWETSLOOT, C.P., JANSEN, J. & SAXENA, P.R. (1992). Antimigraine drug sumatriptan increases blood flow velocity in large cerebral arteries during migraine attacks. *Neurology*, **42**, 1522–1526.

CONNOR, H.E., FENIUK, W. & HUMPHREY, P.P.A. (1989). Characterization of 5-HT receptors mediating contraction of canine and primate basilar artery by use of GR43175, a selective 5-HT_1-like receptor agonist. *Br. J. Pharmacol.*, **96**, 379–387.

CONNOR, H.E., STUBBS, C.M., FENIUK, W. & HUMPHREY, P.P.A. (1992). Effect of sumatriptan, a selective 5-HT_1-like receptor agonist, on pial vessel diameter in anaesthetised cats. *J. Cereb. Blood Flow Metab.*, **12**, 514–519.

DEN BOER, M.O., VILLALÓN, C.M., HEILIGERS, J.P.C., HUMPHREY, P.P.A. & SAXENA, P.R. (1991). Role of 5-HT_1-like receptors in the reduction of porcine cranial arteriovenous anastomotic shunting by sumatriptan. *Br. J. Pharmacol.*, **102**, 323–330.

DIENER, H.-C., PETERS, C., RUDZIO, M., NOE, A., DICHGANS, J., HAUX, R., EHRMANN, R. & TFELT-HANSEN, P. (1991). Ergotamine, flunarizine and sumatriptan do not change cerebral blood flow velocity in normal subjects and migraineurs. *J. Neurol.*, **238**, 245–250.

FENIUK, W., HUMPHREY, P.P.A. & PERREN, M.J. (1989). The selective carotid arterial vasoconstrictor action of GR43175 in anaesthetized dogs. *Br. J. Pharmacol.*, **96**, 83–90.

FRIBERG, L., OLESEN, J., IVERSEN, H.K. & SPERLING, B. (1991). Migraine pain associated with middle cerebral artery dilatation: reversal by sumatriptan. *Lancet*, **338**, 13–17.

GOADSBY, P.J. & EDVINSSON, L. (1993). The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. *Ann. Neurol.*, **33**, 48–56.

GOADSBY, P.J., ZAGAMI, A.S., DONNAN, G.A., SYMINGTON, G., ANTHONY, M., BLADIN, P.F. & LANCE, J.W. (1991). Oral sumatriptan in acute migraine. *Lancet*, **338**, 782–783.

GRODINS, F.S. (1962). Basic concepts in the determination of vascular volumes by indicator-dilution methods. *Circ. Res.*, **10**, 429–446.

HUMPHREY, P.P.A. & FENIUK, W. (1991). Mode of action of the anti-migraine drug sumatriptan. *Trends Pharmacol. Sci.*, **12**, 444–446.

HUMPHREY, P.P.A., FENIUK, W., PERREN, M.J., BERESFORD, I.J.M., SKINGLE, M. & WHALLEY, E.T. (1990). Serotonin and migraine. *Ann. N.Y. Acad. Sci.*, **600**, 587–598.

JANSEN, I., EDVINSSON, L., MORTENSEN, A. & OLESEN, J. (1992). Sumatriptan is a potent vasoconstrictor of human dural arteries via a 5-HT_1-like receptor. *Cephalalgia*, **12**, 202–205.

KOBARI, M., MEYER, J.S. & ICHIJO, M. (1990). Cerebral hemodynamic changes during migraine and cluster headaches: pharmacological implications. *Headache Q. Curr. Treat. Res.*, **1**, 23–37.

MOSKOWITZ, M.A. (1992). Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. *Trends Pharmacol. Sci.*, **13**, 307–311.

PARSONS, A.A. (1991). 5-HT receptors in human and animal cerebrovasculature. *Trends Pharmacol. Sci.*, **12**, 310–315.

PARSONS, A.A., WHALLEY, E.T., FENIUK, W., CONNOR, H.E. & HUMPHREY, P.P.A. (1989). 5-HT_1-like receptors mediate 5-hydroxytryptamine-induced contraction of human isolated basilar artery. *Br. J. Pharmacol.*, **96**, 434–440.

PERRIN, V.L., FÄRKKILÄ, M., GOASGUEN, J., DOENICKE, A., BRAND, J. & TFELT-HANSEN, P. (1989). Overview of initial clinical studies with intravenous and oral GR43175 in acute migraine. *Cephalalgia*, **9**(Suppl 9), 63–72.

SCOTT, A.K., GRIMES, S., NG, K., CRITCHLEY, M., BRECKENRIDGE, A.M., THOMSON, C. & PILGRIM, A.J. (1992). Sumatriptan and cerebral perfusion in healthy volunteers. *Br. J. Clin. Pharmacol.*, **33**, 401–404.

THE SUBCUTANEOUS SUMATRIPTAN INTERNATIONAL STUDY GROUP (1991). Treatment of migraine attacks with sumatriptan. *N. Engl. J. Med.*, **325**, 316–321.

TOMITA, M., GOTOH, F., AMANO, T., TANAHASHI, N., KOBARI, M., SHINOHARA, T. & MIHARA, B. (1983). Transfer function through regional cerebral cortex evaluated by a photoelectric method. *Am. J. Physiol.*, **245**, H385–H398.

TOMITA, M., GOTOH, F., SATO, T., AMANO, T., TANAHASHI, N., TANAKA, K. & YAMAMOTO, M. (1978). Photoelectric method for estimating hemodynamic changes in regional cerebral tissue. *Am. J. Physiol.*, **235**, H56–H63.

TOMITA, M., GOTOH, F., TANAHASHI, N., KOBARI, M., TERAYAMA, Y., MIHARA, B., OHTA, K. & GERDSEN, I. (1988). Comparison between the photoelectric method and H_2 clearance method for measuring cerebrocortical blood flow in cats. *J. Cereb. Blood Flow Metab.*, **8**, 727–732.

ZIERLER, K.L. (1962). Circulation times and the theory of indicator-dilution methods for determining blood flow and volume. In *Handbook of Physiology*, Section 2: Circulation, Volume 1, pp. 585–615, Washington DC: American Physiology Society.

(Received June 14, 1993
Revised August 17, 1993
Accepted August 23, 1993)

Br. J. Pharmacol. (1993), **110**, 1449–1456 © Macmillan Press Ltd, 1993

Homologous and heterologous desensitization of histamine H_1- and ATP-receptors in the smooth muscle cell line, DDT_1MF-2: the role of protein kinase C

[1]John M. Dickenson & Stephen J. Hill

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 The possible role of protein kinase C (PKC) in homologous and heterologous desensitization of histamine H_1- and ATP-receptors has been studied in monolayers of cultured vas deferens smooth muscle cells (DDT_1MF-2). Cells were loaded with the calcium-sensitive fluorescent dye fura-2 and increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) monitored in response to histamine H_1- or ATP-receptor activation.

2 Histamine and ATP stimulated the release of Ca^{2+} from intracellular Ca^{2+} stores and Ca^{2+} influx across the plasma membrane. Activation of PKC with the phorbol ester β-phorbol-12,13 dibutyrate (PDBu; 1 μM) attenuated histamine (100 μM) and ATP (10 μM)-induced release of intracellular Ca^{2+} and Ca^{2+} influx.

3 The selective PKC inhibitor, Ro 31-8220 (10 μM), reversed the PDBu-induced attenuation of histamine (100 μM)-stimulated Ca^{2+} responses.

4 Histamine H_1- and ATP-receptors are readily susceptible to homologous desensitization since short-term exposure to histamine or ATP (450 s) attenuated the Ca^{2+} responses elicited by a second application of the same agonist. Furthermore, H_1-receptor activation-induced heterologous desensitization of ATP stimulated Ca^{2+} responses and *vice versa*.

5 Homologous and heterologous desensitization of histamine and ATP Ca^{2+} responses still occurred in the presence of the PKC inhibitor, Ro 31-8220 (10 μM).

6 These data suggest that PKC activation can attenuate histamine H_1- and ATP-receptor mediated Ca^{2+} responses. However, based on our experimental data, PKC-independent mechanisms appear to be involved in the homologous and heterologous desensitization of histamine H_1- and ATP-receptor mediated Ca^{2+} responses in DDT_1MF-2 cells.

Keywords: Histamine H_1-receptors; intracellular calcium; ATP; nucleotide receptors; protein kinase C; phorbol esters; Ro 31-8220; desensitization

Introduction

Many cells express receptors that are coupled to phospholipase C, via a regulatory G-protein, which upon activation hydrolyses the plasma-membrane phospholipid, phosphatidylinositol 4,5-biphosphate generating the two second messengers D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (Berridge, 1993). $InsP_3$ triggers the release of Ca^{2+} from intracellular stores producing a rise in $[Ca^{2+}]_i$, whereas 1,2,-diacylglycerol activates cytosolic protein kinase C (PKC). Tumour-promoting phorbol esters also activate PKC and numerous studies have used phorbol esters to investigate the role of PKC in the regulation of agonist-stimulated inositol phospholipid hydrolysis. For example, PKC activation (using phorbol esters) has been shown to attenuate bombesin (Brown *et al.*, 1987), α_1-adrenoceptor (Woods *et al.*, 1987) and histamine H_1-receptor (Jones *et al.*, 1990) stimulated inositol phospholipid hydrolysis. Based on these findings it has been proposed that agonist-stimulated phospholipase C activity may be desensitized through a negative feedback loop involving the activation of PKC by diacylglycerol. Interestingly, in bovine adrenal chromaffin cells, the selective PKC inhibitor, Ro 31-8220 (Davis *et al.*, 1989) has been shown to enhance the accumulation of $InsP_3$ elicited by histamine and bradykinin (Boarder & Challis, 1992).

The phenomenon of desensitization is characterized by the loss of an agonist-induced response despite the continued presence of the agonist. The mechanism(s) underlying the desensitization of the β-adrenoceptor have been extensively studied (Hausdorff *et al.*, 1990; Palczewski & Benovic, 1991). The β-adrenoceptor is rapidly desensitized by the concerted action of adenosine 3':5'-cyclic monophosphate (cyclic AMP) -dependent protein kinase A (PKA) and cyclic AMP-independent β-adrenoceptors kinase (βARK). Phosphorylation of the β-adrenoceptor causes the uncoupling of the G-protein-receptor complex. In contrast, the mechanism(s) involved in the desensitization of the histamine H_1-receptor are poorly understood. Data obtained from studies monitoring histamine-induced contractions of guinea-pig intestinal smooth muscle suggest that PKC is not involved in homologous desensitization of the histamine H_1-receptor (Leurs *et al.*, 1991). Similarly in HeLa cells, Smit *et al.* (1992) proposed that homologous desensitization of histamine H_1-receptor-mediated intracellular Ca^{2+} release can occur independently of PKC activation and may involve a specific H_1-receptor kinase, whereas heterologous desensitization (studied using ATP) of the H_1-receptor appeared to involve PKC activation.

Homologous desensitization of histamine H_1- and α_1-adrenoceptor-stimulated inositol phospholipid metabolism has been reported in DDT_1MF-2 cells (Cowlen & Toews, 1988; Mitsuhashi & Payan, 1988; Cowlen *et al.*, 1990). Indeed, Leeb-Lundberg *et al.* (1985; 1987) showed that phorbol esters and continued receptor-occupancy promote α_1-adrenoceptor phosphorylation. Our previous studies have shown that in DDT_1MF-2 cells, histamine H_1- and ATP-receptor activation causes the release of Ca^{2+} from intracellular stores ($InsP_3$-induced) and Ca^{2+} entry (influx) across the plasma membrane (Dickenson & Hill, 1991; 1992; Dickenson *et al.*, 1993). Recently, Felder *et al.* (1992) reported that muscarinic M_3-receptor-operated Ca^{2+} influx in transfected A9 fibroblasts is insensitive to phorbol ester pretreatment, whereas Ca^{2+} re-

[1] Author for correspondence.

lease was inhibited by PKC activation. Similarly, in PC12-64 cells, PKC activation affected carbachol-stimulated intracellular Ca^{2+} release more than Ca^{2+} influx (Clementi *et al.*, 1992).

Therefore, the aims of the present study were: (i) to determine if phorbol esters could selectively inhibit histamine H_1- or ATP-receptor stimulated intracellular Ca^{2+} release or Ca^{2+} influx and (ii) to investigate the role of PKC, using the selective PKC inhibitor, Ro 31-8220 (Davis *et al.*, 1989), in homologous and heterologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses in the smooth muscle cell line, DDT_1MF-2.

Methods

Cell culture

The hamster vas deferens smooth muscle cell line (DDT_1MF-2) was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wiltshire). DDT_1 MF-2 cells were cultured at 37°C in a humidified air/CO_2 (90:10) atmosphere in 75 cm^2/flasks (Costar). The growth medium was Dulbecco's modified Eagle's medium supplemented with 2 mM-L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were passaged twice a week (1/5 split ratio) by vigorous shaking of the flask and placed into 75 cm^2 flasks and fed with fresh growth medium every 48 h. Cells for $[Ca^{2+}]_i$ determinations were grown on 24 mm × 10 mm glass coverslips in 90 mm petri dishes. All experiments were performed on confluent monolayers (passages 4–21, numbers assigned after receiving the cell line).

Measurement of intracellular free calcium

Intracellular free calcium was measured by loading confluent cell monolayers with the calcium-sensitive fluorescent dye fura-2. Individual coverslips were placed in 35 mm petri dishes with 1 ml of physiological buffer (composition, mM: NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1, HEPES 10, $CaCl_2$ 2, pH 7.45) containing 10% FCS (v/v), 3 μM fura-2/AM and incubated for 30 min at 37°C. After this 'loading' period, the fura-2 containing buffer was replaced with fresh buffer that was free of fura-2 and FCS but contained 0.1% bovine serum albumin, and left at 37°C for a further 15 min. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of a polymethacrylate cuvette. Each cuvette contained 2.9 ml of physiological buffer (drugs were added to the cuvettes in 100 μl aliquots) and fluorescent measurements were made at 37°C in a Perkin Elmer LS 50 spectrometer. The excitation wavelengths were 340 and 380 nm, with emission at 500 nm. The slit-widths were set at 10 nm for both the excitation and emission wavelengths and the time taken to switch between 340 and 380 nm was 0.8 s. Intracellular Ca^{2+} was calculated every 1.9 s from the ratio (R) of 340 nm/380 nm fluorescent values using the equation of Grynkiewicz *et al.* (1985).

$$[Ca^{2+}]_i = \frac{(R-R_{min})}{(R_{max.}-R)} \times (S_{380,min.}/S_{380,max.}) \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (224 nM at 37°C) and $S_{380,min.}/S_{380,max.}$ is the ratio (β value) of the fluorescent values obtained at 380 nm in the absence and presence of saturating $[Ca^{2+}]_i$. The maximum and minimum R values ($R_{max.}$ and $R_{min.}$) were determined on separate coverslips under saturating $[Ca^{2+}]_i$ (achieved by increasing the extracellular $[Ca^{2+}]$ to 20 mM followed by 10 μM ionomycin, pH 7.4) and calcium-free (achieved using 8.3 mM EGTA immediately followed by 25 μl of 1.0 M NaOH to compensate for the decrease in pH, in the presence of 10 μM ionomycin) conditions respectively. Corrections for autofluorescence were made by measuring the fluorescence produced by coverslips

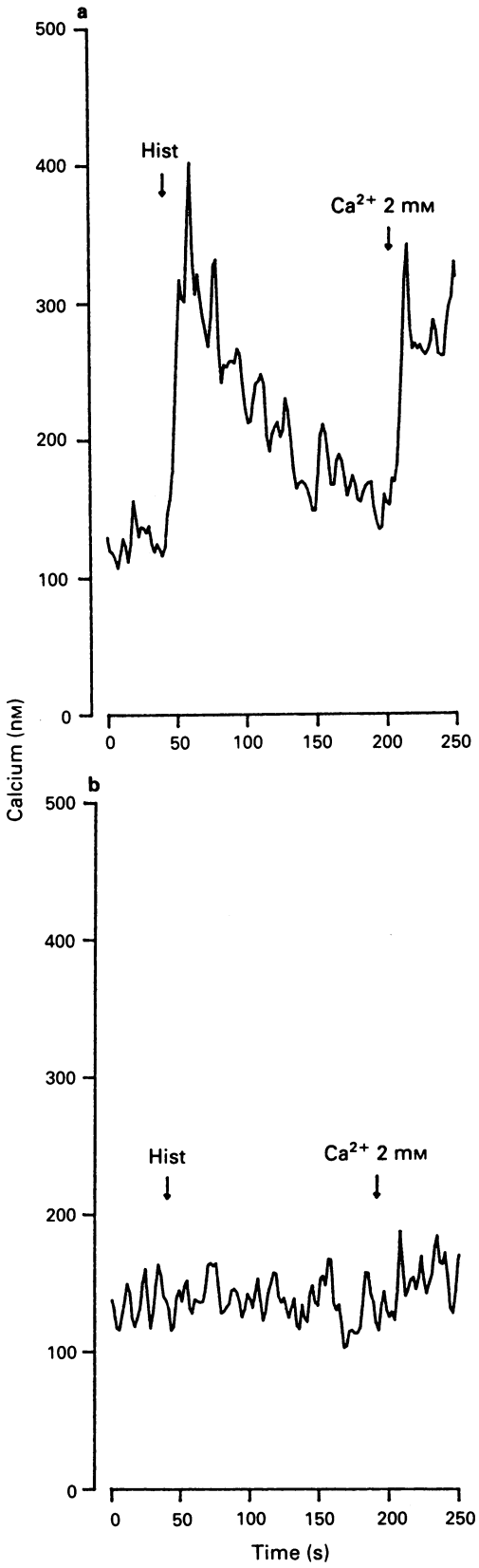


Figure 1 Effect of β-phorbol-12,13 dibutyrate on histamine H_1-receptor mediated increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. (a) Cells were stimulated with histamine (Hist) in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but cells were preincubated for 15 min with 1 μM β-phorbol-12,13-dibutyrate (PDBu). Experiment (a) contained 0.01% dimethyl sulphoxide to control for PDBu vehicle effects. Histamine (100 μM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in eight other experiments.

that had not been loaded with fura-2. Where Ca^{2+}-free conditions were required experiments were performed in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA.

Data analysis

Rises in intracellular free $[Ca^{2+}]$ were evaluated by importing the fluorescence data into the spreadsheet AsEasyAs (TRIUS Inc). Basal $[Ca^{2+}]_i$ levels were determined by calculating the mean of the ten data points (measured every 1.9 s) prior to drug addition, whereas the maximum Ca^{2+} signal was deemed to be the largest Ca^{2+} response obtained immediately after drug addition. Due to the fluctuations in basal $[Ca^{2+}]_i$, the mean of the ten data points measured immediately after drug addition were also calculated to determine whether the measured response was significantly different from basal levels (Mann Whitney U test). Data are shown as means ± s.e.mean. Statistical analysis was performed with Student's unpaired *t* test. A P value <0.05 was considered as statistically significant; n in the text refers to the number of separate experiments.

Chemicals

Fura-2/AM and ionomycin were from Calbiochem. Histamine, bradykinin, adenosine-5'-triphosphate, β-phorbol-12, 13 dibutyrate, and 4α-phorbol were obtained from Sigma Chemical Company Ltd (U.K.). Ro 31-8220 (3-{1-[3-(2-isothioureido)propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,-5-dione) was a kind gift from Dr T. Hallam, Roche Products Ltd. (Welwyn Garden City, Herts). Dulbecco's modified Eagle's medium and foetal calf serum (FCS) were from Northumbria Biologicals (U.K.). All other chemicals were of analytical grade.

Results

Effect of β-phorbol-12,13 dibutyrate on histamine H_1- and ATP-receptor stimulated increases in $[Ca^{2+}]_i$

Experiments monitoring agonist-stimulated increases in $[Ca^{2+}]_i$ were carried out using the Ca^{2+}-free/Ca^{2+}-reintroduction protocol. This involved initially stimulating the cells in Ca^{2+}-free buffer containing 0.1 mM EGTA (to measure intracellular Ca^{2+} release) after which extracellular Ca^{2+} (2 mM) was re-applied (to measure Ca^{2+} influx). Figure 1a shows histamine (100 μM)-stimulated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. Ca^{2+} release increased $[Ca^{2+}]_i$ from 111 ± 6 nM to 408 ± 15 nM ($n = 8$), whereas Ca^{2+} influx increased $[Ca^{2+}]_i$ from 139 ± 9 nM to 390 ± 18 nM ($n = 8$). PDBu pretreatment (1 μM; 15 min) completely abolished H_1-receptor stimulated intracellular Ca^{2+} release and influx ($n = 8$; see Figure 1b). In contrast, the Ca^{2+} responses elicited by maximal concentrations of ATP (100 μM) were insensitive to PDBu pretreatment. The maximum peak Ca^{2+} responses to ATP (100 μM) obtained in Ca^{2+}-free buffer and upon the addition of extracellular Ca^{2+} (2 mM) in PDBu-treated cells were 98 ± 4% ($n = 11$) and 102 ± 6% ($n = 11$) respectively, of those obtained in control experiments (see Table 1). However, if the concentration of ATP was reduced to 10 μM, PDBu pretreatment significantly reduced ATP-induced Ca^{2+} responses (Figure 2). In these experiments the maximum peak Ca^{2+} responses to ATP (10 μM) obtained in Ca^{2+}-free buffer and upon the addition of exogenous Ca^{2+} in PDBu-treated cells were 45 ± 8% ($n = 11$) and 35 ± 5% ($n = 11$) respectively, of those obtained in control experiments (see Table 1). Pretreatment with inactive phorbol 4α-phorbol (1 μM, 15 min) did not inhibit histamine (100 μM)-stimulated intracellular Ca^{2+} release ($[Ca^{2+}]_i$ increased from 108 ± 7 nM to 420 ± 19 nM in control cells and from 103 ± 9 nM to 408 ± 12 nM in 4α-phorbol treated cells; $n = 5$) or Ca^{2+} influx ($[Ca^{2+}]_i$ increased from 125 ± 12 nM to 320 ± 11 nM in control cells and from 118 ± 6 nM to 340 ± 17 nM in 4α-phorbol treated cells; $n = 5$).

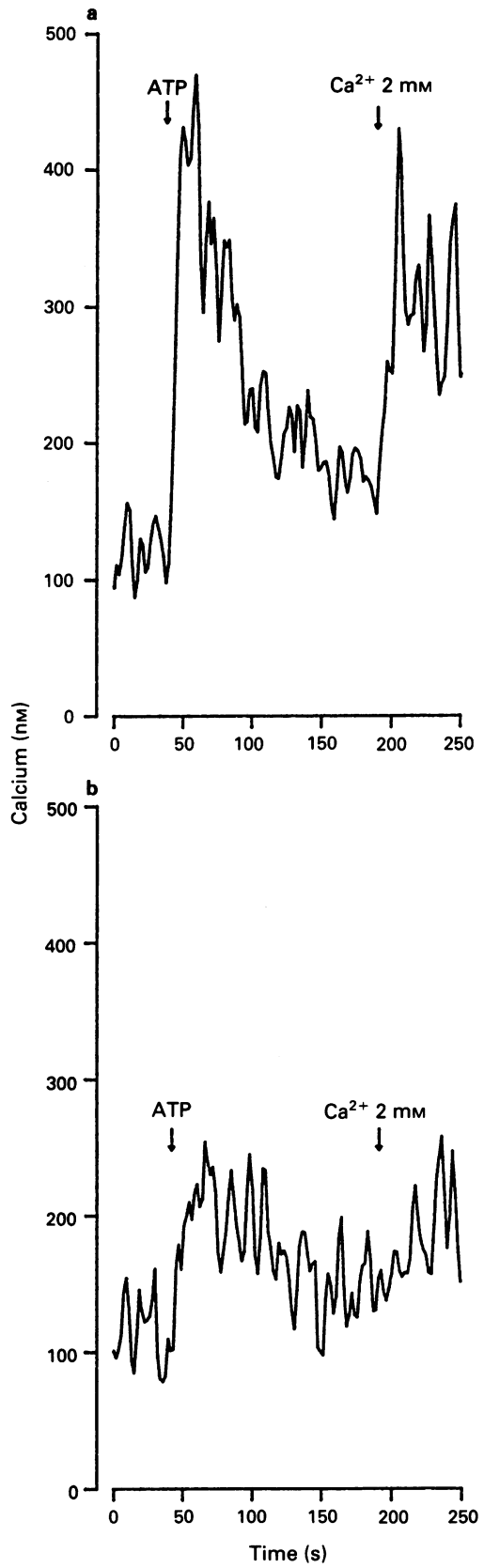


Figure 2 Effect of β-phorbol-12,13 dibutyrate on ATP-receptor mediated increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. (a) Cells were stimulated with ATP in nominally Ca^{2+}-free buffer containing 0.1 mM EGTA after which exogenous Ca^{2+} (2 mM) was re-applied. (b) The same protocol as in (a), but cells were preincubated for 15 min with 1 μM β-phorbol-12,13-dibutyrate (PDBu). Experiment (a) contained 0.01% dimethyl sulphoxide to control for PDBu vehicle effects. ATP (10 μM) and $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in ten other experiments.

Table 1 The effect of β-phorbol-12,13-dibutyrate on ATP-receptor stimulated increases in intracellular calcium

	100 μM ATP			*10 μM ATP*		
	Basal $[Ca^{2+}]_i$ (nM)	*Stimulated*	(*n*)	*Basal* $[Ca^{2+}]_i$ (nM)	*Stimulated*	(*n*)
Ca^{2+} release						
Control	115 ± 6	596 ± 20	11	123 ± 8	450 ± 18	11
PDBu 1 μM	108 ± 9	570 ± 15	11	116 ± 7	263 ± 13*	11
Ca^{2+} influx						
Control	121 ± 8	409 ± 12	11	150 ± 12	435 ± 20	11
PDBu 1 μM	118 ± 6	421 ± 9	11	161 ± 9	270 ± 13*	11

Values represent the mean ± s.e.mean of (*n*) experiments. Statistical analysis was performed (Student's unpaired *t* test) comparing the stimulated Ca^{2+} responses (after subtracting basal $[Ca^{2+}]_i$) obtained in control and treated cells. *Statistically significant, $P<0.05$, from control responses.

Effect of protein kinase C inhibitor Ro 31-8220 on histamine H_1-receptor mediated increases in $[Ca^{2+}]_i$

Preincubation with 500 nM PDBu abolished the histamine (100 μM)-stimulated Ca^{2+} response measured in the presence of extracellular Ca^{2+} (compare Figure 3a with Figure 3b). Pretreatment with Ro 31-8220 (10 μM; 45 min) had no effect on the histamine response (95 ± 7% of control; $n = 6$; Figure 3c). Finally, Ro 31-8220 completely reversed the PDBu-induced attenuation of histamine (100 μM)-stimulated increases in $[Ca^{2+}]_i$ (96 ± 9% of controls; $n = 6$; Figure 3d).

Homologous desensitization of histamine H_1- and ATP-receptor stimulated increases in $[Ca^{2+}]_i$

We performed a series of experiments to study the homologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses in DDT_1MF-2 cells. The experimental protocol involved initially stimulating the cells with maximally-effective concentrations of either agonist in nominally Ca^{2+}-free buffer (for approximately 150 s) after which extracellular Ca^{2+} (2 mM) was added for 300 s in the continued presence of agonist (to allow refilling of the intracellular Ca^{2+} stores). After 500 s the glass coverslip was transferred to a fresh cuvette that contained Ca^{2+}-free buffer (plus 0.1 mM EGTA) before monitoring the Ca^{2+} responses elicited by a second application of the appropriate agonist. Figure 4a shows that short term exposure (450 s) to histamine (100 μM) induces homologous desensitization of histamine H_1-receptor-mediated intracellular Ca^{2+} release and Ca^{2+} influx. An appropriate control for Figure 4a is shown in Figure 4b. In these experiments the cells were briefly exposed to histamine in Ca^{2+}-free buffer (for approximately 150 s) after which the glass coverslip was transferred to a fresh cuvette that contained Ca^{2+}-containing buffer (in the absence of agonist). As we have reported previously in these cells (Dickenson & Hill, 1992) the removal of histamine from its receptor (with mepyramine) prevents Ca^{2+} influx into the cytoplasm which is dependent upon H_1-receptor occupancy (receptor-mediated Ca^{2+} influx) but allows the refilling of the intracellular Ca^{2+} stores (independent of receptor occupancy). In these experiments the second addition of histamine resulted in intracellular Ca^{2+} release (86 ± 6% of the first application; $n = 8$) and Ca^{2+} influx (114 ± 4% of controls; $n = 8$). These data

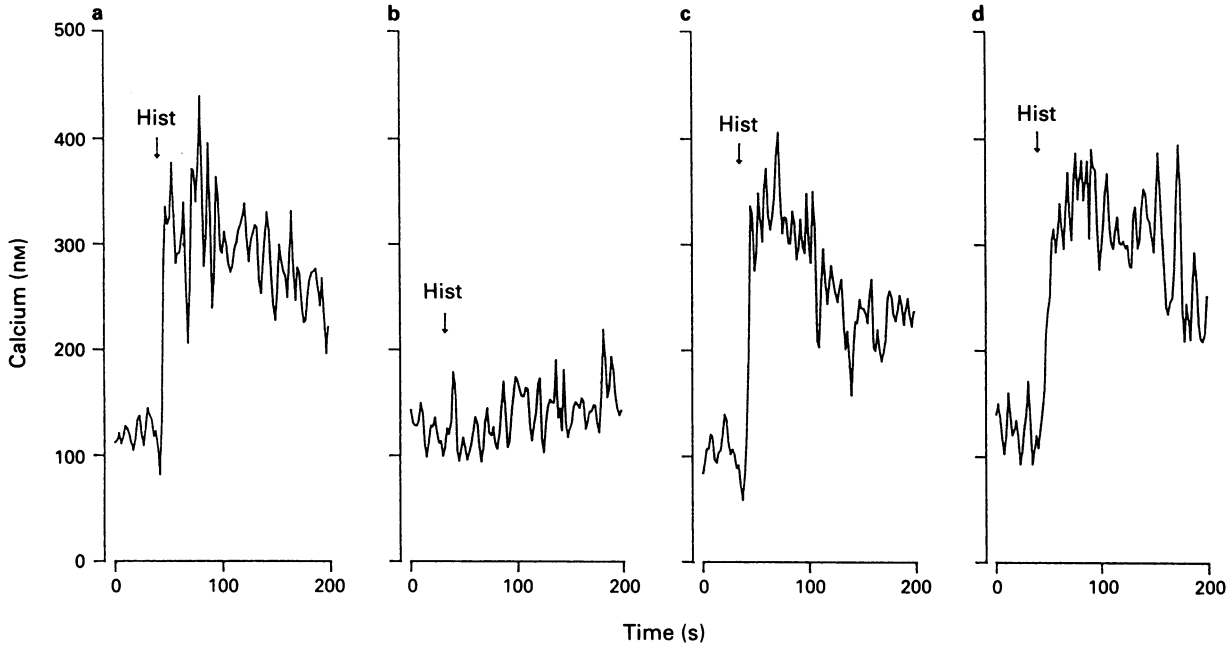


Figure 3 Effect of the selective protein kinase C inhibitor, Ro 31-8220, on β-phorbol-12,13-dibutyrate-induced attenuation of histamine H_1-receptor mediated Ca^{2+} responses. (a) Histamine stimulated increases in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (2 mM). (b) As in (a) but cells were preincubated for 15 min with 500 nM β-phorbol-12,13-dibutyrate (PDBu). (c) Histamine stimulated Ca^{2+} response in the presence of 10 μM Ro 31-8220 (45 min preincubation). (d) Cells incubated for 30 min with 10 μM Ro 31-8220 followed by 15 min with 10 μM Ro 31-8220 in the presence of 500 nM PDBu. Experiments (a) to (d) contained 0.01% dimethyl sulphoxide to control for PDBu and Ro 31-8220 vehicle effects. Histamine (Hist, 100 μM) was added where indicated. Similar results were obtained in five other experiments.

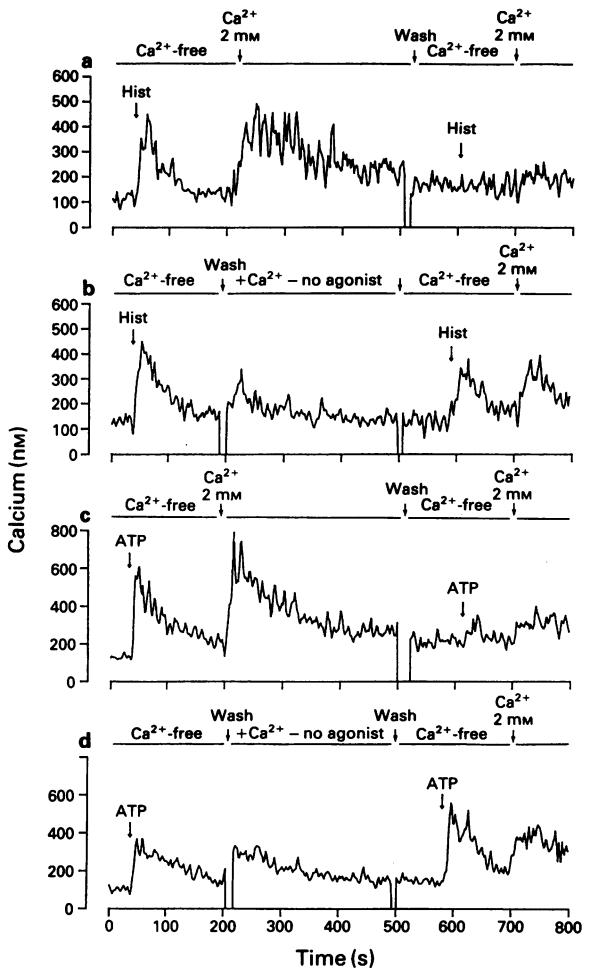


Figure 4 Homologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. (a) Homologous desensitization of histamine H_1-receptor mediated Ca^{2+} responses. (b) Control experiment for (a) to eliminate the possibility that the absence of a second histamine response is not simply a consequence of the intracellular Ca^{2+} stores being empty. (c) Homologous desensitization of ATP-receptor-mediated Ca^{2+} responses. (d) Control experiment for (c) using the same experimental design as in (b). Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. During Ca^{2+}-free periods buffer also contained 0.1 mM EGTA. Similar results were obtained in five other experiments.

suggest that the absence of a second histamine response in Figure 4a is due to homologous desensitization and not a consequence of intracellular Ca^{2+} store depletion or experimental protocol. Furthermore, our previous studies with bradykinin (which does not exhibit heterologous desensitization following H_1-receptor activation) have shown that intracellular Ca^{2+} stores are able to refill in the continued presence of histamine and 2 mM Ca^{2+} (cf. Figure 6c in Dickenson & Hill, 1992).

Similar experiments were performed to determine whether ATP-mediated Ca^{2+} responses are susceptible to homologous desensitization. Figure 4c shows the Ca^{2+} responses obtained by a second application of ATP (100 μM; same protocol as described for Figure 4a) after an initial 450 s exposure to ATP. Both intracellular Ca^{2+} release (11 ± 4% of the first response; $n = 7$) and Ca^{2+} influx (20 ± 7% of the first response; $n = 7$) were significantly attenuated. The relevant control experiment is shown in Figure 4d. In the control experiment the second application of ATP resulted in optimal Ca^{2+} responses for intracellular Ca^{2+} release (96 ± 8% of the first application; $n = 4$) and Ca^{2+} influx (90 ± 7% of controls; $n = 4$).

Heterologous desensitization of histamine H_1- and ATP-receptor-stimulated increases in $[Ca^{2+}]_i$

Having established that both histamine and ATP-receptor mediated Ca^{2+} responses undergo homologous desensitization we investigated the effects of histamine pretreatment on ATP-responses and *vice versa*. Pretreatment with histamine (450 s) markedly reduced the Ca^{2+} responses elicited by ATP (100 μM; Figure 5a). The Ca^{2+} responses attributed to ATP-mediated intracellular Ca^{2+} release and Ca^{2+} influx were 40 ± 6% ($n = 12$) and 52 ± 7% ($n = 12$) respectively, of those obtained in control experiments (see Figure 5b). Similarly, pretreatment with ATP (450 s) significantly attenuated histamine-induced Ca^{2+} responses (Figure 5c). In these experiments histamine-stimulated intracellular Ca^{2+} release and Ca^{2+} influx responses were 32 ± 7% ($n = 12$) and 52 ± 3% ($n = 12$) respectively, of those obtained in control experiments (see Figure 5d). We have previously shown that short term exposure to histamine did not desensitize the response to bradykinin (Dickenson & Hill, 1992). Short term exposure to bradykinin (1 μM; 450 s) also had no effect on histamine (100 μM) Ca^{2+} responses (intracellular Ca^{2+} release 93 ± 4% of controls; Ca^{2+} influx 89 ± 5% of controls, $n = 10$).

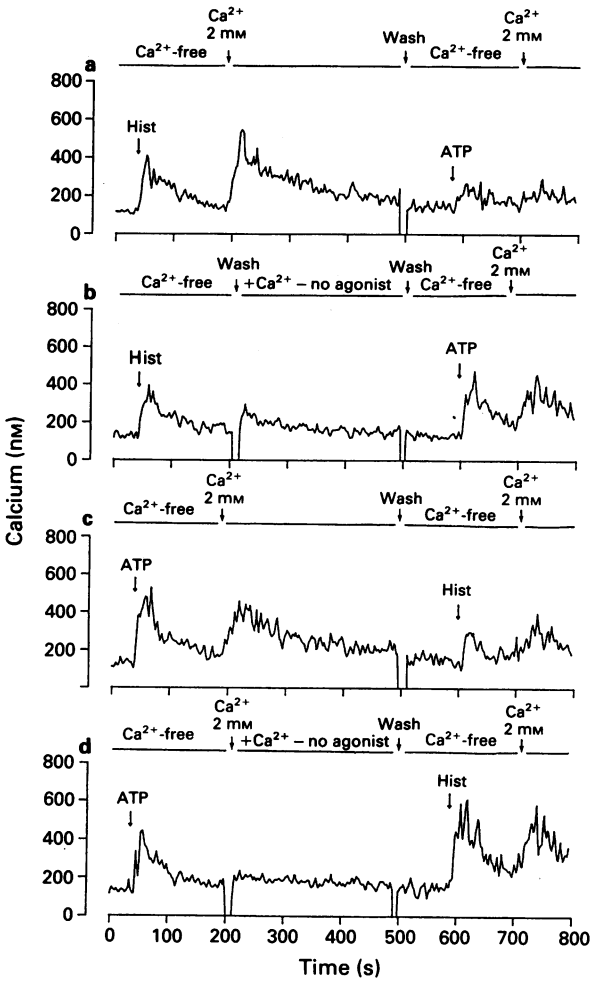


Figure 5 Heterologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. (a) Histamine-induced heterologous desensitization of ATP-receptor responses. (b) Control experiment for (a) to eliminate the possibility that the diminished ATP response is simply a consequence of the intracellular Ca^{2+} stores being empty. (c) ATP induced heterologous desensitization of histamine H_1-receptor Ca^{2+} responses. (d) Control experiment for (c) using the same experimental design as in (b). Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in five other experiments.

Effect of Ro 31-8220 on homologous desensitization of histamine H_1- and ATP-receptor-stimulated increases in $[Ca^{2+}]_i$

To assess the possible involvement of PKC in homologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses, PKC activity was blocked by preincubating DDT_1MF-2 cells with the selective PKC inhibitor, Ro 31-8220. Figure 6a shows homologous desensitization of histamine-stimulated Ca^{2+} responses. The complete desensitization of histamine Ca^{2+} responses was still observed in cells pretreated with Ro 31-8220 (10 μM; 45 min; see Figure 6b). Similarly, preincubation with Ro 31-8220 had no effect on the homologous desensitization of ATP-receptor mediated Ca^{2+} responses (compare Figure 6c with 6d). In these experiments the short-term exposure to ATP (450 s) completely abolished the response to a second ATP challenge (this occurred in approximately 50% of experiments). These data suggest that the homologous desensitization of histamine H_1- and ATP-receptor-mediated Ca^{2+} responses can occur independently of PKC activation.

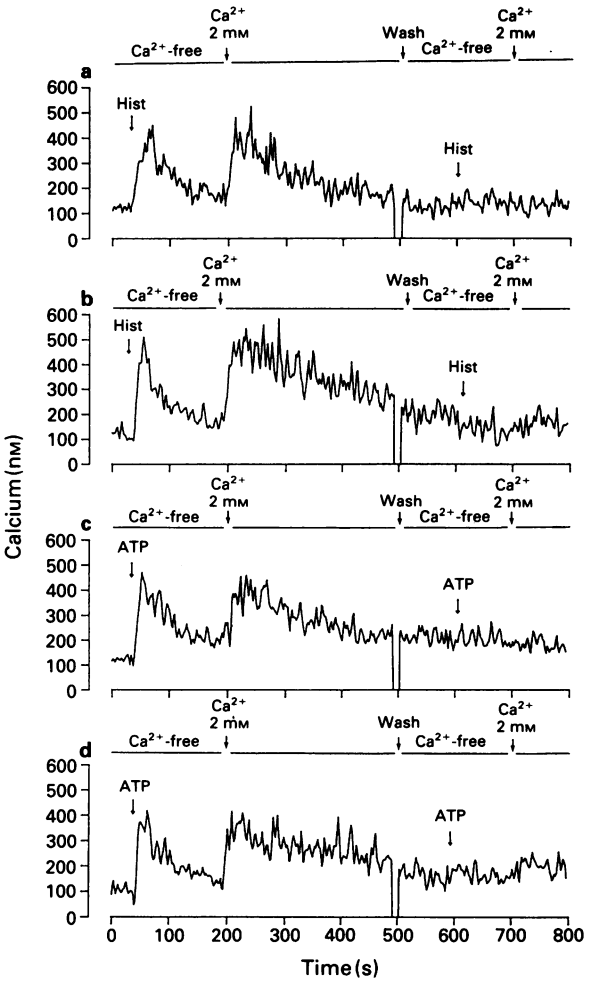


Figure 6 The effect of the protein kinase C inhibitor Ro 31-8220 on homologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. In experiments (b) and (d) the protein kinase C inhibitor Ro 31-8220 (10 μM) was present throughout. (a) Homologous desensitization of histamine H_1-receptor mediated Ca^{2+} responses. (b) The same protocol as in (a), but cells had been preincubated for 45 min with Ro 31-8220 (10 μM). (c) Homologous desensitization of ATP-receptor-mediated Ca^{2+} responses. (d) The same protocol as in (c), but cells had been preincubated for 45 min with Ro 31-8220 (10 μM). Experiments (a) and (c) contained 0.01% dimethyl sulphoxide to eliminate Ro 31-8220 vehicle effects. Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in seven other experiments.

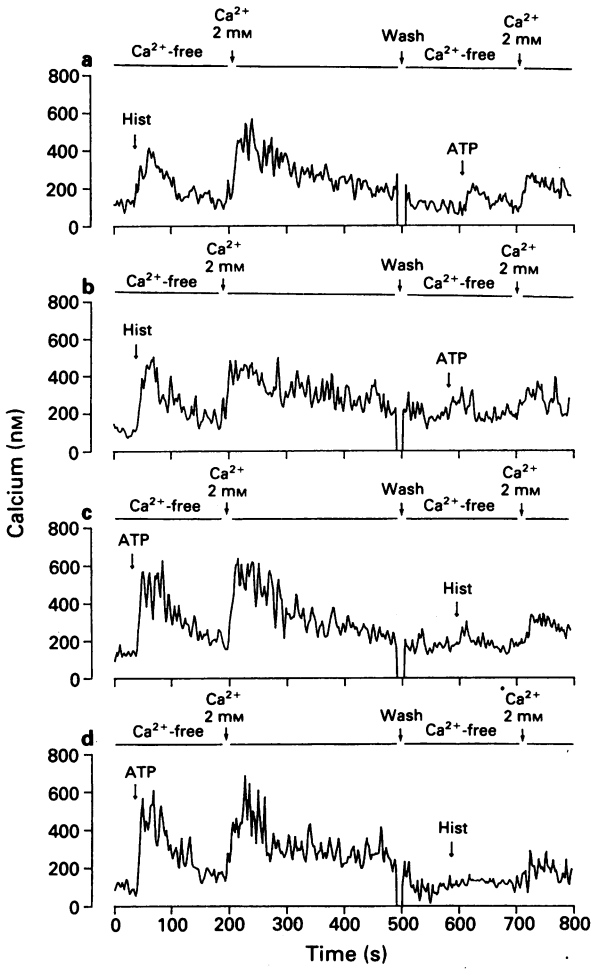


Figure 7 The effect of the protein kinase C inhibitor Ro 31-8220, on heterologous desensitization of histamine H_1- and ATP-receptor mediated increases in $[Ca^{2+}]_i$ in DDT_1MF-2 cells. In experiments (b) and (d) the protein kinase C inhibitor, Ro 31-8220 (10 μM) was present throughout. (a) Histamine induced heterologous desensitization of ATP Ca^{2+} responses. (b) The same protocol as in (a), but cells were preincubated with Ro 31-8220 (10 μM) for 45 min. (c) ATP induced heterologous desensitization of histamine Ca^{2+} responses. (d) The same protocol as in (c), but cells had been preincubated with Ro 31-8220 (10 μM) for 45 min. Experiments (a) and (c) contained 0.01% dimethyl sulphoxide to eliminate Ro 31-8220 vehicle effects. Histamine (Hist, 100 μM), ATP (100 μM) or $CaCl_2$ (2 mM) were added where indicated. Similar results were obtained in seven other experiments.

Effect of Ro 31-8220 on heterologous desensitization of histamine H_1- and ATP-receptor stimulated increases in $[Ca^{2+}]_i$

Recently, Smit *et al.* (1992) hypothesised that in HeLa cells the heterologous desensitization (studied using ATP) of H_1-receptor-mediated increases in $[Ca^{2+}]_i$ (predominantly Ca^{2+} influx) may involve PKC activation. Figure 7b shows that histamine-induced heterologous desensitization of ATP stimulated Ca^{2+} responses still occurred in Ro 31-8220 (10 μM; 45 min)-treated cells (compare Figure 7a with 7b). Similarly, preincubation with Ro 31-8220 had no effect on ATP-induced heterologous desensitization of histamine-mediated Ca^{2+} responses (compare Figure 7c with 7d). It should be noted that ATP-receptor-mediated Ca^{2+} responses are unaffected by Ro-31-8220 pretreatment (intracellular Ca^{2+} release 122 ± 7% of controls; Ca^{2+} influx 103 ± 6% of controls; $n = 8$). The data in Table 2 summarize the effect of Ro 31-8220 on histamine and ATP-receptor heterologous desensitization.

Table 2 The effect of the protein kinase C inhibitor, Ro 31-8220, on heterologous desensitization of histamine and ATP-receptor mediated increases in $[Ca^{2+}]_i$

	Untreated (% of control Ca^{2+} responses)	(*n*)	*Ro 31-8220* (% of control Ca^{2+} responses)	(*n*)
ATP (100 μM)				
Ca^{2+} release	41 ± 8	8	16 ± 9	8
Ca^{2+} influx	45 ± 6	8	46 ± 8	8
Histamine (100 μM)				
Ca^{2+} release	16 ± 9	8	3 ± 2	8
Ca^{2+} influx	48 ± 4	8	41 ± 3	8

Values represent heterologous desensitization of Ca^{2+} responses for histamine H_1- and ATP-receptor stimulation in control and Ro 31-8220 (10 μM)-treated cells (obtained after the initial stimulation with ATP or histamine respectively) and are expressed as a percentage of the Ca^{2+} responses elicited by histamine and ATP-receptor stimulation in control experiments, i.e. the same protocol as that used for histamine and ATP Ca^{2+} responses in Figures 5d and 5b respectively. Values represent the mean ± s.e.mean of (*n*) experiments.

Discussion

In this study we have shown that histamine H_1- and ATP-receptor-mediated increases in $[Ca^{2+}]_i$ are susceptible to homologous and heterologous desensitization (see Figures 4 and 5). However, the mechanisms involved in histamine H_1-receptor desensitization are poorly understood. Therefore, one of the aims of this work was to investigate the possible role PKC may have in agonist-induced desensitization of the histamine H_1-receptor.

The data presented in this study have shown that in DDT_1MF-2 cells, histamine H_1- and ATP-receptor-mediated increases in $[Ca^{2+}]_i$ (both intracellular Ca^{2+} release and Ca^{2+} influx) can be inhibited by PKC activation (with the phorbol ester PDBu). Furthermore, using the selective PKC inhibitor Ro 31-8220, we were able to reverse the inhibitory effects of PDBu on agonist-stimulated increases in $[Ca^{2+}]_i$, confirming the involvement of PKC in these events. The recent cloning of both the bovine (Yamashita *et al.*, 1991) and rat (Fujimoto *et al.*, 1993) histamine H_1-receptor has revealed several potential PKC phosphorylation sites in the amino acid sequence of the H_1-receptor. The availability of a selective PKC inhibitor, Ro 31-8220 (Davis *et al.*, 1989) has enabled us to investigate the role of PKC in the desensitization of histamine H_1- or ATP-receptor Ca^{2+} responses. The data presented show that homologous (see Figure 6) and heterologous (see Figure 7) desensitization of histamine and ATP-receptor-mediated increases in $[Ca^{2+}]_i$ still occurred in the presence of Ro 31-8220. Therefore, based on our experimental data, it would appear that PKC is not involved in either the homologous or heterologous desensitization of these receptors.

These data are similar to those recently reported in HeLa cells (Smit *et al.*, 1992) where, although the H_1-receptor-mediated increases in $[Ca^{2+}]_i$ (intracellular Ca^{2+} release and Ca^{2+} influx) are phorbol ester-sensitive, the homologous desensitization of H_1-receptor-mediated intracellular Ca^{2+} release appears to involve a mechanism which is PKC-independent. Similarly this group (Leurs *et al.*, 1991) have suggested that PKC is not involved in the homologous desensitization of H_1-receptor-induced contractions of guinea-pig intestinal smooth muscle (again phorbol ester-sensitive). In contrast to the data presented in this paper, Smit *et al.* (1992) also proposed that the non-selective inhibition (heterologous desensitization) of histamine and ATP-receptor-mediated Ca^{2+} influx may involve a PKC-dependent mechanism. These observations and the data presented in this paper suggest that, although initial H_1-receptor-mediated functional responses (i.e. contractions, Ca^{2+} release) are phorbol ester-sensitive, PKC-independent mechanisms appear to be involved in the homologous desensitization of the H_1-receptor.

Extensive research has shown that β-adrenoceptor desensitization is mediated by PKA and a receptor-specific β-adrenoceptor kinase (βARK; Hausdorff *et al.*, 1990; Palczewski & Benovic, 1991). Our previous studies (Dickenson *et al.*, 1993) have shown that the activation of cyclic AMP-dependent protein kinase A (PKA; achieved by elevating cytosolic cyclic AMP levels with forskolin) in DDT_1MF-2 cells attenuates histamine H_1-receptor-mediated inositol phospholipid hydrolysis and Ca^{2+} mobilization (the ATP responses are unaffected by PKA). Furthermore, in DDT_1MF-2 cell monolayers prelabelled with $[^3H]$-adenine, both histamine and ATP failed to stimulate the accumulation of $[^3H]$-cyclic AMP (data not shown). These data suggest that PKA is not involved in the desensitization of histamine H_1- or ATP-mediated Ca^{2+} responses.

There are several further possible mechanisms by which the desensitization of histamine and ATP Ca^{2+} responses is occurring. One explanation is that the $InsP_3$ receptor is being desensitized (possibly via autophosphorylation; Ferris *et al.*, 1992), this would account for both homologous and heterologous desensitization (assuming histamine and ATP release Ca^{2+} from the same intracellular Ca^{2+} store). However, the finding that bradykinin does not desensitize histamine Ca^{2+} responses and *vice versa* (Dickenson & Hill, 1992) suggest that $InsP_3$ receptor desensitization is not occurring. These data would also seem to rule out the possibility that a calcium-calmodulin dependent kinase is involved. An alternative explanation is that receptor-activated kinases may be involved in the homologous desensitization of histamine and ATP response, analogous to the βARK. Interestingly, it has been shown that in DDT_1MF-2 cells, β-adrenoceptor activation leads to the translocation of βARK activity from the cytosol to the plasma membrane (Strasser *et al.*, 1986). However to date, there is little evidence for the existence of other G-protein-coupled receptor kinases. If a histamine H_1-receptor specific kinase is responsible for homologous desensitization, the histamine-induced heterologous desensitization of ATP responses (and *vice versa*) may be due to the absence of receptor kinase specificity. The βARK has been shown to phosphorylate other G-protein coupled receptors, for example α_2-adrenoceptor and muscarinic M_2-receptors (Benovic *et al.*, 1987; Kwatra *et al.*, 1989). Smit *et al.* (1992) have also proposed that the homologous desensitization of the H_1-receptor in HeLa cells is associated with activation of a H_1-receptor kinase.

In conclusion, the present study has shown that PKC activation can inhibit histamine H_1- and ATP-receptor mediated Ca^{2+} responses (intracellular Ca^{2+} release and Ca^{2+} influx). However, PKC activation does not appear to be involved in homologous or heterologous desensitization of histamine or ATP-receptor mediated increases in $[Ca^{2+}]_i$.

The authors thank the Wellcome Trust for financial support.

References

BENOVIC, J.L., REGAN, J.W., MATSUI, H., MAYOR, F., COTECCHIA, S., LEEB-LUNDBERG, L.M.F., CARON, M.G. & LEFKOWITZ, R.J. (1987). Agonist-dependent phosphorylation of the α_2-adrenergic receptor by the β-adrenergic receptor kinase. *J. Biol. Chem.*, **262**, 17251–17253.

BERRIDGE, M.J. (1993). Inositol triphosphate and calcium signalling. *Nature*, **361**, 315–325.

BOARDER, M.R. & CHALLIS, R.A.J. (1992). Role of protein kinase C in the regulation of histamine and bradykinin stimulated inositol polyphosphate turnover in adrenal chromaffin cells. *Br. J. Pharmacol.*, **107**, 1140–1145.

BROWN, K.D., BLAKELEY, D.M., HAMON, M.H., LAURIE, M.S. & CORPS, A.N. (1987). Protein kinase C mediated negative-feedback inhibition of unstimulated and bombesin-stimulated polyphosphoinositide hydrolysis in Swiss mouse 3T3 cells. *Biochem. J.*, **245**, 631–639.

CLEMENTI, E., SCHEER, H., ZACCHETTI, D., FASOLATO, C., POZZAN, T. & MELDOLESI, J. (1992). Receptor-activated Ca^{2+} influx: Two independently regulated mechanisms of influx stimulation coexist in neurosecretory PC12 cells. *J. Biol. Chem.*, **267**, 2164–2172.

COWLEN, M.S., BARNES, M.R. & TOEWS, M.L. (1990). Regulation of histamine H_1 receptor-mediated phosphoinositide hydrolysis by histamine and phorbol esters in DDT_1MF-2 cells. *Eur. J. Pharmacol.*, **188**, 105–112.

COWLEN, M.S. & TOEWS, M.L. (1988). Evidence for alpha-1 adrenergic receptor internalization in DDT_1MF-2 cells following exposure to agonists plus protein kinase C activators. *Mol. Pharmacol.*, **34**, 340–346.

DAVIS, P.D., HILL, C.H., KEECH, E., LAWTON, G., NIXON, J.S., SEDGWICK, A.D., WADSWORTH, J., WESTMACOTT, D. & WILKINSON, S.E. (1989). Potent selective inhibitors of protein kinase C. *FEBS Lett.*, **259**, 61–63.

DICKENSON, J.M. & HILL, S.J. (1991). Histamine-stimulated increases in intracellular calcium in the smooth muscle cell line, DDT_1MF-2. *Biochem. Pharmacol.*, **42**, 1545–1550.

DICKENSON, J.M. & HILL, S.J. (1992). Histamine H_1-receptor mediated calcium influx in DDT_1MF-2 cells. *Biochem. J.*, **284**, 425–431.

DICKENSON, J.M., WHITE, T.E. & HILL, S.J. (1993). The effects of elevated cyclic AMP levels on histamine H_1-receptor stimulated inositol phospholipid hydrolysis and calcium mobilization in the smooth muscle cell line, DDT_1MF-2. *Biochem. J.*, **292**, 409–417.

FELDER, C.C., POULTER, M.O. & WESS, J. (1992). Muscarinic receptor-operated Ca^{2+} influx in transfected fibroblast cells is independent of inositol phosphates and release of intracellular Ca^{2+}. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 509–513.

FERRIS, C.D., CAMERON, A.M., BREDT, D.S., HUGANIR, R.L. & SNYDER, S.H. (1992). Autophosphorylation of inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem.*, **267**, 7036–7041.

FUJIMOTO, K., HORIO, Y., SUGAMA, K., ITO, S., LUI, Y.Q. & FUKUI, H. (1993). Genomic cloning of the rat histamine H_1-receptor. *Biochem. Biophys. Res. Commun.*, **190**, 294–301.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HAUSDORFF, W.P., CARON, M.C. & LEFKOWITZ, R.J. (1990). Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB J.*, **4**, 2881–2889.

JONES, J.A., OWEN, P.J. & BOARDER, M.R. (1990). Influence of phorbol esters, and diacylglycerol kinase and lipase inhibitors on noradrenaline release and phosphoinositide hydrolysis in chromaffin cells. *Br. J. Pharmacol.*, **101**, 521–526.

KWATRA, M.M., BENOVIC, J.L., CARON, M.G., LEFKOWITZ, R.J. & HOSEY, M.M. (1989). Phosphorylation of chick heart muscarinic cholinergic receptors by the β-adrenergic receptor kinase. *Biochemistry*, **28**, 4543–4547.

LEEB-LUNDBERG, L.M.F., COTECCHIA, S., DEBLASI, A., CARON, M.G. & LEFKOWITZ, R.J. (1987). Regulation of adrenergic receptor function by phosphorylation. I. Agonist-promoted desensitization and phosphorylation of alpha-1 adrenergic receptors coupled to inositol phospholipid metabolism in DDT_1MF-2 smooth muscle cells. *J. Biol. Chem.*, **262**, 3098–3105.

LEEB-LUNDBERG, L.M.F., COTECCHIA, S., LOMASNEY, J.W., DE BERNARDIS, J.F., LEFKOWITZ, R.J. & CARON, M.G. (1985). Phorbol esters promote alpha-1 adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 5651–5655.

LEURS, R., SMIT, M.J., BAST, A. & TIMMERMAN, H. (1991). Is protein kinase C involved in histamine H_1-receptor desensitization? In *New Perspectives in Histamine Research*. ed. Van der Goot, H. & Timmerman, H. pp. 393–403. Berlin: Birkhauser Verlag.

MITSUHASHI, M. & PAYAN, D.G. (1988). Phorbol ester-mediated desensitization of histamine H_1 receptors on a cultured smooth muscle cell line. *Life Sci.*, **43**, 1433–1440.

PALCZEWSKI, K. & BENOVIC, J.L. (1991). G-protein coupled receptor kinases. *Trends Biol. Sci.*, **16**, 387–391.

SMIT, M.J., BLOEMERS, S.M., LEURS, R., TERTOOLEN, L.G.T., BAST, A., DE LAAT, S.W. & TIMMERMAN, H. (1992). Short-term desensitization of the histamine H_1 receptor in human HeLa cells: involvement of protein kinase C dependent and independent pathways. *Br. J. Pharmacol.*, **107**, 448–455.

STRASSER, R.H., BENOVIC, J.L., CARON, M.G. & LEFKOWITZ, R.J. (1986). Beta-agonist and prostaglandin E1-induced translocation of the beta-adrenergic receptor kinase: evidence that the kinase may act on multiple adenylate cyclase-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6362–6366.

WOODS, N.M., CUTHBERTSON, K.S.R. & COBBOLD, P.H. (1987). Phorbol-ester-induced alterations of free calcium ion transients in single rat hepatocytes. *Biochem. J.*, **246**, 619–623.

YAMASHITA, M., FUKUI, H., SUGAMA, K., HORIO, Y., ITO, S., MIZUGUCHI, H. & WADA, H. (1991). Expression cloning of a cDNA encoding the bovine histamine H_1-receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11515–11519.

(Received June 1, 1993
Revised July 26, 1993
Accepted August 24, 1993)

Br. J. Pharmacol. (1993), **110**, 1457–1464 © Macmillan Press Ltd, 1993

Regional and cardiac haemodynamic effects of N^G, N^G,dimethyl-L-arginine and their reversibility by vasodilators in conscious rats

[1]S.M. Gardiner, P.A. Kemp, T. Bennett, *R.M.J. Palmer & *S. Moncada

Department of Physiology & Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH and *Wellcome Research Laboratories, Beckenham, Kent BR3 3BS

1 A series of experiments was carried out on 3 separate groups of male Long Evans rats, chronically instrumented for the measurement of regional haemodynamics, to compare the effects of N^G,N^G, dimethyl-L-arginine (ADMA) and N^G-monomethyl-L-arginine (L-NMMA), and their reversibility by the nitric oxide donors, S-nitroso-*N*-acetyl-penicillamine (SNAP), S-nitroso-glutathione (SNOG), sodium nitroprusside (SNP), and the vasodilator, hydralazine.

2 As previously reported for L-NMMA, ADMA (1–100 mg kg^{-1}) caused dose-dependent pressor and bradycardic effects, accompanied by renal, mesenteric and hindquarters vasoconstrictions. The magnitude and duration of these effects were similar for ADMA and L-NMMA, consistent with their being equipotent inhibitors of nitric oxide synthase.

3 Infusion of SNAP or SNOG (300 $\mu g\ kg^{-1}\ h^{-1}$) after injection of ADMA or L-NMMA (100 mg kg^{-1}) reversed the pressor but did not abolish the vasoconstrictor, effects of ADMA or L-NMMA. However, a higher dose of SNAP (3 mg $kg^{-1}\ h^{-1}$) caused complete reversal of the pressor and mesenteric haemodynamic effects of ADMA (100 mg kg^{-1}), although its renal and hindquarters vasoconstrictor effects were not abolished.

4 Infusion of SNP (300 $\mu g\ kg^{-1}\ h^{-1}$) after administration of L-NMMA (100 mg kg^{-1}), caused complete reversal of its pressor and mesenteric and hindquarters haemodynamic effects, and reduced substantially its renal vasoconstrictor action; hydralazine (7.5 mg $kg^{-1}\ h^{-1}$) was almost as effective as SNP in reversing all these variables.

5 In animals chronically instrumented for the measurement of cardiac haemodynamics, ADMA (100 mg kg^{-1}) caused a pressor effect accompanied by a rise in central venous pressure, and reductions in heart rate, cardiac index, stroke index, peak aortic flow, maximum rate of rise of aortic flow and total peripheral conductance. The reversal of the pressor effect of ADMA by SNAP (300 $\mu g\ kg^{-1}\ h^{-1}$) was accompanied by a reduction of central venous pressure below resting levels and a further diminution of stroke index; all other variables showed an increase, but they still remained below resting levels (with the exception of heart rate).

6 Thus, following inhibition of NO synthesis, pharmacological intervention with NO donors, or other vasodilators, may cause normalisation of the mean arterial pressure without necessarily returning all associated cardiovascular variables to normal.

Keywords: N^G,N^G-dimethyl-L-arginine; S-nitroso-*N*-acetyl-penicillamine; S-nitroso-glutathione; sodium nitroprusside; hydralazine; haemodynamics

Introduction

It was reported recently, that N^G,N^G-dimethyl-L-arginine (asymmetrical dimethyl-L-arginine, ADMA), an inhibitor of nitric oxide (NO) synthase, is a constituent of normal human plasma, being present in a concentration about 10 fold greater than N^G-monomethyl-L-arginine (L-NMMA) (Vallance *et al.*, 1992). L-NMMA is also an inhibitor of NO synthase (see Moncada *et al.*, 1991), which, on acute i.v. administration to conscious rats, causes a hypertensive response accompanied by widespread regional vasoconstriction (Gardiner *et al.*, 1990b). Vallance *et al.* (1992) reported that i.v. injection of ADMA in anaesthetized guinea-pigs induced dose-dependent pressor effects. However, it is not known whether the profiles of effect of ADMA and L-NMMA, on regional haemodynamics, are similar. Therefore, our first objective was to compare the regional haemodynamic effects of ADMA and L-NMMA in the same conscious rats.

At present, it is not known whether NO donors, or other vasodilators, are able to reverse the hypertensive and haemodynamic effects which result from systemic inhibition of NO in conscious rats. Therefore, our second objective was to assess the influence of the NO donors, S-nitroso-*N*-acetyl penicillamine (SNAP) (Ignarro *et al.*, 1981; Boughton-Smith *et al.*, 1990; Lopez-Belmonte *et al.*, 1993), S-nitroso-glutathione (SNOG) (Kowaluk & Fung, 1990; Radomski *et al.*, 1992), and sodium nitroprusside (SNP) on the pressor and regional haemodynamic effects of ADMA and/or L-NMMA and to determine if hydralazine, a vasodilator which is not an NO donor, was also able to influence the effects of NO synthase inhibition in a similar fashion.

Finally, since we found that the vasodilators studied could reverse the pressor effects of the NO synthase inhibitors without necessarily abolishing their vasoconstrictor effects, we measured the cardiac haemodynamic responses to ADMA, and the influence of SNAP upon them, to determine the extent to which changes in cardiac function contributed to the ability of SNAP to reverse the hypertensive effects of ADMA. Some of these results have been presented to the British Pharmacological Society (Bennett *et al.*, 1993; Gardiner *et al.*, 1993).

[1] Author for correspondence.

Methods

All experiments were carried out on male, Long Evans rats (350–450 g), bred in the Animal Unit at Nottingham. All surgery was carried out under sodium methohexitone anaesthesia (40–60 mg kg^{-1}, i.p., supplemented as required).

Measurement of regional haemodynamics

Miniaturised pulsed Doppler probes (Haywood *et al.*, 1981) were sutured around the left renal and superior mesenteric arteries, and the distal abdominal aorta (to monitor flow to the hindquarters). Probe wires were led subcutaneously to emerge at the back of the neck where their ends were fixed to the skin by a suture. Animals were given ampicillin (Penbritin, Beecham; 7 mg kg^{-1}, i.m.) and returned to their home cages with free access to food and water. One to 2 weeks later, animals were anaesthetized again (sodium methohexitone, 40 mg kg^{-1}, i.p.) and the signals from the flow probes were checked. Animals with acceptable signals from all 3 probes had intravascular catheters implanted (1 in the distal abdominal aorta via the ventral caudal artery and 3 in the right jugular vein). The catheters were led subcutaneously to emerge at the same point as the probe wires. The latter were soldered into a 6-way microconnector (Microtech Inc., Boothwyn, U.S.A.) that was clamped into a harness, and the catheters ran through the spring for protection. An extension of the lead to the Doppler flowmeter (a modified (Gardiner *et al.*, 1990a) Crystal Biotech VF-1 mainframe) was taped to the spring and the whole assembly was supported by a counterbalanced lever system to allow the animal free movement. Rats were housed in their home cages with free access to food and water throughout the experiment protocols, which began at least 1 day after catheter implantation.

Throughout the experiments, continuous recordings were made of phasic and mean arterial blood pressure, instantaneous heart rate and phasic and mean Doppler shift signals from the renal, mesenteric and hindquarters probes. Percentage changes in the mean Doppler shift signals were taken as an index of flow changes (Haywood *et al.*, 1981) and percentage changes in vascular conductance were calculated from the mean Doppler shift and mean arterial blood pressure signals (Gardiner *et al.*, 1990d).

Measurement of cardiac haemodynamics

An electromagnetic flow probe (Skalar, Delft) was implanted around the ascending thoracic aorta via a transthoracic approach (Smith & Hutchins 1979; Smits *et al.*, 1982; Gardiner *et al.*, 1990a,c,d). Animals were given ampicillin (as above) and left to recover for at least 7 days. At that time, they were anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.) and had intra-arterial and intravenous catheters implanted; one of the latter was designed and positioned so as to permit recording of central venous pressure (Gardiner *et al.*, 1990c,d). At least 1 day after catheter implantation, when animals were fully conscious, the output from the thoracic aortic probe was connected to a Skalar MDL 1401 flowmeter. The output from this device, together with pressure signals were digitised on-line using a custom-built microprocessor (University of Limburg, Department of Instrument Services; Schoemaker, 1989; Gardiner *et al.*, 1990c,d). The microprocessor provided values for mean systemic arterial blood pressure and central venous pressure; from the aortic flow signal it determined the integrated aortic flow, peak aortic flow and maximum rate of rise of aortic flow (dF/dt_{max}). The integrated aortic flow (which represents cardiac output minus coronary flow) was divided by heart rate to give an estimate of stroke volume, and the microprocessor also calculated total peripheral conductance by dividing integrated aortic flow by mean systemic arterial blood pressure. The microprocessor was set to perform these procedures for two consecutive cardiac cycles every 2 s, and then to average the data before storing them on disk. Off-line, data were retrieved and averaged (using the microprocessor system) over 40–50 s across the times of interest. These data for each animal were then entered into a spread-sheet that calculated cardiac index (i.e. integrated aortic flow 100 g^{-1} body weight), stroke index (stroke volume 100 g^{-1} body weight) and also factored peak aortic flow, dF/dt_{max} and total peripheral conductance by body weight before calculating means and s.e.mean for all variables, including mean systemic arterial blood pressure. While dF/dt_{max} is a useful index of contractility (de Wildt & Sangster, 1983) it is not independent of preload or afterload.

Experiments were performed, in random order, over several days. The treatment groups were as follows:-

Group 1 Haemodynamic interactions between ADMA or L-NMMA and SNAP or SNOG In 8 conscious Long Evans rats incremental bolus doses (1–100 mg kg^{-1}, i.v.) of ADMA or L-NMMA were given at intervals of at least 60 min to allow all variables to return to baseline before administration of the next dose. Five min after administration of the highest dose of ADMA or L-NMAA, an isotonic saline (154 nmol l^{-1} NaCl) infusion was begun and continued for 10 min as a control.

The same group of animals, on separate occasions were given continuous infusions of SNAP or SNOG (300 μg kg^{-1} h^{-1}) for 10 min.

Again, using the same group of animals on other occasions, bolus injections of ADMA or L-NMMA (100 mg kg^{-1}) were administered. Five min later, an infusion of SNAP or SNOG (300 μg kg^{-1} h^{-1}) was begun and was continued for 10 min. The changes observed were compared to those seen when saline was administered following the injection of ADMA or L-NMMA (see earlier).

Group2 Haemodynamic interactions between ADMA and a high concentration of SNAP In a separate group of animals ($n = 7$) SNAP was administered at a 10 fold higher dose (i.e. 3 mg kg^{-1} h^{-1}) for 10 min. On a separate occasion these animals received a bolus injection of ADMA (100 mg kg^{-1}) followed 5 min later by SNAP at a dose of 3 mg kg^{-1} h^{-1} for 10 min.

Group 3 Haemodynamic interactions between L-NMMA and SNP or hydralazine A third group of animals ($n = 8$) were given 10 min infusions of SNP (300 μg kg^{-1} h^{-1}) or hydralazine (7.5 mg kg^{-1} h^{-1}).

On separate experimental days, the animals in this group were given 10 min infusions of SNP or hydralazine beginning 5 min after bolus injection of L-NMMA (100 mg kg^{-1}).

Group 4 Cardiac haemodynamic responses to ADMA and SNAP A fourth group of animals ($n = 8$), that had previously had an electromagnetic flow probe implanted around the ascending thoracic aorta, received a 10 min infusion of SNAP (300 μg kg^{-1} h^{-1}).

At least 60 min later these animals were given ADMA (100 mg kg^{-1}) followed 5 min later by a 10 min infusion of SNAP.

Group 5 Haemodynamic responses to SDMA The final group of animals ($n = 5$) received bolus injections (100 mg kg^{-1}, i.v.) of N^G,N^G-dimethyl-L-arginine (symmetrical dimethyl-L-arginine; SDMA).

Plasma levels of ADMA

In order to determine resting plasma levels of endogenous ADMA and the effects of i.v. bolus injection of exogenous ADMA, Long Evans rats ($n = 2$) were anaesthetized (sodium methohexitone 40 mg kg^{-1}, i.p.) and had catheters implanted in the distal abdominal aorta and right jugular vein. At least 24 h later, an arterial blood sample (500 μl) was taken 30 min prior to i.v. injection of ADMA (100 mg kg^{-1}), and blood

Table 1 Resting cardiovascular variables in 3 of the groups of animals in which regional haemodynamics were studied (see Methods)

	Group 1 ($n = 8$)	*Group 2* ($n = 7$)	*Group 3* ($n = 8$)
Heart rate (beats min^{-1})	312 ± 10	343 ± 11	356 ± 12
Mean arterial blood pressure (mmHg)	103 ± 2	101 ± 2	98 ± 2
Renal flow (kHz)	8.8 ± 0.5	7.6 ± 0.7	7.4 ± 0.6
Mesenteric flow (kHz)	5.7 ± 0.2	7.2 ± 0.6	6.9 ± 0.6
Hindquarters flow (kHz)	3.5 ± 0.1	5.5 ± 0.5	4.9 ± 0.2
Renal conductance ([kHz $mmHg^{-1}$]10^3)	85 ± 5	75 ± 8	76 ± 6
Mesenteric conductance ([kHz $mmHg^{-1}$]10^3)	56 ± 3	71 ± 6	71 ± 7
Hindquarters conductance ([kHz $mmHg^{-1}$]10^3)	35 ± 2	54 ± 5	50 ± 3

Values are mean ± s.e.mean.

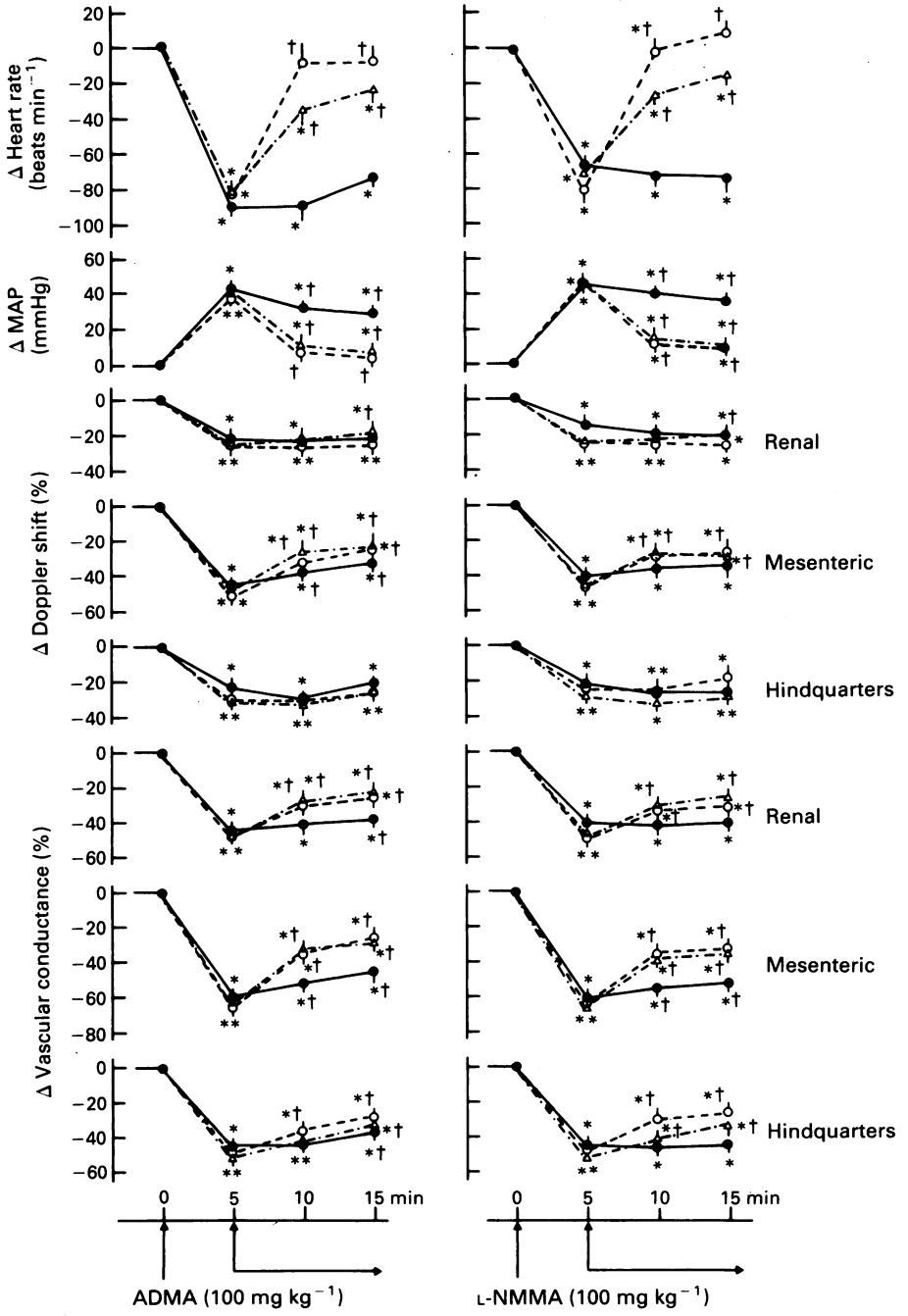


Figure 1 Cardiovascular responses to N^G,N^G-dimethyl-L-arginine (ADMA, 100 mg kg^{-1}, left-hand panels) or N^G-monomethyl-L-arginine (L-NMMA, 100 mg kg^{-1}, right-hand panels) followed after 5 min by infusion of saline (●) or S-nitroso-*N*-acetyl-penicillamine (SNAP, 300 μg kg^{-1} h^{-1}; ○) or S-nitroso-glutathione (SNOG, 300 μg kg^{-1} h^{-1}; Δ) in conscious, Long Evans rats (Group 1, $n = 8$). Values are mean ± s.e.mean; $*P < 0.05$ versus baseline; $\dagger P < 0.05$ versus value at 5 min.

samples were taken at 5, 15 and 60 min after ADMA injection. Plasma ADMA concentrations were measured with the technique described by Vallance et al. (1992), relative to a spiked standard.

Data analysis

Responses to the incremental bolus doses of ADMA or L-NMMA were assessed from the maximum changes, which occurred about 5 min after the injection.

The responses to SNAP, SNOG, SNP or hydralazine were estimated from the average values recorded across the 5 and 10 min time points during the infusions.

In order to assess the effects of SNAP or SNOG on responses to ADMA or L-NMMA, measurements were made around 5, 10 and 15 min following injection of the 100 mg kg^{-1} dose of ADMA or L-NMMA in the presence of saline, SNAP or SNOG. The effects of SNP or hydralazine on responses to L-NMMA were assessed in the same way.

All within-group responses were analysed by Friedman's test applied to changes relative to baseline. A P value <0.05 was taken as significant.

Drugs

With the exception of SNP and hydralazine (Sigma, UK), all compounds were synthesized by Dr Harold Hodson (Wellcome Research Laboratories). They were dissolved in sterile, isotonic saline (154 nmol l^{-1} NaCl); bolus injections were given in a volume of 100 µl and flushed in with 100 µl saline while infusions were given at a rate of 0.3 ml h^{-1}. Administration of vehicle alone in these volumes had no consistent cardiovascular effects.

Results

Resting values for cardiovascular variables in the three major groups of animals studied in the experiments involving measurement of regional haemodynamics are shown in Table 1.

Regional haemodynamic responses to ADMA or L-NMMA

ADMA and L-NMMA caused dose-dependent rises in mean arterial blood pressure and reductions in heart rate and renal, mesenteric and hindquarters flow and vascular conductance (Figure 1). There were no significant differences between the responses to ADMA and L-NMMA (Figures 1 and 2).

SDMA had no significant cardiovascular effects (data not shown).

In the two separate animals in which plasma ADMA levels were measured, resting values were 3 and 4 µmol l^{-1}. Five min after injection of ADMA plasma levels had increased to 1800 and 1500 µmol l^{-1}, respectively; at 15 min they were 800 and 740 µmol l^{-1}, respectively, and by 60 min they had fallen to 290 and 140 µmol l^{-1}, respectively.

Regional haemodynamic responses to SNAP or SNOG

The 10 min infusions of 300 µg kg^{-1} h^{-1} of SNAP or SNOG, alone, caused slight hypotension and tachycardia (Table 2). There were small and variable reductions in regional blood flows; only during SNAP infusion was there a slight increase in mesenteric vascular conductance (Table 2).

During the 10 min infusion of SNAP at a dose of 3 mg kg^{-1} h^{-1} there was a fall in mean arterial blood pressure, similar to that seen during infusion of the lower dose of SNAP, but the tachycardia was greater (Tables 2 and 3). Moreover, the higher dose of SNAP caused significant reductions in renal, mesenteric and hindquarters flows, although these changes were not accompanied by significant changes in vascular conductances (Table 3).

Effects of SNAP or SNOG on regional haemodynamic responses to ADMA or L-NMMA

The pressor and bradycardic effects of ADMA and L-NMMA were almost completely reversed by SNOG and, particularly, SNAP (Figures 1 and 3). However, the pressure changes were accompanied by relatively small effects on the reductions in flows and vascular conductances elicited by the NO synthase inhibitors (Figures 1 and 3).

Infusion of the higher dose of SNAP following ADMA reversed the hypertensive and bradycardic effects of the latter and caused slight hypotension and tachycardia (Table 3, Figure 4). In addition, the reduction in mesenteric flow caused by ADMA was almost abolished, and the accompanying reduction in mesenteric vascular conductance was completely inhibited (Table 3, Figure 4). In contrast, SNAP had no significant effect on the reductions in renal and hindquarters flow caused by ADMA, and the diminutions in conductances in these vascular beds were not reversed completely (Table 3, Figure 4).

Regional haemodynamic responses to SNP or hydralazine

The 10 min infusion of SNP alone caused tachycardia and a modest hypotension accompanied by a progressive fall in renal flow and a transient increase in mesenteric flow; the latter was associated with a significant rise in vascular conductance (Table 4).

Infusion of hydralazine alone caused a tachycardia and a

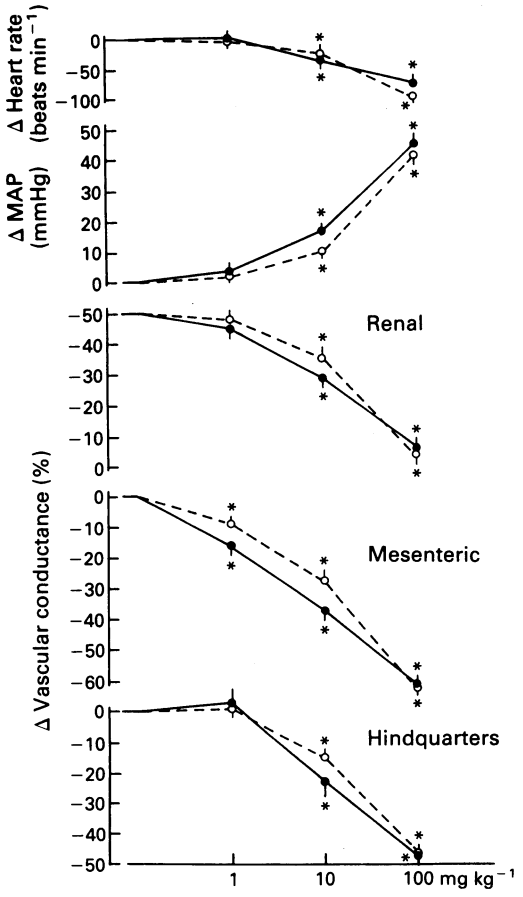


Figure 2 Maximum changes in heart rate, mean arterial blood pressure and renal mesenteric and hindquarters vascular conductances in the same conscious Long Evans rats (Group 1, $n = 8$) after i.v. injection of increasing bolus doses of N^G-monomethyl-L-arginine (L-NMMA, ●) or N^G,N^G,dimethyl-L-arginine (ADMA, ○). Values are mean ± s.e.mean; $*P<0.05$ versus baseline.

Table 2 Cardiovascular changes during 10 min infusions of S-nitroso-*N*-acetyl-penicillamine (SNAP; 300 μg kg^{-1} h^{-1}) or S-nitroso-glutathione (SNOG; 300 μg kg^{-1} h^{-1}) in conscious, Long Evans rats (Group 1, $n = 8$)

	SNAP		*SNOG*	
Time	5 min	10 min	5 min	10 min
Δ Heart rate (beats min^{-1})	31 ± 11*	39 ± 12*	15 ± 5*	16 ± 6*
Δ Mean blood pressure (mmHg)	−10 ± 1*	−9 ± 1*	−5 ± 1*	−4 ± 2*
Δ Renal flow (%)	−10 ± 3*	−8 ± 4	−6 ± 2*	−7 ± 2*
Δ Mesenteric flow (%)	−3 ± 2	−3 ± 3	−2 ± 3	−8 ± 2*
Δ Hindquarters flow (%)	−11 ± 2*	−10 ± 3*	−3 ± 2	−10 ± 4
Δ Renal conductance (%)	0 ± 3	0 ± 4	−2 ± 2	−3 ± 2
Δ Mesenteric conductance (%)	8 ± 2*	6 ± 4	2 ± 3	−5 ± 2
Δ Hindquarters conductance (%)	−1 ± 2	−2 ± 3	2 ± 3	−6 ± 4

Values are mean ± s.e.mean; *$P < 0.05$ for change.

Table 3 Cardiovascular changes during 10 min infusions of S-nitroso-*N*-acetyl-penicillamine (SNAP, 3 mg kg^{-1} h^{-1}) alone or starting 5 min after administration of N^G,N^G,dimethyl-L-arginine (ADMA 100 mg kg^{-1}) in conscious Long Evans rats (Group 2, $n = 7$)

	SNAP		*ADMA*	*ADMA + SNAP*	
Time	5 min	10 min	5 min	5 min	10 min
Δ Heart rate (beats min^{-1})	93 ± 12*	89 ± 17*	−92 ± 12*	72 ± 16*†	85 ± 19*†
Δ Mean blood pressure (mmHg)	−10 ± 2*	−10 ± 1*	41 ± 5*	−8 ± 2*†	−10 ± 2*†
Δ Renal flow (%)	−23 ± 4*	−21 ± 6*	−24 ± 3*	−23 ± 3*	−26 ± 3*
Δ Mesenteric flow (%)	−12 ± 3*	−11 ± 4*	−44 ± 2*	−12 ± 2*†	−12 ± 4*†
Δ Hindquarters flow (%)	−17 ± 3*	−12 ± 5	−41 ± 3*	−34 ± 6*	−32 ± 6*
Δ Renal conductance (%)	−13 ± 5	−13 ± 6	−45 ± 3*	−15 ± 4*†	−18 ± 3*†
Δ Mesenteric conductance (%)	−2 ± 5	−1 ± 5	−60 ± 2*	−4 ± 2†	−2 ± 5†
Δ Hindquarters conductance (%)	−8 ± 3	−2 ± 5	−58 ± 3*	−27 ± 6*†	−25 ± 7*†

Values are mean ± s.e.mean; *$P < 0.05$ for change relative to baseline; †$P < 0.05$ versus ADMA alone.

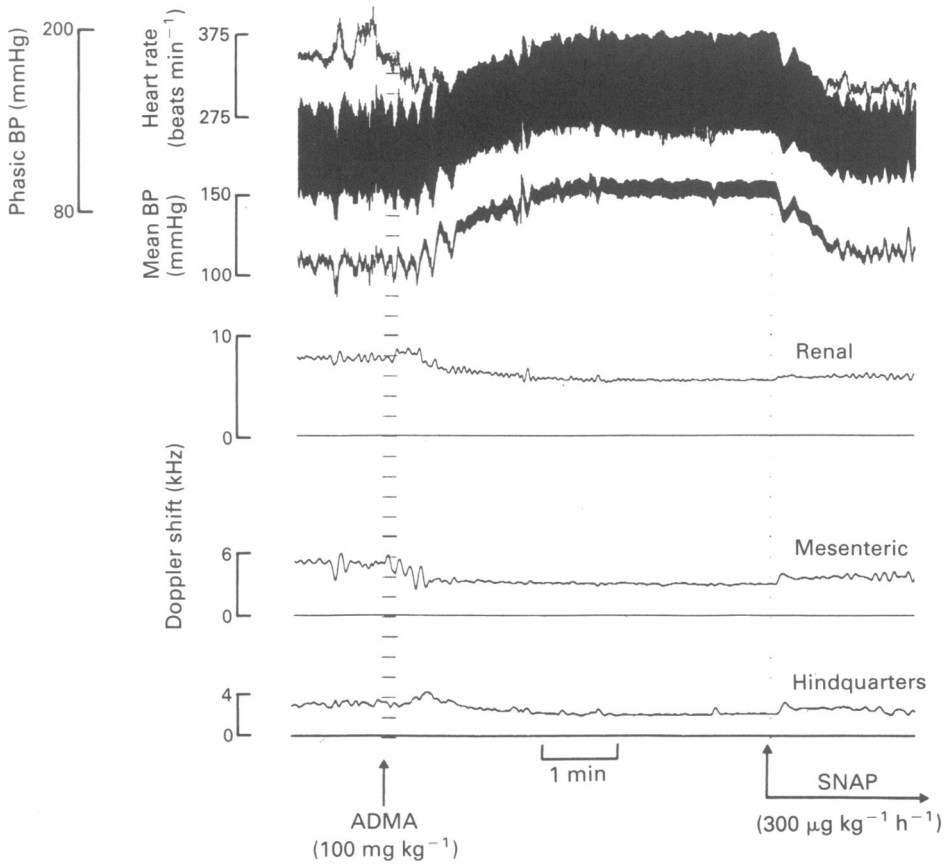


Figure 3 Cardiovascular responses at the onset of S-nitroso-*N*-acetyl-penicillamine (SNAP) infusion (300 μg kg^{-1} h^{-1}) 5 min after i.v. bolus injection of N^G,N^G,dimethyl-L-arginine (ADMA, 100 mg kg^{-1}) in a conscious, Long Evans rat.

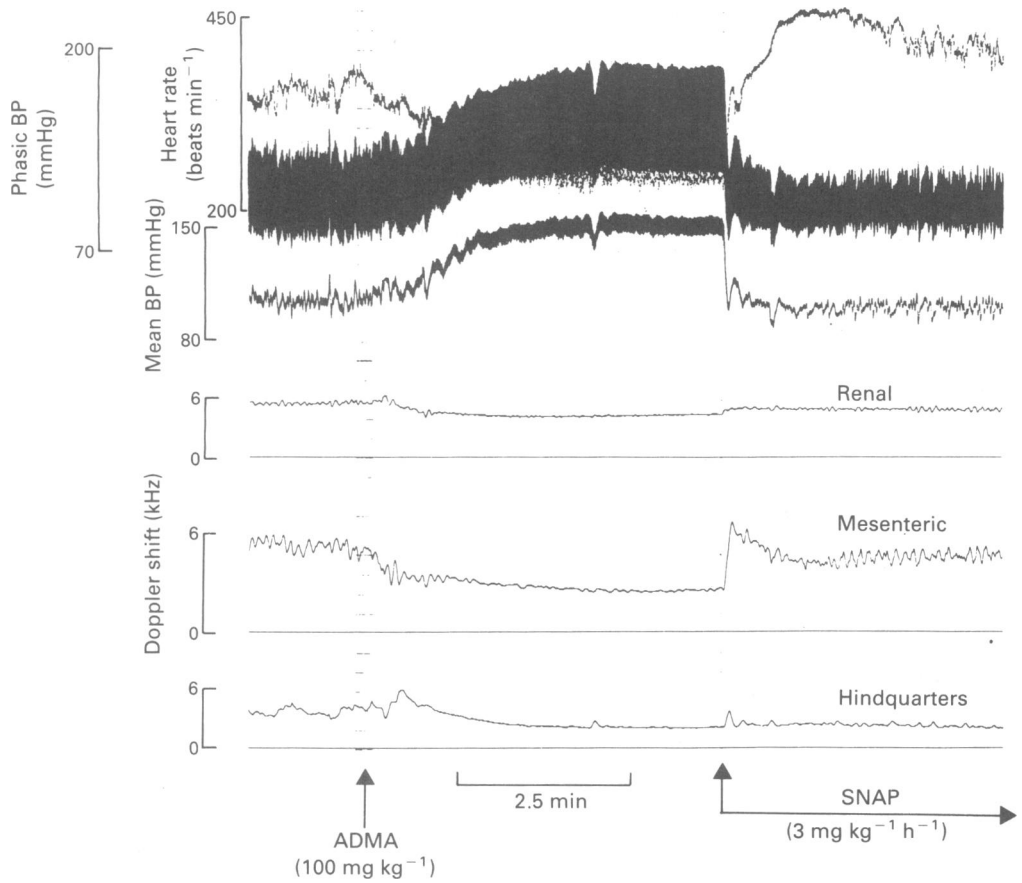


Figure 4 Cardiovascular responses at the onset of S-nitroso-*N*-acetyl-penicillamine (SNAP) infusion (3 mg kg^{-1} h^{-1}) 5 min after administration of N^G,N^G,dimethyl-L-arginine (ADMA, 100 mg kg^{-1}) in a conscious, Long Evans rat.

Table 4 Cardiovascular changes during 10 min infusion of sodium nitroprusside (SNP, 300 μg kg^{-1} h^{-1}) or hydralazine (7.5 mg kg^{-1} h^{-1}) in the same conscious Long Evans rats ($n = 8$, Group 3)

	SNP		*Hydralazine*	
Time	5 min	10 min	5 min	10 min
Δ Heart rate (beats min^{-1})	69 ± 14*	56 ± 12*	95 ± 16*	122 ± 16*
Δ Mean arterial blood pressure (mmHg)	−12 ± 2*	−9 ± 3*	−9 ± 2*	−15 ± 3*
Δ Renal flow (%)	−8 ± 4	−14 ± 4*	8 ± 3*	4 ± 6
Δ Mesenteric flow (%)	12 ± 2*	6 ± 3	33 ± 6	41 ± 7*
Δ Hindquarters flow (%)	−4 ± 5	−3 ± 8	24 ± 4*	28 ± 7*
Δ Renal conductance (%)	5 ± 5	−5 ± 6	19 ± 3*	22 ± 6*
Δ Mesenteric conductance (%)	28 ± 5*	16 ± 6*	46 ± 8*	66 ± 10*
Δ Hindquarters conductance (%)	10 ± 7	7 ± 9	35 ± 5*	51 ± 10*

Values are mean ± s.e.mean; *$P < 0.05$ for change.

Table 5 Cardiovascular variables under resting conditions and 5 and 10 min after onset of infusion of S-nitroso-*N*-acetyl-penicillamine (SNAP, 300 μg kg^{-1} h^{-1}) alone or SNAP starting 5 min after administration of N^G,N^G-dimethyl-L-arginine (ADMA, 100 mg kg^{-1}) in conscious, Long Evans rats (Group 4, $n = 8$)

		SNAP		*ADMA + SNAP*		
	Resting	5 min	10 min	ADMA	5 min	10 min
Heart rate (beats min^{-1})	364 ± 8	369 ± 10*	389 ± 8*	285 ± 9*	355 ± 15†	367 ± 13†
Mean arterial blood pressure (mmHg)	102 ± 2	95 ± 3*	97 ± 2	148 ± 9*	113 ± 2*†	110 ± 1*†
Central venous pressure (cmH_2O)	3.6 ± 0.2	3.0 ± 0.2*	2.9 ± 0.2*	4.7 ± 0.5*	2.7 ± 0.3*†	2.5 ± 0.3*†
Cardiac index (ml min^{-1} 100 g^{-1})	25.8 ± 0.9	24.5 ± 0.8	25.2 ± 0.7	17.6 ± 0.7*	19.4 ± 0.9*	20.7 ± 0.9*†
Peak thoracic aortic flow (ml min^{-1} 100 g^{-1})	104 ± 3	106 ± 3	106 ± 2	76 ± 3*	88 ± 3*†	92 ± 3*†
Maximum rate of rise of aortic flow (1 min^{-2} 100 g^{-1})	439 ± 10	458 ± 13	452 ± 10	276 ± 10*	364 ± 16*†	384 ± 17*†
Stroke index (μl beat^{-1} 100 g^{-1})	71 ± 3	62 ± 3*	65 ± 3*	62 ± 2*	55 ± 3*†	57 ± 3*†
Total peripheral conductance (μl min^{-1} mmHg^{-1} 100 g^{-1})	253 ± 72	260 ± 15	260 ± 9	120 ± 6*	173 ± 10*†	189 ± 9*†

Values are mean ± s.e.mean; *$P < 0.05$ versus resting; †$P < 0.05$ versus ADMA alone.

progressive hypotension; there was a transient increase in renal flow, but an incremental increase in mesenteric flow and a sustained increase in hindquarters flow (Table 4). There were significant increases in conductance in renal, hindquarters and, particularly, mesenteric vascular beds (Table 4).

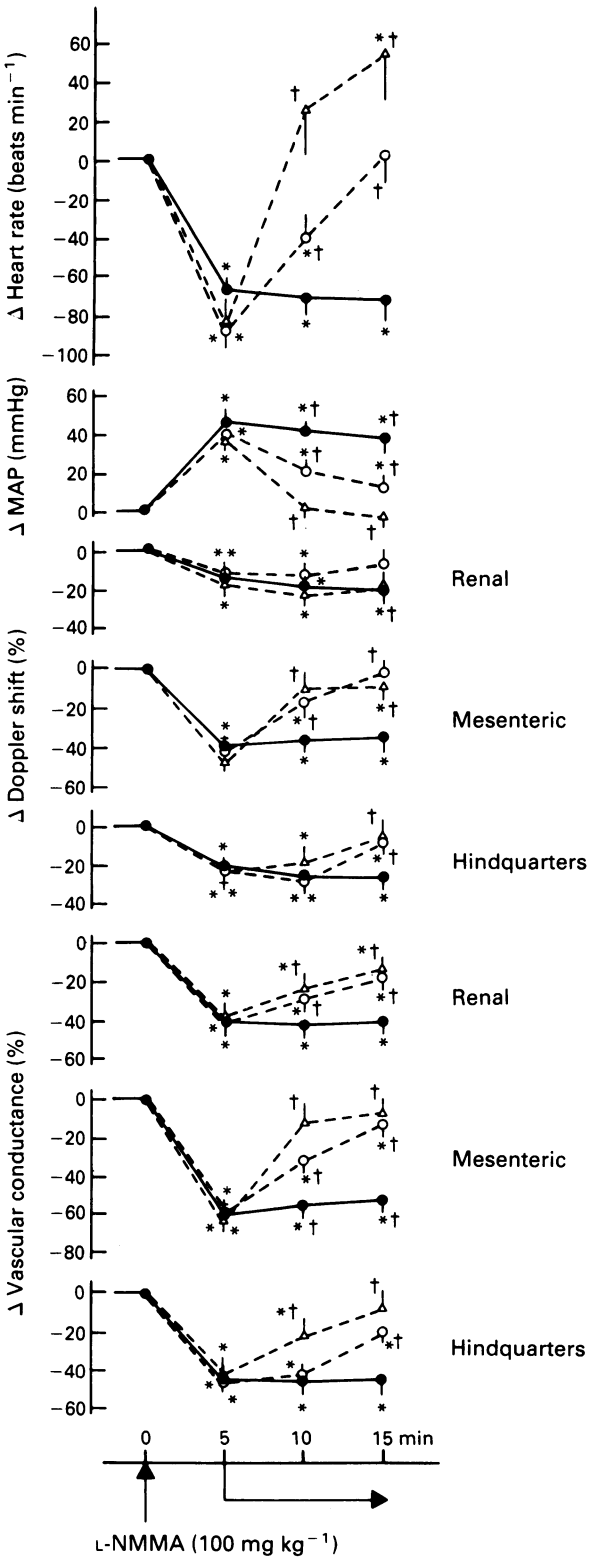


Figure 5 Cardiovascular responses to N^G-monomethyl-L-arginine (L-NMMA 100 mg kg^{-1}) followed 5 min later by a 10 min infusion of saline (●—●) or (SNP, 300 μg kg^{-1} h^{-1}; Δ–Δ) or hydralazine (7.5 mg kg^{-1} h^{-1}; O—O). The results for L-NMMA followed by saline are those shown in Figure 1; those for L-NMMA followed by SNP or hydralazine were obtained in a separate group of conscious Long Evans rats (Group 3, $n = 8$). Values are mean ± s.e.mean; *$P < 0.05$ versus baseline; †$P < 0.05$ versus value at 5 min.

Effects of SNP or hydralazine on regional haemodynamic responses to L-NMMA

SNP abolished the pressor effect of L-NMMA and converted the bradycardia to a tachycardia (Figure 5). SNP also reversed completely the L-NMMA-induced reductions in flow and conductance in the mesenteric and hindquarters, but not in the renal, vascular beds (Figure 5).

In spite of the fact that hydralazine alone had a more marked hypotensive and tachycardic effect than SNP (Table 4), hydralazine did not reverse completely the pressor effect of L-NMMA, although it abolished the bradycardic action (Figure 5). Hydralazine caused some reversal of the reductions in flow and conductance in the renal, mesenteric and hindquarters vascular beds caused by L-NMMA (Figure 5).

Cardiac haemodynamic responses to SNAP

During infusion of SNAP there was a tachycardia and slight transient hypotension (Table 5), similar to the changes seen in animals instrumented for measurement of regional haemodynamics (Table 2). These changes were accompanied by significant reductions only in central venous pressure and stroke index (Table 5).

Effects of SNAP on cardiac haemodynamic responses to ADMA

The pressor and bradycardic effects of ADMA were accompanied by an increase in central venous pressure, but reductions in all other variables (Table 5). In the presence of ADMA, SNAP caused marked inhibition of the pressor effect and complete reversal of the bradycardia (Table 5), and central venous pressure was reduced below resting levels (Table 5). Although SNAP caused some reversal of the ADMA-induced reductions in all other variables, they still remained significantly below the resting values (Table 5).

Discussion

The three major objectives of this study were:- (1) to delineate the regional haemodynamic effects of ADMA, and to compare them with those of L-NMMA in the same conscious rats; (2) to determine the extent to which the NO donors, SNAP, SNOG and SNP, and the NO-independent vasodilator, hydralazine, were capable of reversing the effects of ADMA or L-NMMA; and (3) to quantitate the cardiac haemodynamic effects of ADMA, and to assess how cardiac variables changed when the pressor effects of ADMA were reversed by SNAP.

The present results, showing dose-dependent pressor, bradycardic and regional vasoconstrictor effects of L-NMMA in conscious rats, are consistent with our earlier findings (Gardiner *et al.*, 1990b) and were included in the current protocols to represent a proper comparison for the effects of ADMA. It is clear from our results that the pressor responses to ADMA were due to haemodynamic changes similar to those seen with L-NMMA, and most likely attributable to inhibition of synthesis of NO by endothelial cells (Gardiner *et al.*, 1990b). The lack of effects of SDMA, and the potent actions of ADMA, corroborate the observations of Vallance *et al.* (1992).

Bolus i.v. injection of ADMA (100 mg kg^{-1}) increased resting plasma levels from 3–4 μmol l^{-1} to a peak of 1500–1800 μmol l^{-1}. It could be claimed that such plasma levels of endogenous ADMA would never be encountered, even in pathological conditions. However, the recent finding that endothelial cells produce ADMA (Fickling *et al.*, 1993),

raises the possibility that inhibition of NO synthase by elevation of local levels of ADMA could occur in the absence of any notable change in plasma levels of ADMA. Thus, injection of exogenous ADMA might be much less effective at inhibiting NO synthase than would local production of endogenous ADMA.

Although all the vasodilator compounds tested were capable of abolishing the mean arterial pressor effects of ADMA or L-NMMA, this action was accompanied by varying degrees of restitution of the normal regional haemodynamic profile. In all cases, the vasodilators were most effective in normalising mesenteric haemodynamics. In the presence of ADMA or L-NMMA, the more marked vasodilator effects of SNAP and SNOG on the mesenteric, than on the renal or hindquarters, vascular bed could have been due to a greater sensitivity of this vascular bed to the vasodilator effects of exogenous NO in the presence of NO synthase inhibitors. However, we found that SNP or hydralazine could reverse almost all regional haemodynamic effects of L-NMMA. Thus, although the doses of the vasodilators were chosen for their equi-antihypertensive effects they were clearly not matched for their regional vasodilator actions. It is feasible that the differential regional vasodilator responses were due to activation of the renin-angiotensin system, for example, opposing renal vasodilatation. However, we (S.M. Gardiner, P.A. Kemp & T. Bennett, unpublished observations) have found that the AT_1-receptor antagonist, losartan, does not render the renal vascular bed any more susceptible to the vasodilator effects of SNP in the presence of L-NMMA.

It is clear from the present findings that substantial changes in cardiac haemodynamics must have been occurring in the various experimental conditions studied. This was confirmed by direct measurement, showing that the mean arterial pressure effects of ADMA were accompanied by an increase in preload (i.e. central venous pressure), but reductions in all other variables. It is likely that the marked reductions in cardiac index, peak aortic flow and dF/dt_{max} in the presence of ADMA were probably due to the in-increase in afterload, rather than any direct negative inotropic effects (De Wildt & Sangster, 1983). Likewise, the partial reversal of the cardiac haemodynamic actions of ADMA by SNAP were probably due to the (incomplete) reduction in afterload. The failure of cardiac output to return to baseline levels under this condition could be attributed to the persistent increase in afterload. However, it is notable that, in the presence of ADMA, SNAP caused a significant reduction in preload (i.e., central venous pressure), indicating that venodilatation may have prevented cardiac output from rising further.

As pointed out elsewhere, the magnitude of the vasoconstrictor effect due to inhibition of NO synthase is underestimated on account of the concurrent reduction in cardiac function (Gardiner *et al.*, 1990d). Here, we show that the extent of reversal of the regional vasoconstrictor effects of ADMA by SNAP are overestimated by using normalisation of mean arterial pressure as an index of this effect, because the 'normal' arterial pressure is accompanied by a total peripheral conductance which is still below control levels, together with a reduced cardiac output.

In conclusion, it is clear that normalisation of blood pressure by NO donors, or by hydralazine, in the presence of NO synthase inhibitors, can occur in spite of persistent vasoconstriction and decreased cardiac function. This observation may have important clinical implications, for it indicates that organ perfusion, in conditions in which NO synthase is inhibited, may not necessarily be improved by administration of NO donors, or other vasodilators.

We are very grateful to Dr Anna Leone (Wellcome Research Laboratories) for the measurement of plasma ADMA levels.

References

BENNETT, T., GARDINER, S.M., KEMP, P.A., PALMER, R.M.J. & MONCADA, S. (1993). Regional haemodynamic responses to N^G,N^G-dimethyl-L-arginine (ADMA) or N^G-monomethyl-L-arginine (L-NMMA) in conscious rats. *Br. J. Pharmacol.*, **108**, 4P.

BOUGHTON-SMITH, N.K., HUTCHESON, I.R., DEAKIN, A.M., WHITTLE, B.J.R. & MONCADA, S. (1990). Protective effect of S-nitroso-N-acetyl-penicillamine in endotoxin-induced acute intestinal damage in the rat. *Eur. J. Pharmacol.*, **191**, 485–488.

DE WILDT, D.J. & SANGSTER, B. (1983). An evaluation of derived aortic flow parameters as indices of myocardial contractility in rats. *J. Pharmacol. Methods*, **10**, 55–64.

FICKLING, S.A., LEONE, A.M., NUSSEY, S.S., VALLANCE, P. & WHITLEY, G.StJ. (1993). Synthesis of N^G,N^G-dimethylarginine by human endothelial cells. *Endothelium*, (in press).

GARDINER, S.M., COMPTON, A.M., BENNETT, T. & HARTLEY, C.J. (1990a). Can pulsed Doppler technique measure changes in aortic blood flow in conscious rats? *Am. J. Physiol.*, **259**, H448–H456.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990b). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, **15**, 486–492.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990c). Cardiac output effects of endothelin-1, -2 and -3 and sarafotoxin S6b in conscious rats. *J. Auton. Nerv. Syst.*, **30**, 143–148.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990d). Regional and cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats. *Br. J. Pharmacol.*, **101**, 625–631.

GARDINER, S.M., KEMP, P.A., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1993). S-nitroso-N-acetyl-pencillamine (SNAP) abolishes the pressor, but not the vasoconstrictor, effects of N^G,N^G-dimethyl-L-arginine (ADMA) in conscious rats. *Br. J. Pharmacol.*, **108**, 5P.

HAYWOOD, J.R., SHAFFER, R.A., FASTENOW, C., FINK, G.D. & BRODY, M.J. (1981). Regional blood flow measurement with pulsed Doppler flow meter in conscious rat. *Am. J. Physiol.*, **241**, H273–H278.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanisms of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218**, 739–749.

KOWALUK, E.A. & FUNG, H.L. (1990). Spontaneous liberation of nitric oxide cannot account for *in vitro* vascular relaxation by S-nitrosothiols. *J. Pharmacol. Exp. Ther.*, **255**, 1256–1264.

LOPEZ-BELMONTE, J., WHITTLE, B.J.R. & MONCADA, S. (1993). The actions of nitric oxide donors in the prevention or induction of injury to the rat gastric mucosa. *Br. J. Pharmacol.*, **108**, 73–78.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

RADOMSKI, M.W., REES, D.D., DUTRA, A. & MONCADA, S. (1992). S-nitroso-glutathione inhibits platelet activation *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **107**, 745–749.

SCHOEMAKER, R. (1989). Experimental heart failure in rats. *PhD Thesis, University of Limburg, Maastricht, Netherlands.*

SMITH, T.L. & HUTCHINS, P.M. (1979). Central hemodynamics in the developmental stage of spontaneous hypertension in the unanaesthetised rat. *Hypertension*, **1**, 508–517.

SMITS, T.F.M., COLEMAN, T.G., SMITH, T.L., KASBERGEN, C.M., VAN ESSEN, H. & STRUYKER-BOUDIER, H.A.J. (1982). Antihypertensive effect of propranolol in conscious spontaneously hypertensive rats; central haemodynamics, plasma volume, and renal function during β-blockade with propranolol. *J. Cardiovasc. Pharmacol.*, **4**, 903–914.

VALLANCE, P., LEONE, A., CALVER, COLLIER, J. & MONCADA, S. (1992). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*, **339**, 572–575.

(Received May 5, 1993
Revised August 17, 1993
Accepted August 26, 1993)

Br. J. Pharmacol. (1993), **110**, 1465–1472 © Macmillan Press Ltd, 1993

Prejunctional modulation of noradrenaline release in mouse and rat vas deferens: contribution of P_1- and P_2-purinoceptors

Katharina Kurz, [1]Ivar von Kügelgen & Klaus Starke

Pharmakologisches Institut, Hermann-Herder-Strasse 5, D-79104 Freiburg i. Br., Germany

1 Prejunctional purinoceptors modulating the release of noradrenaline were compared in mouse and rat vas deferens. Tissue slices were preincubated with [^{3}H]-noradrenaline and then superfused and stimulated electrically, in most experiments by trains of 60 pulses, 1 Hz.

2 In mouse vas deferens, 2-chloroadenosine (IC_{50} 0.24 μM), β,γ-methylene-ATP (IC_{50} 3.8 μM), α,β-methylene-ATP (IC_{50} 2.9 μM) and 2-methylthio-ATP (only 30 μM tested) reduced the evoked overflow of tritium. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 10 nM, antagonized the effect of 2-chloroadenosine (apparent pK_B 10.2) as well as of β,γ-methylene-ATP (apparent pK_B 9.6) and α,β-methylene-ATP. Suramin, 300 μM, attenuated the effect of 2-chloroadenosine at best very slightly, antagonized the effect of β,γ-methylene-ATP (apparent pK_B 4.5) and, when combined with DPCPX 10 nM, caused a further marked shift to the right of the concentration-response curve of β,γ-methylene-ATP beyond the shift produced by DPCPX alone.

3 In rat vas deferens, 2-chloroadenosine (IC_{50} 0.20 μM), β,γ-methylene-ATP (IC_{50} 4.8 μM), α,β-methylene-ATP (IC_{50} 3.0 μM) and 2-methylthio-ATP (only 30 μM tested) also reduced the evoked overflow of tritium. DPCPX, 10 nM, antagonized the effect of 2-chloroadenosine (apparent pK_B 9.7) as well as of β,γ-methylene-ATP (apparent pK_B 9.6) and α,β-methylene-ATP. Suramin, 300 μM, did not change the effect of 2-chloroadenosine, attenuated the effect of β,γ-methylene-ATP at best very slightly and, when combined with DPCPX, caused at best a very small shift to the right of the concentration-response curve of β,γ-methylene-ATP beyond the shift produced by DPCPX alone.

4 It is concluded that prejunctional purinoceptor mechanisms in mouse and rat vas deferens are similar. In either species, both nucleosides such as adenosine and nucleotides such as β,γ-methylene-ATP activate a common release-inhibiting receptor which is a P_1- or, more specifically, A_1-purinoceptor. There seems to be no need to postulate the existence of a novel prejunctional P_3-purinoceptor. Moreover, the sympathetic terminal axons possess an additional P_2-purinoceptor in both species which is activated by some nucleotides such as β,γ-methylene-ATP and 2-methylthio-ATP, although the activation of the P_2-purinoceptor by β,γ-methylene-ATP is difficult to demonstrate in the rat.

Keywords: Mouse vas deferens; rat vas deferens; P_1-purinoceptor; P_2-purinoceptor; P_3-purinoceptor; prejunctional receptors; noradrenaline release; adenine nucleotides; 2-chloroadenosine; β,γ-methylene-ATP

Introduction

Adenosine inhibits action potential-evoked neuronal release of noradrenaline (Hedqvist & Fredholm, 1976; reviewed by Fredholm & Hedqvist, 1980; Stone, 1981; Olsson & Pearson, 1990). The same holds true for nucleotides such as ATP (Fredholm, 1974; Clanachan *et al.*, 1977; Enero & Saidman, 1977). It was thought for some time that the nucleotides act only indirectly, by way of their degradation product adenosine (e.g. Clanachan *et al.*, 1977; Stone, 1985). However, direct effects on noradrenergic axons are now established (Lukacsko & Blumberg, 1982; Shinozuka *et al.*, 1988; von Kügelgen *et al.*, 1989; 1992a; Forsyth *et al.*, 1991; Fuder *et al.*, 1992). Where do adenosine and the nucleotides act?

It has been suggested that two separate prejunctional, release-inhibiting purinoceptors operate in the vas deferens of the mouse: a classical P_1-purinoceptor, activated mainly but not exclusively by the nucleotide adenosine, and a P_2-purinoceptor, activated only by nucleotides (von Kügelgen *et al.*, 1989). The main evidence was that adenosine, ATP, its metabolically more stable derivative adenosine 5'-O-(3-thio)-triphosphate (ATPγS) and UTP all inhibited the release of noradrenaline; that the P_1-purinoceptor antagonist 8-(*p*-sulphophenyl)theophylline attenuated the effect of adenosine much more than the effects of ATP and ATPγS; that the P_2-purinoceptor antagonist suramin (Dunn & Blakeley, 1988) attenuated only the effect of ATPγS but not that of adenosine; and that the effect of the nucleotide UTP was attenuated by both 8-(*p*-sulphophenyl)theophylline and suramin. In contrast, in rat tail artery and vas deferens, it has been suggested that adenosine and nucleotides act through a common 'hybrid' prejunctional purinoceptor of a novel type, called P_3 (Shinozuka *et al.*, 1988; 1990; Forsyth *et al.*, 1991). The main evidence was that adenosine and 2-chloroadenosine as well as ATP, its metabolically more stable derivative β,γ-methylene-ATP and UTP all reduced the release of noradrenaline; and that 8-(*p*-sulphophenyl)theophylline and α,β-methylene-ATP, known as a P_{2X}-purinoceptor agonist, both acted as antagonists against nucleosides as well as nucleotides. In the experiments of Shinozuka *et al.* (1988, 1990), von Kügelgen *et al.* (1989) and Forsyth *et al.* (1991), release of noradrenaline was measured as overflow. Similar previous suggestions, either of a common prejunctional receptor for nucleosides and nucleotides (Lukacsko & Blumberg, 1982) or of separate prejunctional P_1- and P_2-purinoceptors (Taylor *et al.*, 1983), were based on postjunctional response measurements which are ambiguous, mainly due to possible desensitization by nucleotides of postjunctional P_2-purinoceptors (p. 437 of Burnstock & Kennedy, 1985).

The suggestion of different prejunctional purinoceptor mechanisms in the same tissue – vas deferens – of the rat and the mouse is intriguing. Is there in fact a species difference? Or are the prejunctional purinoceptors in the two species more similar than proposed? In order to answer the question, we compared the effects of 2-chloroadenosine, β,γ-methylene-ATP, α,β-methylene-ATP, the selective P_{2Y}-purinoceptor agonist 2-methylthio-ATP, the P_1 antagonist 8-cyclopentyl-

[1] Author for correspondence.

1,3-dipropylxanthine (DPCPX) and suramin on the release of [^{3}H]-noradrenaline in slices of mouse and rat vas deferens under identical conditions. Some of these results have been reported to the Deutsche Gesellschaft für Pharmakologie und Toxikologie (Kurz *et al.*, 1993).

Methods

Male NMRI mice weighing 35–40 g or male Wistar rats weighing 250–300 g (Savo, Kisslegg, Germany) were killed by cervical dislocation and exsanguination. The vasa deferentia were dissected out and desheathed. Slices, 5–6 mg, were prepared from the prostatic portions. Four slices were preincubated at 37°C for 30 min in each of two vials containing 2 ml medium with (−)-[^{3}H]-noradrenaline 0.1 μM, specific activity 1.62–2.11 TBq mmol^{-1}. Following incubation with [^{3}H]-noradrenaline, the slices were washed three times with 30 ml [^{3}H]-noradrenaline-free medium. One slice was transferred to each of eight glass superfusion chambers where it was held by a polypropylene mesh between platinum plate electrodes, 18 mm apart. The slices were superfused with [^{3}H]-noradrenaline-free medium at a rate of 1.25 ml min^{-1} at 37°C. A Stimulator I (Hugo Sachs Elektronik, March-Hugstetten, Germany) operating in the constant mode was used for electrical field stimulation. Six periods of stimulation were applied (rectangular pulses of 1 ms width and 50 mA current strength). The first, delivered after 30 min of superfusion (18 pulses, 1 Hz), was not used for determination of tritium outflow. The following stimulation periods (S_1–S_5) began after 96, 117, 138, 159 and 180 min of superfusion; each consisted of 60 pulses delivered at 1 or 8 Hz (identical parameters at S_1–S_5 in each single experiment); unless stated otherwise, the frequency was 1 Hz. The collection of successive 3-min superfusate samples began 6 min before S_1. Adenosine deaminase, DPCPX, suramin and (when used for interaction experiments) α,β-methylene-ATP were added to the medium 36 min before S_1 and maintained for the remainder of the experiment. Other drugs (including α,β-methylene-ATP in some experiments) were added either at a constant concentration from 3 or 6 min before S_3 for the remainder of the experiment, or at increasing concentrations from 6 min before to 15 min after the onset of S_3, S_4 and S_5; the increasing concentrations differed between slices so that concentration-response curves in Figures 3–7 are based on up to seven concentrations. After superfusion, each slice was solubilized in 0.5 ml Soluene-350 (Canberra Packard, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized slices by liquid scintillation counting.

The superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 0.9, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. The medium used for preincubation contained $CaCl_2$ 0.2 mM instead of $CaCl_2$ 2.5 mM because otherwise the basal efflux of tritium increased during the course of the experiment (see also Limberger *et al.*, 1992). Media were saturated with 5% CO_2 in O_2. The superfusion but not the preincubation medium also contained desipramine 1 μM, corticosterone 10 μM and yohimbine 1 μM in order to block the cellular uptake of [^{3}H]-noradrenaline and α_2-autoreceptors, respectively.

The outflow of tritium was expressed as fractional rate (min^{-1}), i.e., (tritium outflow per 3 min)/[3(tritium present in the slice at the onset of the respective collection period)]. The electrically evoked overflow was calculated as the difference between the total overflow during the 12 min after onset of stimulation, and the estimated basal outflow; the basal outflow was assumed to decline linearly from the 3-min interval before, to the interval 12–15 min after, the start of stimulation. The difference (total minus basal; Bq) was expressed as a percentage of the tritium content (Bq) of the slice at the onset of stimulation. S_2 served as the reference stimulation period in each tissue slice. The ratio of the overflow of tritium evoked by S_1 to the overflow evoked by S_2 was calculated as an internal control. Only experiments with an S_1/S_2 ratio of 0.9 to 1.1 were evaluated further. Effects of drugs that were added before S_3 to S_5 on basal tritium efflux were evaluated as ratios of the fractional rate of outflow immediately before S_3, S_4 and S_5 and immediately before S_2 (b_n/b_2). Effects of drugs that were added before S_3 to S_5 on electrically evoked overflow were evaluated as ratios of the overflow elicited by S_3, S_4 and S_5 and the overflow elicited by S_2 (S_n/S_2). S_n/S_2 ratios obtained in individual tissue slices were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent added before S_3 to S_5; '% of control' in Figure 1 and Figures 3–7).

For calculation of maximal inhibitions produced by 2-chloroadenosine, β,γ-methylene-ATP and α,β-methylene-ATP and of their IC_{50} values (concentrations that caused 50% of the maximal inhibition), the sigmoid-shaped function No. 25 of Waud (1976) was fitted to the averaged concentration-response data (Figures 3–7). This function was also fitted to averaged agonist concentration-response data from experiments carried out in the presence of antagonists (DPCPX or suramin); since maximal effects of agonists often were not reached in the presence of antagonists (e.g. Figure 6a), the maximal inhibition was taken as that obtained in the absence of antagonist. DPCPX and suramin pK_B ($-\log K_B$) values were calculated from the increase in IC_{50} values (equation No. 16 of Waud, 1976); since only one antagonist concentration was tested and a competitive character of the antagonism was not verified, the values are apparent pK_B values.

The following drugs were used: suramin hexasodium salt (Bayer, Wuppertal, Germany), (−)-[ring-2,5,6-^{3}H]-noradrenaline (Du Pont, Dreieich, Germany), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-methylthioadenosine 5′-triphosphate tetrasodium salt (2-methylthio-ATP) (Research Biochemicals, Natick, MA, U.S.A.), yohimbine HCl (Roth, Karlsruhe, Germany), adenosine deaminase type VII from calf intestinal mucosa (EC 3.5.4.4), 2-chloroadenosine, corticosterone, desipramine HCl, α,β-methylene adenosine 5′-triphosphate lithium salt (α,β-methylene-ATP, APCPP), β,γ-methylene adenosine 5′-triphosphate sodium salt (β,γ-methylene-ATP, APPCP), tetrodotoxin (Sigma, Deisenhofen, Germany). Solutions of drugs were prepared with either distilled water or (corticosterone) 1,2-propanediol (Sigma; final concentration about 8.7 mM) or (DPCPX) dimethyl sulphoxide (Roth; final concentration about 0.4 mM) or (tetrodotoxin) sodium acetate buffer (0.1 M, pH 4.8). Dimethyl sulphoxide was added to superfusion media 36 min before S_1 (when administration of DPCPX began) in all experiments except those of Figure 1; since it caused no change, b_2 and S_2 values with and without dimethyl sulphoxide were pooled.

Means ± s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test. $P < 0.05$ or lower was taken as the criterion of statistical significance. For multiple comparisons with the same control, P levels were adjusted according to Bonferroni. n is the number of tissue slices.

Results

Slices of mouse or rat vas deferens were preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine 1 μM, corticosterone 10 μM and yohimbine 1 μM. Electrical stimulation by 60 pulses, 1 Hz markedly increased the outflow of tritium (see Figure 2 below). Values for the overflow elicited by the reference stimulation period S_2 are summarized in Table 1. When no drug was present (except desipramine, corticosterone and yohimbine), the overflow at S_2 averaged 1.009% of tissue tritium in the mouse and 0.774% in the rat. Adenosine deaminase (not tested in the mouse), DPCPX and suramin, as well as DPCPX and suramin combined, increased the evoked overflow of tritium (except DPCPX in the mouse), whereas α,β-methylene-ATP caused a decrease. The fractional rate of

Table 1 Electrically evoked overflow of tritium from slices of mouse and rat vas deferens

Drugs added 36 min before S_1	*Mouse* S_2 (% of tissue tritium)	*n*	*Rat* S_2 (% of tissue tritium)	*n*
–	1.009 ± 0.032[a]	99	0.774 ± 0.019[b]	120
Adenosine-deaminase 0.3 u ml^{-1}			0.979 ± 0.019**	15
DPCPX 10 nM	0.999 ± 0.028	59	0.845 ± 0.026*	43
Suramin 300 μM	1.234 ± 0.047**	33	0.963 ± 0.028**	53
DPCPX 10 nM + Suramin 300 μM	1.366 ± 0.086**	15	0.959 ± 0.062**	17
α,β-Methylene-ATP 30 μM	0.786 ± 0.030**	39	0.657 ± 0.024**	33

S_2 is the second period of electrical stimulation (60 pulses, 1 Hz). DPCPX, 8-cyclopentyl-1,3-dipropylxanthine. Means ± s.e.mean of *n* experiments.
[a]Corresponding to 30.4 ± 1.4 Bq.
[b]Corresponding to 41.1 ± 1.6 Bq.
Significant differences from corresponding experiments without drugs added before S_1: *$P<0.05$ and **$P<0.01$.

tritium efflux immediately before S_2 (b_2) was 0.00211 ± 0.00006 min^{-1} ($n = 99$) in the mouse and 0.00162 ± 0.00003 min^{-1} ($n = 120$) in the rat. It was slightly reduced by the compounds listed in Table 1 except adenosine deaminase and DPCPX (rat; not shown).

In experiments without drugs (except desipramine, corticosterone and yohimbine), the basal outflow of tritium as well as the overflow response to electrical stimulation remained fairly constant (see Figure 2a and 2d below). In mouse vas deferens, the b_3/b_2, b_4/b_2, and b_5/b_2 ratios were 0.95 ± 0.02, 0.92 ± 0.02 and 0.95 ± 0.04, and S_3/S_2, S_4/S_2, and S_5/S_2 ratios were 0.99 ± 0.02, 0.97 ± 0.05 and 0.94 ± 0.06, respectively ($n = 8$). Average b_n/b_2 ratios also were slightly below unity, and S_n/S_2 ratios close to unity, when the compounds listed in Table 1 were present in the medium from 36 min before S_1 but no second drug was added later (not shown). The same was true for rat vas deferens.

In initial experiments, 2-chloroadenosine, 2-methylthio-ATP, α,β-methylene-ATP and β,γ-methylene-ATP were added 6 min before S_3 at a concentration of 30 μM and maintained throughout. All reduced the evoked overflow of tritium in both mouse and rat vas deferens, and for all the effect was approximately constant from S_3, after 6 min of exposure, to S_5, after 48 min of exposure (Figure 1; β,γ-methylene-ATP not tested in the mouse). 2-Chloroadenosine caused similar inhibition, irrespective of whether it was added 6 min (Figure 1) or 3 min before S_3 (not shown), and the same was true for α,β-methylene-ATP ($n = 3$–4 for the 3-min experiments). The effects of α,β- and β,γ-methylene-ATP were not changed when the superfusion medium contained adenosine deaminase (rat; Figure 1c). Tetrodotoxin practically abolished the overflow response (Figure 1). An exposure time of 6 min before the respective stimulation period was chosen for all subsequent experiments.

When added before S_3, S_4 and S_5 at increasing concentrations, 2-chloroadenosine progressively reduced the evoked overflow of tritium (mouse: Figure 2b; rat: Figure 2e). The IC_{50} (maximal inhibition) values obtained by fitting a sigmoid to the averaged concentration-response curves were 0.24 μM (47%) in mouse vas deferens slices (closed symbols in Figure 3) and 0.20 μM (70%) in rat vas deferens slices (closed symbols in Figure 4). In both species, DPCPX 10 nM caused a large shift of the concentration-response curve to the right without a depression of the maximal inhibition (Figures 3a and 4a). The apparent pK_B value (see Methods) of DPCPX against 2-chloroadenosine was 10.2 in the mouse and 9.7 in the rat. In contrast to DPCPX, suramin 300 μM caused little if any change of the concentration-response curve of 2-chloroadenosine in the mouse (Figure 3b) and no change whatsoever in the rat (Figure 4b). α,β-Methylene-ATP 30 μM antagonized the effect of 2-chloroadenosine in the mouse, apparently with a depression of the maximal inhibition, but not, or not significantly, in the rat (Figures 3c and 4c).

β,γ-Methylene-ATP also progressively reduced the evoked overflow of tritium when added before S_3, S_4 and S_5 at increasing concentrations (mouse: Figure 2c; rat: Figure 2f). The IC_{50} (maximal inhibition) values were 3.8 μM (48%) in mouse vas deferens slices (closed symbols in Figure 5) and 4.8 μM (84%) in rat vas deferens slices (closed symbols in

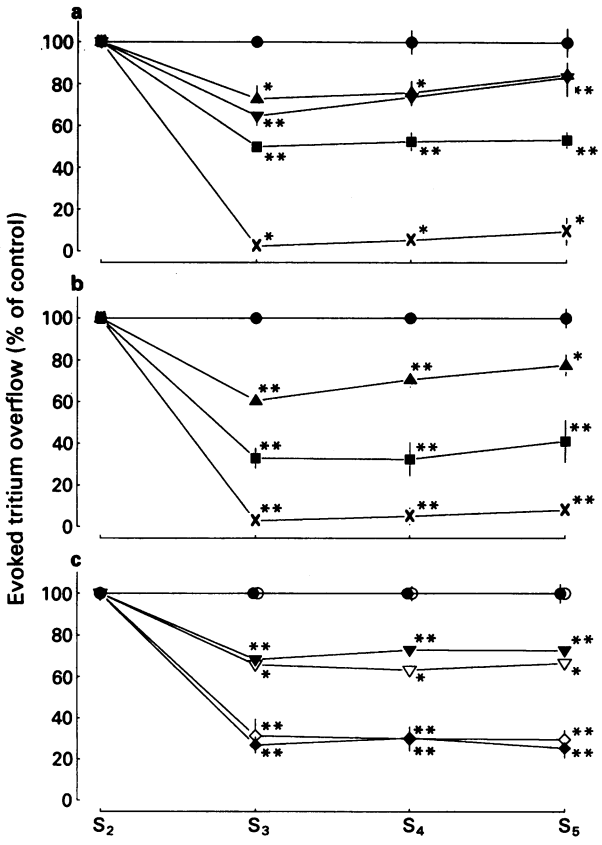


Figure 1 Effects of purinoceptor agonists and tetrodotoxin on electrically evoked overflow of tritium from slices of mouse (a) and rat vas deferens (b, c). Slices were stimulated five times by 60 pulses, 1 Hz (S_1–S_5). Solvent (●, ○), 2-chloroadenosine 30 μM (■), 2-methylthio-ATP 30 μM (▲), α,β-methylene-ATP 30 μM (▼, ▽), β,γ-methylene-ATP 30 μM (◆, ◇) or tetrodotoxin 0.5 μM (X) was added 6 min before S_3 for the remainder of the experiment. Open symbols (c) represent experiments in which the medium contained adenosine deaminase 0.3 u ml^{-1} from 36 min before S_1 onwards. Ordinates, evoked tritium overflow: S_n/S_2 ratios obtained in individual tissue slices were calculated as a percentage of the corresponding mean S_n/S_2 ratio in the appropriate control (solvent) group. Means ± s.e.mean of 4–11 experiments. Significant differences from corresponding control (solvent): *$P<0.05$ and **$P<0.01$.

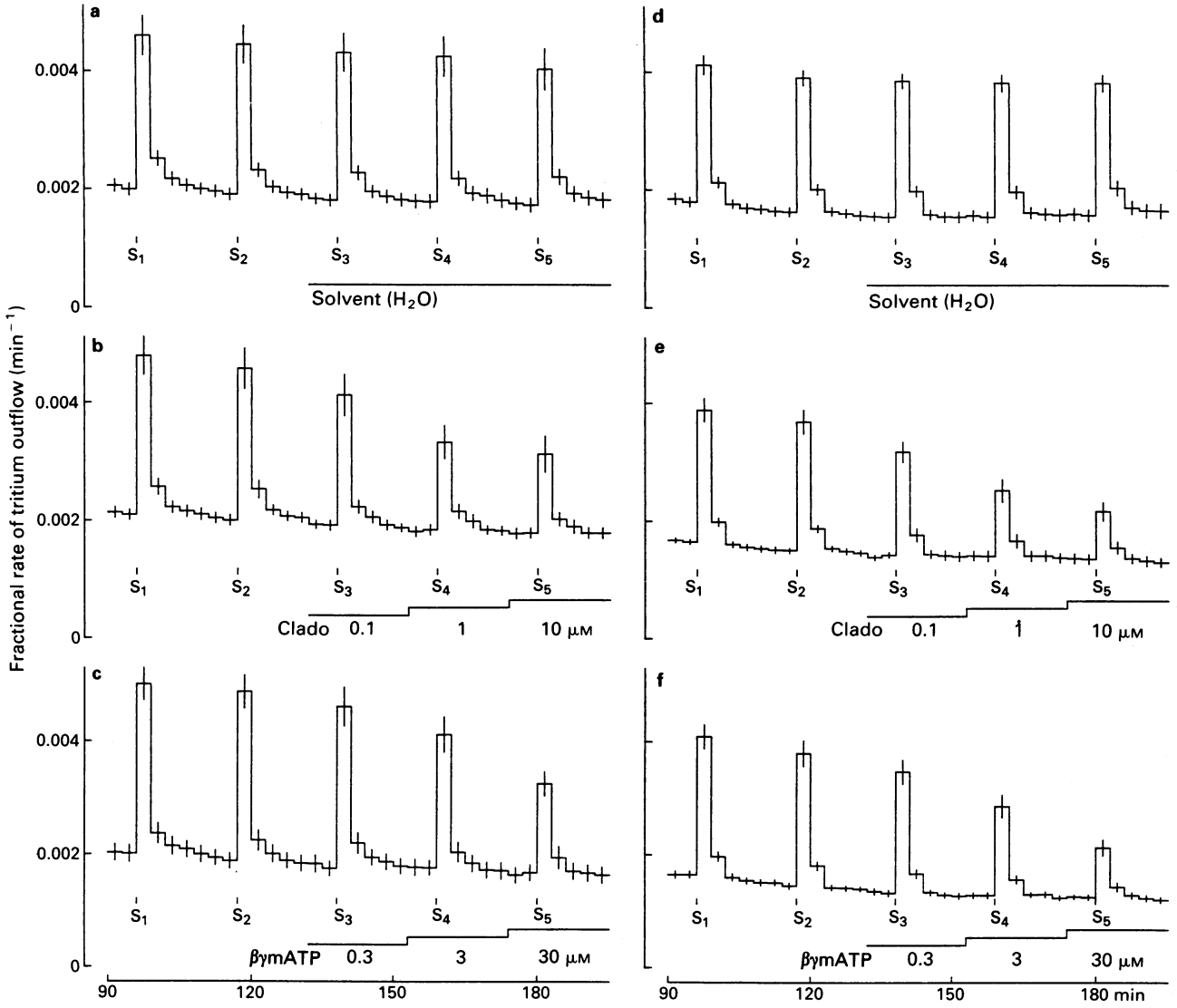


Figure 2 Time course of tritium outflow from slices of mouse (a–c) and rat (d–f) vas deferens and effects of 2-chloroadenosine (b, e, Clado) and β,γ-methylene-ATP (c, f, βγmATP). Slices were stimulated five times with 60 pulses, 1 Hz (S_{1-5}). Solvent, 2-chloroadenosine and β,γ-methylene-ATP were added as indicated. Means ± s.e.mean of 10–15 experiments.

Figure 6). As in the case of 2-chloroadenosine, DPCPX (10 nM) shifted the concentration-response curve of β,γ-methylene-ATP to the right in both species (open diamonds in Figures 5a and 6a). The apparent pK_B value (see Methods) of DPCPX against β,γ-methylene-ATP was 9.6 in both species, similar to the apparent pK_B values against 2-chloroadenosine. Suramin (300 μM) displayed greater antagonist activity against β,γ-methylene-ATP than against 2-chloroadenosine at least in the mouse: there was a clear shift of the concentration-response curve of β,γ-methylene-ATP, with an apparent pK_B of 4.5 (Figure 5b); in the rat, the antagonism was questionable (Figure 6b). α,β-Methylene-ATP (30 μM) antagonized the effect of β,γ-methylene-ATP in the mouse, but not, or not significantly, in the rat (Figures 5c and 6c) as had been the case for 2-chloroadenosine. The effect of DPCPX (10 nM) combined with suramin (300 μM) against β,γ-methylene-ATP was also examined. In the mouse, the combination caused a large shift beyond that produced by DPCPX alone (open circles in Figure 5a), approximately as large as the shift produced by suramin (300 μM) alone (Figure 5b). In the rat, there was at best a very small shift beyond that produced by DPCPX alone (open circles in Figure 6a).

Finally, concentration-response curves were determined for α,β-methylene-ATP. The IC_{50} (maximal inhibition) values were 2.9 μM (27%) in the mouse (closed symbols in Figure 7a) and 3.0 μM (33%) in the rat (closed symbols in Figure 7b). DPCPX (10 nM) abolished the effect in both species (Figure 7). In some experiments, the 60 pulses of each stimulation period were applied at 8 instead of 1 Hz. The overflow of tritium at S_2 was then 1.023% of the tritium content of the tissue in mouse vas deferens ($n = 16$; as compared with 1.009% at 1 Hz; Table 1) and 1.197% in rat vas deferens ($n = 12$; as compared with 0.774% at 1 Hz; Table 1). α,β-Methylene-ATP 3, 30 and 300 μM, when administered before S_3, S_4 and S_5, failed to change the overflow of tritium evoked by 60 pulses, 8 Hz in both mouse and rat ($n = 6-10$).

2-Chloroadenosine (Figure 2b and e), β,γ-methylene-ATP (Figure 2c and f) and α,β-methylene-ATP did not change the basal efflux of tritium (b_n/b_2) except for a decrease caused by α,β-methylene-ATP (300 μM) in the rat (not shown).

Discussion

The results indicate that the prejunctional purinoceptor mechanisms in mouse and rat vas deferens, although not identical, are similar.

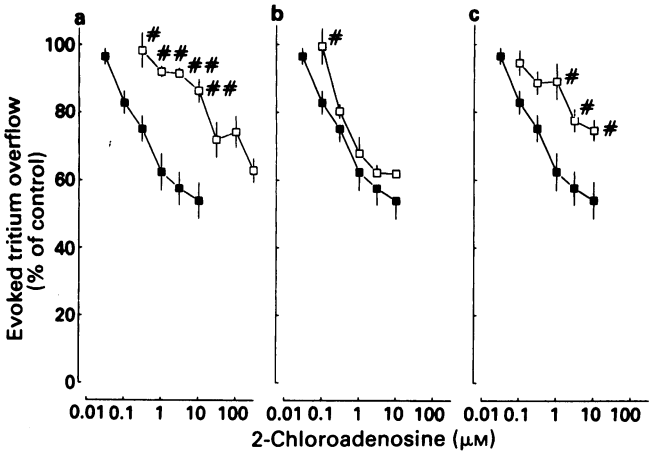


Figure 3 Interaction of 2-chloroadenosine with 8-cyclopentyl-1,3-dipropylxanthine (a, DPCPX), suramin (b) and α,β-methylene-ATP (c) on electrically evoked tritium overflow from slices of mouse vas deferens. Slices were stimulated five times with 60 pulses, 1 Hz. 2-Chloroadenosine was added at increasing concentrations from 6 min before to 15 min after the onset of S_3, S_4 and S_5. Closed symbols represent experiments in which 2-chloroadenosine was given alone. Open symbols represent experiments in which the medium contained DPCPX 10 nM (a), suramin 300 μM (b) or α,β-methylene-ATP 30 μM (c) from 36 min before S_1 onwards. Abscissae, concentration of 2-chloroadenosine. Ordinates, evoked tritium overflow: S_n/S_2 ratios obtained in individual tissue slices were calculated as a percentage of the corresponding mean S_n/S_2 ratio in the appropriate control group (solvent instead of 2-chloroadenosine). Means ± s.e.mean of 5–11 experiments. Significant differences from experiments without DPCPX, suramin and α,β-methylene-ATP: $\#P<0.05$ and $\#\#P<0.01$.

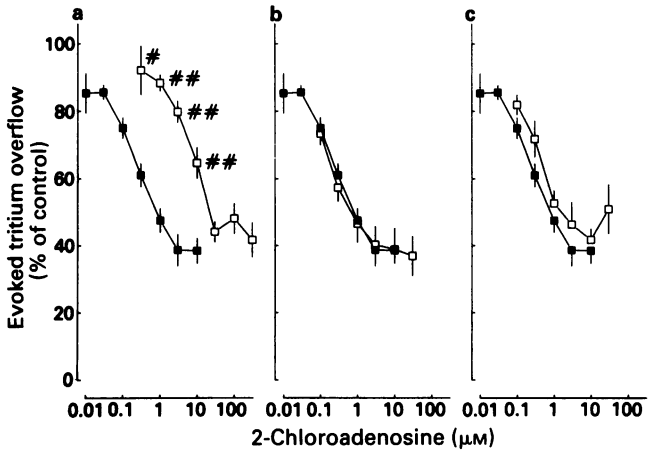


Figure 4 Interaction of 2-chloroadenosine with 8-cyclopentyl-1,3-dipropylxanthine (a, DPCPX), suramin (b) and α,β-methylene-ATP (c) on electrically evoked tritium overflow from slices of rat vas deferens. Means ± s.e.mean of 4–13 experiments. Other details as explained in legend to Figure 3.

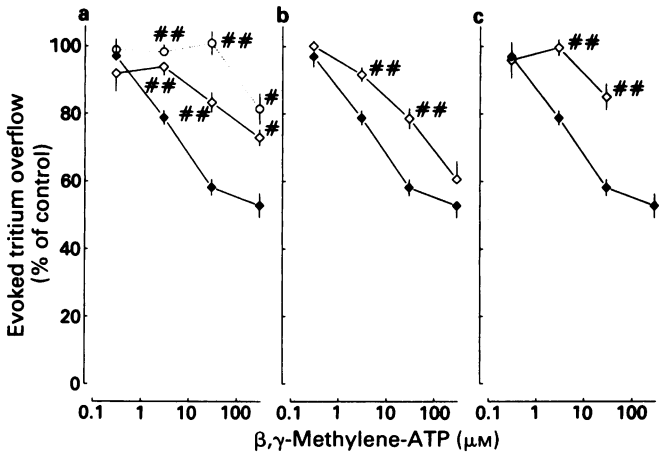


Figure 5 Interaction of β,γ-methylene-ATP with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or DPCPX combined with suramin (a), suramin (b) and α,β-methylene-ATP (c) on electrically evoked tritium overflow from slices of mouse vas deferens. Slices were stimulated five times with 60 pulses, 1 Hz. β,γ-Methylene-ATP was added at increasing concentrations from 6 min before to 15 min after the onset of S_3, S_4 and S_5. Closed symbols represent experiments in which β,γ-methylene-ATP was given alone. Open diamonds represent experiments in which the medium contained DPCPX 10 nM (a), suramin 300 μM (b) or α,β-methylene-ATP 30 μM (c) from 36 min before S_1 onwards. Open circles represent experiments in which the medium contained both DPCPX 10 nM and suramin 300 μM from 36 min before S_1 onwards (a). Abscissae, concentration of β,γ-methylene-ATP. Ordinates, evoked tritium overflow: S_n/S_2 ratios obtained in individual tissue slices were calculated as a percentage of the corresponding mean S_n/S_2 ratio in the appropriate control group (solvent instead of β,γ-methylene-ATP). Means ± s.e.mean of 5–17 experiments. Significant differences from experiments without DPCPX, suramin and α,β-methylene-ATP: $^{\#}P<0.05$ and $^{\#\#}P<0.01$.

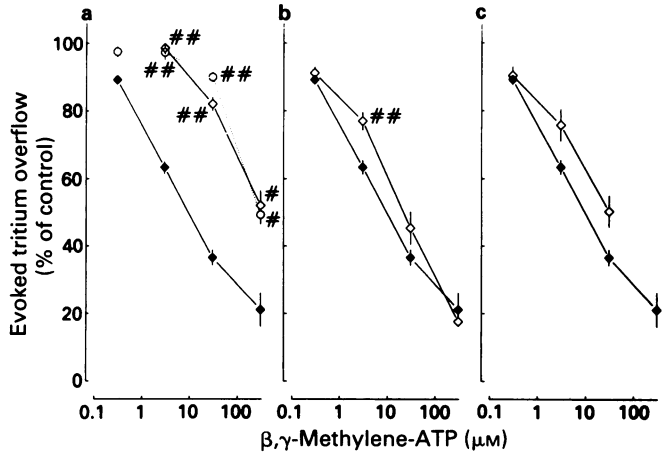


Figure 6 Interaction of β,γ-methylene-ATP with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or DPCPX combined with suramin (a), suramin (b) and α,β-methylene-ATP (c) on electrically evoked tritium overflow from slices of rat vas deferens. Means ± s.e.mean of 5–17 experiments. Other details as explained in legend to Figure 5.

Mouse vas deferens

The operation of prejunctional P_1-purinoceptors in mouse vas deferens (von Kügelgen *et al.*, 1989) is confirmed in the present study by the agonist effect of 2-chloroadenosine (see Blakeley *et al.*, 1988) and the antagonism exerted against this effect by DPCPX (Figure 3a). DPCPX is highly selective for the A_1 subtype of P_1-purinoceptor. Its apparent pK_B value against 2-chloroadenosine (10.2) is similar to values found at A_1-receptors (Bruns *et al.*, 1987; Lohse *et al.*, 1987; Sebastião *et al.*, 1990) and, hence, identifies the prejunctional receptor as A_1. DPCPX also, and with a very similar apparent pK_B value (9.6), antagonized the effect of β,γ-methylene-ATP, indicating that β,γ-methylene-ATP also acted to a great extent through the A_1-receptor (Figure 5a). The A_1 character of the receptor will be discussed further in the section on the rat vas deferens.

The results obtained with β,γ-methylene-ATP also confirm

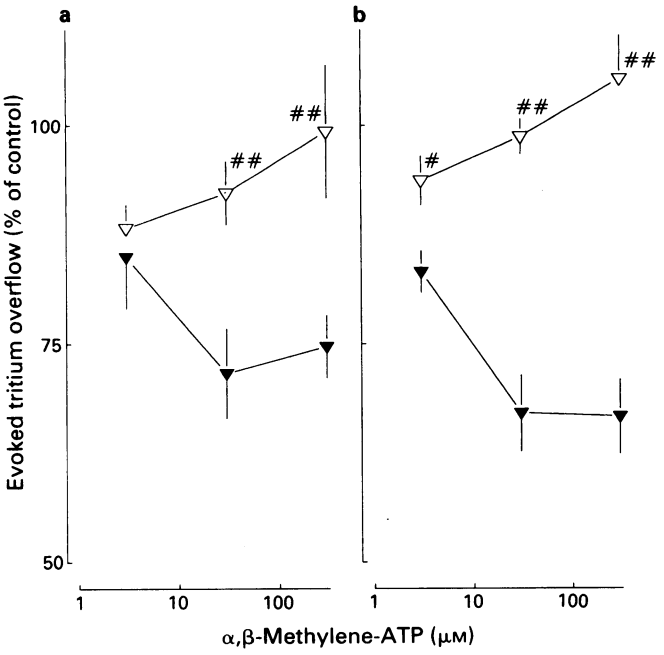


Figure 7 Interaction of α,β-methylene-ATP with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on electrically evoked tritium overflow from slices of mouse (a) and rat vas deferens (b). Slices were stimulated five times with 60 pulses, 1 Hz. α,β-Methylene-ATP was added at increasing concentrations from 6 min before to 15 min after the onset of S_3, S_4 and S_5. Closed symbols represent experiments in which α,β-methylene-ATP was given alone. Open symbols represent experiments in which the medium contained DPCPX 10 nM from 36 min before S_1 onwards. Abscissae, concentration of α,β-methylene-ATP. Ordinates, evoked tritium overflow: S_n/S_2 ratios obtained in individual tissue slices were calculated as a percentage of the corresponding mean S_n/S_2 ratio in the appropriate control group (solvent instead of α,β-methylene-ATP). Means ± s.e.mean of 5–6 experiments. Significant differences from experiments without DPCPX: $^{\#}P<0.05$ and $^{\#\#}P<0.01$.

the operation of prejunctional P_2-purinoceptors in mouse vas deferens. Under conditions that left the concentration-response curve of 2-chloroadenosine unchanged or almost unchanged (Figure 3b), the P_2 antagonist, suramin, clearly shifted the curve of β,γ-methylene-ATP to the right (Figure 5b), with an apparent pK_B value (4.5) close to that found at other P_2-purinoceptors (Hoyle *et al.*, 1990; Leff *et al.*, 1990; von Kügelgen *et al.*, 1990; Inoue *et al.*, 1991; Hourani *et al.*, 1992). A shift of similar magnitude was seen when the concentration-response curve of β,γ-methylene-ATP in the presence of suramin plus DPCPX was compared with the curve determined in the presence of DPCPX alone (Figure 5a); if DPCPX and suramin had blocked the same receptor, the combination should have caused a much smaller shift beyond that caused by DPCPX alone. It should be noted that the dual antagonism by both a xanthine and suramin against β,γ-methylene-ATP need not extend to all nucleotides. In our previous study, both 8-(*p*-sulphophenyl) theophylline and suramin antagonized the release-inhibiting effect of UTP. However, only suramin but not 8-(*p*-sulphophenyl) theophylline attenuated significantly the effect of ATPγS (von Kügelgen *et al.*, 1989); possibly the contribution of P_1-receptors, relative to the contribution of P_2-purinoceptors, is weaker in the case of ATPγS than of β,γ-methylene-ATP and UTP.

Two additional observations support the existence of prejunctional P_2-purinoceptors in mouse vas deferens. The ATP analogue 2-methylthio-ATP reduced the release of noradrenaline (Figure 1a). 2-Methylthio-ATP does not activate A_1-purinoceptors (Bailey & Hourani, 1990; von Kügelgen *et al.*, 1992a). Therefore, its effect in all likelihood was mediated by P_2-purinoceptors. Since 2-methylthio-ATP is selective for the P_{2Y} subtype, the prejunctional P_2-receptors may be P_{2Y}-like as proposed (von Kügelgen *et al.*, 1989). The second observation is the increase, by suramin, of the release of noradrenaline elicited by the second stimulation period S_2 (Table 1). Such changes must be considered with caution because this kind of study is planned to compare release *ratios* (S_n/S_2 here) rather than single stimulation periods (Wichmann *et al.*, 1989). However, suramin also increases release *ratios* when properly administered and we have shown that it does so by blockade of a release-inhibiting, negative feedback, effect of endogenous ATP at prejunctional P_2-purinoceptors (von Kügelgen *et al.*, 1993).

α,β-Methylene-ATP reduced the release of noradrenaline elicited by stimulation at 1 but not at 8 Hz, possibly due to the general inverse relationship between stimulation frequency and the extent of prejunctional modulation (e.g. Kirpekar *et al.*, 1975). Antagonism by DPCPX suggests that the effect was A_1-purinoceptor-mediated (Figure 7a) and the lower maximum in comparison with 2-chloroadenosine and β,γ-methylene-ATP suggests that α,β-methylene-ATP acted at the A_1-receptor with low efficacy. If so, partial antagonism at the A_1-receptor may have been the reason for the attenuation, by α,β-methylene-ATP, of the inhibition produced by 2-chloroadenosine and β,γ-methylene-ATP (Figures 3c, 5c).

Rat vas deferens

When the results obtained in the mouse are compared with those in the rat vas deferens, the similarity in the effects of 2-chloroadenosine (Figure 3a *versus* 4a) and β,γ-methylene-ATP (Figure 5a *versus* 6a) as well as in their interaction with DPCPX (same Figures) is striking. The inhibition of noradrenaline release in rat vas deferens by both 2-chloroadenosine and β,γ-methylene-ATP, and the attenuation of the inhibition by a xanthine derivative, confirm the findings of Westfall's group as well as their conclusion that the nucleoside and the nucleotide act at a common site (Forsyth *et al.*, 1991; see also Shinozuka *et al.*, 1988; 1990). Westfall and his colleagues suggested that the common site in the rat was a novel, P_3-purinoceptor. Here we disagree: the more complete evidence now available rather indicates a P_1 or, more specifically, A_1 character. First, the common site is blocked by 8-(*p*-sulphophenyl)theophylline, a known P_1-purinoceptor antagonist, although affinity constants of the antagonist were not determined (Shinozuka *et al.*, 1988; 1990; Forsyth *et al.*, 1991). Second, the apparent pK_B values of DPCPX against 2-chloroadenosine and β,γ-methylene-ATP in the rat, 9.7 and 9.6, on the one hand support the identity of the two agonists' sites of action, and on the other hand are very close to pK_B values of DPCPX at A_1-receptors, in support of an A_1 character of the common site, as already discussed for the mouse vas deferens. Third, a reason for the P_3 postulate was that nucleotides usually are considered to be poor agonists at P_1-purinoceptors (e.g. Forsyth *et al.*, 1991). However, there is now evidence showing that this may not be true. For example, nucleotides including β,γ-methylene-ATP potently inhibit cholinergic neuroeffector transmission in the guinea-pig ileum (Wiklund *et al.*, 1985; Wiklund & Gustafsson, 1986), contract the rat colon muscularis mucosae (Bailey & Hourani, 1990; Bailey *et al.*, 1992), inhibit the efferent function of capsaicin-sensitive neurones in guinea-pig atria (Rubino *et al.*, 1992), and inhibit the release of noradrenaline from rabbit brain noradrenergic axons (von Kügelgen *et al.*, 1992a) – all effects mediated by P_1- (A_1-) purinoceptors.

The prejunctional P_2-purinoceptor was more difficult to detect in the rat than in the mouse. Although suramin did not change the concentration-response curve of 2-chloroadenosine in the rat (Figure 4b), it also caused little, if any, shift to the right of the concentration-response curve of β,γ-methylene-ATP (Figure 6b) and produced little additional shift when it was combined with DPCPX (Figure 6a). Possibly the P_2 agonist activity of β,γ-methylene-ATP, relative to

its A_1 agonist activity, is weaker in the rat than in the mouse. As in the mouse, however, 2-methylthio-ATP reduced the release of noradrenaline in the rat vas deferens (Figure 1b), again indicating both the occurrence of prejunctional P_2-purinoceptors and their P_{2Y}-like character. Finally, as in the mouse, suramin increased the release of noradrenaline at S_2 (Table 1), and it also increased release *ratios* when properly administered, apparently by antagonism against endogenous ATP at P_2-purinoceptors (Kurz, von Kügelgen & Starke, unpublished observations).

The effect of α,β-methylene-ATP in the rat is a final example of similarity to, but not identity with, the mouse: as in the mouse, α,β-methylene-ATP reduced the release of noradrenaline at 1 but not 8 Hz, an effect blocked by DPCPX (Figure 7b) and with a lower maximum than found for 2-chloroadenosine and β,γ-methylene-ATP; in contrast to the mouse, however, α,β-methylene-ATP at best tended to attenuate the inhibition produced by 2-chloroadenosine and β,γ-methylene-ATP (Figures 4c, 6c). In the study of Forsyth *et al.* (1991), α,β-methylene-ATP did not change the release of noradrenaline in rat vas deferens but did antagonize the effect of β,γ-methylene-ATP; the difference may be due to the frequency of stimulation which was 2 Hz in Forsyth *et al.* (1991). Generally speaking, effects of α,β-methylene-ATP on the release of noradrenaline have been examined with discrepant results, from slight decrease via no change to increase (Ishikawa, 1985; Stjärne & Åstrand, 1985; Miyahara & Suzuki, 1987; von Kügelgen *et al.*, 1989; 1992a; Shinozuka *et al.*, 1990; Forsyth *et al.*, 1991; Sperlagh & Vizi, 1991). We suspected that the release-inhibiting A_1 effect of α,β-methylene-ATP might be indirect, due to release of adenosine in the vas deferens slices. However, the inhibition remained unchanged in the presence of adenosine deaminase (Figure 1) which, under similar conditions, has been shown to destroy exogenous as well as endogenous adenosine (Forsyth *et al.*, 1991; von Kügelgen *et al.*, 1992a,b). The A_1 effect of α,β-methylene-ATP, hence, seems to be direct. It should be noted, however, that α,β-methylene-ATP has little, if any, agonist or antagonist effect at other A_1-purinoceptors (Bailey & Hourani, 1990; Bailey *et al.*, 1992; von Kügelgen *et al.*, 1992a).

Conclusion

Previous studies led to different conclusions concerning prejunctional purinoceptor mechanisms in the vas deferens of the mouse and the rat (see Introduction). The present direct comparison shows, however, that the mechanisms in the two species are similar. Simple inspection of the Figures makes the similarity obvious (compare Figure 3, mouse, with Figure 4, rat; compare Figure 5, mouse, with Figure 6, rat). Specifically, the results indicate that nucleosides and nucleotides have an important common prejunctional site of action in the vas deferens of either species, as originally suggested for the rat (Forsyth *et al.*, 1991; see also Lukacsko & Blumberg, 1982; Shinozuka *et al.*, 1988; 1990). The common site is a P_1-purinoceptor; there seems to be no need to postulate a novel P_3-purinoceptor. However, the results also demonstrate a second, P_2, prejunctional purinoceptor in both species, as originally suggested for the mouse (von Kügelgen *et al.*, 1989), although the nucleotide mainly used in the present study, β,γ-methylene-ATP, acts to a smaller extent through the P_2-receptor in the rat than in the mouse.

This study was supported by the Jung-Stiftung, Hamburg, and the Deutsche Forschungsgemeinschaft (SFB 325). We thank Bayer, Wuppertal, for suramin.

References

BAILEY, S.J., HICKMAN, D. & HOURANI, S.M.O. (1992). Characterization of the P_1-purinoceptors mediating contraction of the rat colon muscularis mucosae. *Br. J. Pharmacol.*, **105**, 400–404.

BAILEY, S.J. & HOURANI, S.M.O. (1990). A study of the purinoceptors mediating contraction in the rat colon. *Br. J. Pharmacol.*, **100**, 753–756.

BLAKELEY, A.G.H., DUNN, P.M. & PETERSEN, S.A. (1988). A study of the actions of P_1-purinoceptor agonists and antagonists in the mouse vas deferens *in vitro*. *Br. J. Pharmacol.*, **94**, 37–46.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 59–63.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CLANACHAN, A.S., JOHNS, A. & PATON, D.M. (1977). Presynaptic inhibitory actions of adenine nucleotides and adenosine on neurotransmission in the rat vas deferens. *Neuroscience*, **2**, 597–602.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

ENERO, M.A. & SAIDMAN, B.Q. (1977). Possible feed-back inhibition of noradrenaline release by purine compounds. *Naunyn-Schmied. Arch. Pharmacol.*, **297**, 39–46.

FORSYTH, K.M., BJUR, R.A. & WESTFALL, D.P. (1991). Nucleotide modulation of norepinephrine release from sympathetic nerves in the rat vas deferens. *J. Pharmacol. Exp. Ther.*, **256**, 821–826.

FREDHOLM, B.B. (1974). Vascular and metabolic effects of theophylline, dibuturyl cyclic AMP and dibuturyl cyclic GMP in canine subcutaneous adipose tissue in situ. *Acta Physiol. Scand.*, **90**, 226–236.

FREDHOLM, B.B. & HEDQVIST, P. (1980). Modulation of neurotransmission by purine nucleotides and nucleosides. *Biochem. Pharmacol.*, **29**, 1635–1643.

FUDER, H., BRINK, A., MEINCKE, M. & TAUBER, U. (1992). Purinoceptor-mediated modulation by endogenous and exogenous agonists of stimulation-evoked [^{3}H]noradrenaline release on rat iris. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 417–423.

HEDQVIST, P. & FREDHOLM, B.B. (1976). Effects of adenosine on adrenergic neurotransmission; prejunctional inhibition and postjunctional enhancement. *Naunyn-Schmied. Arch. Pharmacol.*, **293**, 217–223.

HOURANI, S.M.O., HALL, D.A. & NIEMAN, C.J. (1992). Effects of the P_2-purinoceptor antagonist, suramin, on human platelet aggregation induced by adenosine 5'-diphosphate. *Br. J. Pharmacol.*, **105**, 453–457.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

INOUE, K., NAKAZAWA, K., OHARA-IMAIZUMI, M., OBAMA, T., FUJIMORI, K. & TAKANAKA, A. (1991). Selective and competitive antagonism by suramin of ATP-stimulated catecholamine-secretion from PC12 phaeochromocytoma cells. *Br. J. Pharmacol.*, **102**, 581–584.

ISHIKAWA, S. (1985). Actions of ATP and α,β-methylene ATP on neuromuscular transmission and smooth muscle membrane of the rabbit and guinea-pig mesenteric arteries. *Br. J. Pharmacol.*, **86**, 777–787.

KIRPEKAR, S.M., PRAT, J.C. & WAKADE, A.R. (1975). Effect of calcium on the relationship between frequency of stimulation and release of noradrenaline from the perfused spleen of the cat. *Naunyn-Schmied. Arch. Pharmacol.*, **287**, 205–212.

KURZ, K., VON KÜGELGEN, I. & STARKE, K. (1993). Purinoceptors modulating release of [^{3}H]-noradrenaline in mouse and rat vas deferens – evidence for a P_2-receptor-mediated autoinhibition. *Naunyn-Schmied. Arch. Pharmacol.*, **347**, R120.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly-equilibrating but competitive antagonist at P_{2X}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645–649.

LIMBERGER, N., TRENDELENBURG, A.U. & STARKE, K. (1992). Pharmacological characterization of presynaptic α_2-autoreceptors in rat submaxillary gland and heart atrium. *Br. J. Pharmacol.*, **107**, 246–255.

LOHSE, M.J., KLOTZ, K.N., LINDENBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **336**, 204–210.

LUKACSKO, P. & BLUMBERG, A. (1982). Modulation of the vasoconstrictor response to adrenergic stimulation by nucleosides and nucleotides. *J. Pharmacol. Exp. Ther.*, **222**, 344–349.

MIYAHARA, H. & SUZUKI, H. (1987). Pre- and post-junctional effects of adenosine triphosphate on noradrenergic transmission in the rabbit ear artery. *J. Physiol.*, **389**, 423–440.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoceptors. *Physiol. Rev.*, **70**, 761–845.

RUBINO, A., AMERINI, S., LEDDA, F. & MANTELLI, L. (1992). ATP modulates the efferent function of capsaicin-sensitive neurones in guinea-pig isolated atria. *Br. J. Pharmacol.*, **105**, 516–520.

SEBASTIÃO, A.M., STONE, T.W. & RIBERIO, J.A. (1990). The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines. *Br. J. Pharmacol.*, **101**, 453–459.

SHINOZUKA, K., BJUR, R.A. & WESTFALL, D.P. (1988). Characterization of prejunctional purinoceptors on adrenergic nerves of the rat caudal artery. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 221–227.

SHINOZUKA, K., BJUR, R.A. & WESTFALL, D.P. (1990). Effects of α,β-methylene ATP on the prejunctional purinoceptors of the sympathetic nerves of the rat caudal artery. *J. Pharmacol. Exp. Ther.*, **254**, 900–904.

SPERLAGH, B. & VIZI, E.S. (1991). Effect of presynaptic P_2 receptor stimulation on transmitter release. *J. Neurochem.*, **56**, 1466–1470.

STJÄRNE, L. & ÅSTRAND, P. (1985). Relative pre- and postjunctional roles of noradrenaline and adenosine 5′-triphosphate as neurotransmitters of the sympathetic nerves of guinea-pig and mouse vas deferens. *Neuroscience*, **14**, 929–946.

STONE, T.W. (1981). Physiological roles for adenosine and adenosine 5′-triphosphate in the nervous system. *Neuroscience*, **6**, 523–555.

STONE, T.W. (1985). The activity of phosphorothioate analogues of ATP in various smooth muscle systems. *Br. J. Pharmacol.*, **84**, 165–173.

TAYLOR, D.A., WIESE, S., FAISON, E.P. & YARBROUGH, G.G. (1983). Pharmacological characterization of purinergic receptors in the rat vas deferens. *J. Pharmacol. Exp. Ther.*, **224**, 40–45.

VON KÜGELGEN, I., BÜLTMANN, R. & STARKE, K. (1990). Interaction of adenine nucleotides, UTP and suramin in mouse vas deferens: suramin-sensitive and suramin-insensitive components in the contractile effect of ATP. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 198–205.

VON KÜGELGEN, I., KURZ, K. & STARKE, K. (1993). Axon terminal P_2-purinoceptors in feedback control of sympathetic transmitter release. *Neuroscience*, **56**, 263–267.

VON KÜGELGEN, I., SCHÖFFEL, E. & STARKE, K. (1989). Inhibition by nucleotides acting at presynaptic P_2-receptors of sympathetic neuro-effector transmission in the mouse isolated vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 522–532.

VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992a). Stable adenine nucleotides inhibit [^{3}H]-noradrenaline release in rabbit brain cortex slices by direct action at presynaptic adenosine A_1-receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 187–196.

VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992b). Adenosine but not an adenine nucleotide mediates tonic purinergic inhibition, as well as inhibition by glutamate, of noradrenaline release in rabbit brain cortex slices. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 677–684.

WAUD, D.R. (1976). Analysis of dose-response relationships. In *Advances in General and Cellular Pharmacology*. ed. Narahashi, T. & Bianchi, C.P. pp. 145–178. New York: Plenum.

WICHMANN, T., LIMBERGER, N. & STARKE, K. (1989). Release and modulation of release of serotonin in rabbit superior colliculus. *Neuroscience*, **32**, 141–151.

WIKLUND, N.P. & GUSTAFSSON, L.E. (1986). Neuromodulation by adenine nucleotides, as indicated by experiments with inhibitors of nucleotide inactivation. *Acta Physiol. Scand.*, **126**, 217–223.

WIKLUND, N.P., GUSTAFSSON, L.E. & LUNDIN, J. (1985). Pre- and postjunctional modulation by cholinergic neuroeffector transmission by adenine nucleotides. Experiments with agonist and antagonist. *Acta Physiol. Scand.*, **125**, 681–691.

(Received July 7, 1993
Revised August 18, 1993
Accepted August 27, 1993)

Br. J. Pharmacol. (1993), **110**, 1473–1478 © Macmillan Press Ltd, 1993

EDRF release from canine coronary artery by lectins

Joseph F. Kleha, [1]Pierre Devesly & [2]Anthony Johns

Cardiovascular Research, Berlex Biosciences, Richmond, California 94804, U.S.A.

1 The effect of wheat germ agglutinin (WGA), concanavalin A (Con A) and lentil lectin agglutinin (LCA) were investigated on pre-contracted canine coronary artery rings *in vitro*.

2 In endothelium-intact canine coronary artery, contracted with the thromboxane A_2-analogue, U46619, WGA relaxed the tissue in a concentration-dependent manner, with an inhibitory concentration (IC_{50}) of 112 ± 17 nM ($n = 6$). In the absence of an endothelium, WGA did not cause any relaxation of the tissue.

3 In endothelium-intact canine coronary artery, contracted with the thromboxane A_2-analogue, U46619, LCA relaxed the tissue in a concentration-dependent manner, with an inhibitory concentration (IC_{50}) of 423.1 ± 41 nM ($n = 6$). In the absence of an endothelium, LCA produced a 20.1 ± 1.1% ($n = 6$) relaxation at the highest concentration tested (3 μM).

4 Concanavalin A (Con A) relaxed canine coronary artery in a partial endothelium-dependent manner with an IC_{50} of 104 ± 19 nM on endothelium-intact coronary artery and an IC_{50} of 1.3 ± 0.3 μM ($n = 6$) on endothelium-denuded tissues.

5 The relaxation effects of WGA were attenuated by 1 mM N^G-monomethyl L-arginine (L-NMMA) and completely inhibited by haemoglobin (3 μM), methylene blue (10 μM) and LY 83583 (30 μM). Ibuprofen had no effect on WGA-induced relaxation.

6 The relaxant effects of WGA were reversed by addition of 20 mM N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-galactosamine (GalNAc) but not by α-mannose, D-(+)-galactose, and β-lactose, whereas the endothelin-dependent relaxations to LCA and Con A were unaffected.

7 The endothelium-dependent relaxation induced by the lectins was unaffected by pretreatment of the tissue with 1 μM atropine.

8 In the absence of extracellular calcium, WGA was also able to release EDRF suggesting that WGA acts through a second messenger system to release intracellular calcium.

9 We suggest that WGA acts as an agonist to release EDRF from endothelial cells possibly by binding to a sugar moiety, specific receptor or adhesion molecules on the endothelial cell surface.

Keywords: EDRF; nitric oxide; endothelium; lectins; wheat germ agglutinin; calcium; canine coronary artery

Introduction

Lectins have been used to characterize and label the vascular endothelial cells without labelling the underlying smooth muscle cells (Raub *et al.*, 1990). Lectins have the ability to bind to saccharide groups of glycoproteins with this binding being reversed by specific sugars (Grulich-Henn *et al.*, 1988) and have recently been shown to be important in mediating cell to cell interactions by regulating biological processes in living organisms (Sharon & Lis, 1989). The characterization of cell surface glycoproteins including growth factor receptors (Sinnett-Smith *et al.*, 1988), and cell adhesion molecules on endothelial cells (Walz *et al.*, 1990) have also used the unique properties of different lectins.

Endothelium-derived relaxing factor (EDRF), a very labile compound with a rapid biological action is released by the vascular endothelium in response to some vasodilators (Furchgott, 1984). EDRF has been shown to be indistinguishable from nitric oxide (NO) with respect to biological activity (Palmer *et al.*, 1987). Vascular responses to EDRF are similar to the responses of nitrovasodilators in that both stimulate guanylate cyclase, which, by increasing smooth muscle guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels, causes smooth muscle relaxation (Furchgott, 1984). This endothelium-dependent vascular relaxation is effectively blocked by haemoglobin, methylene blue, (Martin *et al.*, 1985), N^G-monomethyl-L-arginine (L-NMMA) (Rees *et al.*, 1989) and LY 83583 (6-anilino-5,8-quinolinedione) (Mulsch *et al.*, 1988). In cultured endothelial cells, the stimulation of the same receptors causes a coupled release of EDRF and prostacyclin (PGI_2) which results in vasodilatation (De Nucci *et al.*, 1988). Thrombin stimulates prostacyclin (PGI_2) synthesis in human umbilical vein endothelial cells (HUVEC) (Weksler *et al.*, 1978) and it has been shown that wheat germ agglutinin (WGA) blocks thrombin-induced stimulation of $[Ca^{2+}]_i$, [^{3}H]-arachidonate release, and prostacyclin production in cultured HUVECs (Grulich-Henn *et al.*, 1988). We have suggested that, in the rabbit aorta, WGA binds to a receptor or a cell surface glycoprotein which, via the release of EDRF from the endothelial cell, causes smooth muscle relaxation (Kleha *et al.*, 1991). The purpose of the present investigation was to determine, in more detail, the mechanism(s) involved in the release of EDRF by WGA (Kleha *et al.*, 1991). Lentil lectin agglutinin (LCA) and concanavalin A (ConA) were also investigated to determine if other lectins, with different sugar specificities, caused similar effects to wheat germ agglutinin on vascular tissue.

Methods

Isometric muscle tension bath system

Mongrel dogs were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}) by intravenous injection into the saphenous vein. The heart and carotid arteries were excised and placed in cooled HEPES buffered physiological salt solution (PSS). The PSS had the following composition (mM): NaCl 140, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, D-glucose 10, N-[2-

[1] Present address: Berlex Laboratories, 300 Fairfield Road, Wayne, New Jersey, 07470-7358, U.S.A.
[2] Author for correspondence.

hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (HEPES) 2.7, sodium N-[2-hydroxyethyl] piperazine-N'-[2-thanessulfonic acid] (Na HEPES) 2.3, ethylenediamine-tetraacetic acid (EDTA) 10 μM and ibuprofen 10 μM. This solution had a pH 7.4 at 37°C when bubbled with air and is identical to that used previously to determine the pharmacological, biochemical and electrophysiological characteristics of cultured endothelial cells (Johns et al., 1987; Freay et al., 1989; Laskey et al., 1990). The circumflex branch of the coronary artery was isolated and any traces of adhering fascia removed by careful dissection. Rings of approximately 3 mm in length were used with the endothelium layer intact or the endothelium was removed by gently rubbing the intimal surface of the blood vessel with a wooden toothpick.

Tissues were suspended in 5 ml isometric muscle tension baths under 4 g of tension in PSS bubbled with room air at 37°C. The tension was measured with a force transducer (Grass FT03C) and displayed on a Beckman polygraph (R-611). The vessels were allowed to equilibrate for 2 h, during which time the maximum contractile response was measured by the addition of 40 mM KCl every 30 min. A constant basal tension of 4 g was maintained during this period. The presence of an endothelial cell layer was confirmed by the addition of 300 nM acetylcholine (ACh), which is known to cause a maximum relaxation (DeMey & Vanhoutte, 1981), following development of a maintained contraction to added KCl (20 mM).

In order to determine the effects of the different lectins the canine coronary artery was contracted with 50 nM of the thromboxane A_2-analogue, U46619. After a sustained contraction developed, concentration-response curves to the lectins were constructed.

Calcium-free (EGTA) experiments

Endothelium-intact and endothelium-denuded canine coronary arteries were prepared as described above. After equilibration, the PSS was replaced with calcium-free PSS containing 2 mM EGTA. The calcium-free PSS (2 mM EGTA) solution was prepared by substitution of calcium chloride with 2 mM of the sodium salt of ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA). After incubation in this solution for 2 min the tissues were contracted by addition of endothelin-1 (final concentration 30 nM). After a further 7 min, WGA was added to the baths (final concentration 900 nM). After the solution bathing the tissues had been replaced with the calcium-free (2 mM EGTA) PSS the tissues were not again exposed to calcium containing PSS. Data are expressed as percentage relaxation of endothelin-1 contraction and shown as mean ± s.e.mean.

Superfusion bioassay

The bioassay tissue (endothelium-denuded canine coronary artery) was mounted between two stainless steel hooks under a resting tension of 3 g. The tissue was superfused with prewarmed, aerated (37°C) PSS at a rate of 4 ml min^{-1}. The tissue was allowed to equilibrate for 2 h during which time responses to addition of 40 mM KCl to the PSS were determined. The absence of a functional endothelium on the canine coronary artery was confirmed by the lack of a relaxation of the bioassay tissue to acetylcholine (300 nM) following a 20 mM KCl-induced contraction.

Canine carotid arteries were mounted on stainless steel cannulae in a jacketed organ bath. The carotid artery (donor tissue) was perfused with PSS at a rate of 4 ml min^{-1} at 37°C. The bioassay tissue was contracted by changing the solution perfusing the donor to one containing 10 nM U46619. Basal release of EDRF was measured by moving the canine carotid artery (donor) over the canine coronary artery and allowing the perfusate to superfuse the bioassay tissue. Stimulated release of EDRF was measured by perfusing the carotid artery with a solution containing 900 nM WGA and 10 nM U46619 in PSS and allowing the perfusate to superfuse the bioassay tissue. Data are expressed as percentage relaxation of thromboxane A_2-analogue U46619 and shown as mean ± s.e.mean.

Drugs

All the chemicals used were of analytical grade and obtained from Sigma Chemical Co., St Louis, MO., U.S.A. The following drugs were used: N-acetyl-D-galactosamine (GalNAc, Sigma Chemical Co., St Louis, MO., U.S.A.), N-acetyl-D-glucosamine (GlcNAc, Sigma Chemical Co., St Louis, MO., U.S.A.), acetylcholine chloride (ACh, Sigma Chemical Co., St Louis, MO., U.S.A.), 6-anilino-5,8-quinolinedione (LY 83583, Lilly, U.S.A.), atropine sulphate (ICN, U.S.A.), concanavalin A lectin (Con A, Boehringer Mannheim Biochemicals, U.S.A.), 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619, Sigma Chemical Co., St Louis, MO., U.S.A.), endothelin (ET-1, Mid-West Biotech, U.S.A.), D-(+)-galactose (Sigma Chemical Co., St Louis, MO., U.S.A.), haemoglobin (prepared as described by Martin et al., 1985) (Hb, Sigma Chemical Co., St Louis, MO., U.S.A.), ibuprofen, free acid (Ibu, Sigma Chemical Co., St Louis, MO., U.S.A.), β-lactose (Sigma Chemical Co., St Louis, MO., U.S.A.), *Lens culinaris* (Lentil) agglutinin lectin (LCA, Boehringer Mannheim Biochemicals, U.S.A.), α-mannose (Sigma Chemical Co., St Louis, MO., U.S.A.), N^G monomethyl-L-arginine acetate (L-NMMA, Sigma Chemical Co., St Louis, MO., U.S.A.), methylene blue (Mb, Sigma Chemical Co., St Louis, MO., U.S.A.), wheat germ agglutinin lectin (WGA, Boehringer Mannheim Biochemicals, U.S.A.).

Statistical analysis

Results are expressed as mean ± standard error of the mean (s.e.mean). Statistical significance between data points was tested by Student's unpaired *t* test. Probability of $P<0.05$ was considered significant and indicated as *.

Results

The effects of WGA on endothelium-intact and endothelium-denuded canine coronary arteries are shown in Figure 1. Representative tension recordings of paired canine coronary arteries are shown in Figure 1a. WGA was added to the tissue preparations in a concentration-dependent manner from 30 nM up to 900 nM as described in Methods. WGA caused endothelium-dependent relaxations with a half maximal inhibitory concentration (IC_{50}) of 112 ± 17 nM ($n = 6$). No vasorelaxation occurred in the endothelium-denuded preparations as shown in Figure 1b ($n = 6$).

Concentration-dependent responses to the lectin LCA on endothelium-intact and endothelium-denuded preparations of canine coronary artery are shown in Figure 2. In six experiments, LCA caused the endothelium-intact tissues to relax with the maximum concentration (3 μM) yielding a 101.2 ± 3.5% relaxation and an IC_{50} of 423.1 ± 41 nM. LCA caused the endothelium-denuded tissue to relax 20.1 ± 1.1% at the maximum concentration tested (3 μM) but did not reach an IC_{50}.

The lectin, Con A, caused both endothelium-intact and endothelium-denuded canine coronary artery preparations to relax, however the extent of vasodilatation was more pronounced in the endothelium-intact preparations. Concentration-dependent responses to Con A on canine coronary arteries with and without endothelium are shown in Figure 3. At the maximum concentration tested (1.4 μM) on endothelium-intact preparations, Con A gave a relaxation response of 102 ± 3.3% with an IC_{50} of 104 ± 19 nM as compared to a 61.8 ± 7.9% on endothelium-denuded tissues ($n = 6$).

Figure 4 demonstrates the stimulated release of EDRF

from the endothelium induced by WGA. The effects of WGA were specifically inhibited by haemoglobin, methylene blue, and LY 83583. The recovery response of 10 μM haemoglobin on WGA-induced relaxations is shown in Figure 4a. In 6 experiments, haemoglobin caused a 114.9 ± 6.5% recovery when compared to the U46619 (50 nM)-induced contraction. Methylene blue at 3 μM reversed the WGA-induced relaxations by 89.7 ± 10.5% ($n = 6$), with a representative tracing shown in Figure 4b. Figure 4c shows a representative trace of the effects of the guanylate cyclase inhibitor, LY 83583, at a concentration of 30 μM. LY 83583 produced a 94.9 ± 6.7% reversal of the WGA-induced relaxation ($n = 6$) of paired canine coronary arteries. WGA binds specifically to N-acetyl-D-glucosamine moieties with a high affinity. To show the effects of WGA were due to the lectin effect and not a possible contaminant in the solution, N-acetyl-D-glucosamine was added to the bath after WGA-induced relaxation. The specific effect of GlcNAc on relaxations induced by WGA is

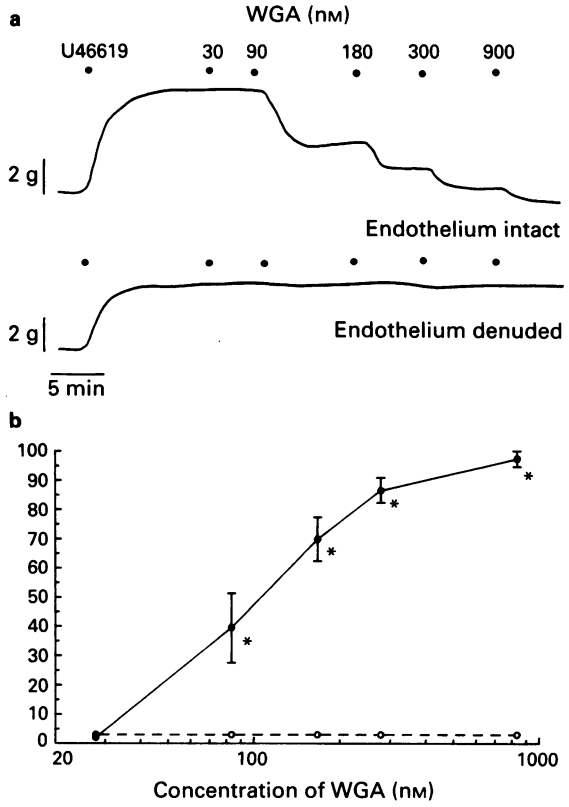


Figure 1 (a) The effects of wheat germ agglutinin (WGA) on canine coronary artry. Endothelium-intact and denuded rings of canine coronary arteries were contracted with 50 nM U46619. Concentration-responses to WGA were performed in the following sequence: 30 nM, 90 nM, 180 nM, 300 nM, 900 nM. Representative traces of coronary artery rings showing endothelium-dependent relaxations to wheat germ agglutinin (WGA) in a concentration-dependent manner. (b) The effects of different concentrations of WGA on rings of canine coronary artery in the presence (●) and absence (○) of an endothelium. The values shown are mean ± s.e.mean relaxations of a 50 nM U46619 contraction (6 experiments). All relaxations induced by concentrations of WGA above 30 nM were significantly ($P < 0.05$) different and indicated as (*).

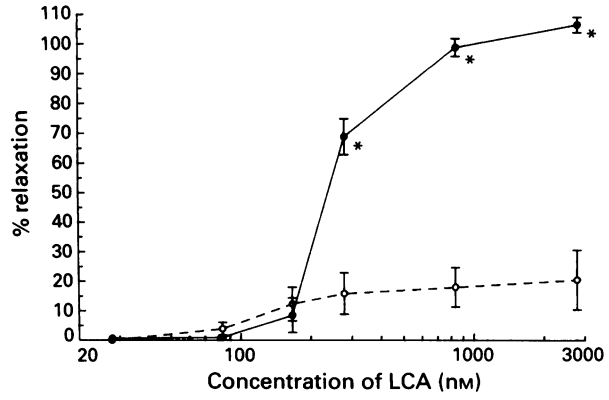


Figure 2 The effects of different concentrations of lentil (*Lens culinaris*) agglutinin (LCA) on coronary artery rings in the presence (●) and absence (○) of an endothelium. The values shown are mean ± s.e.mean relaxations of a 50 nM U46619-induced contraction (6 experiments). Significantly ($P < 0.05$) different points are indicated (*).

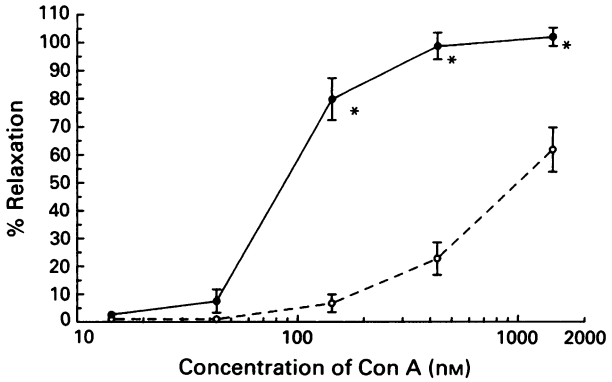


Figure 3 The effects of different concentrations of concanavalin (Con A) on canine coronary artery rings in the presence (●) and absence (○) of endothelium. The values shown are mean ± s.e.mean relaxations of a 50 nM U46619-induced contraction (6 experiments). Significantly ($P < 0.05$) different points are indicated (*).

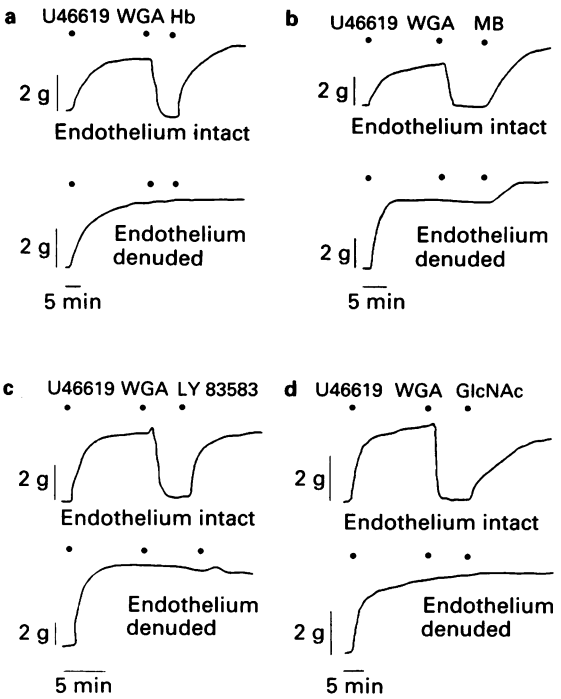


Figure 4 The effects of haemoglobin (Hb), methylene blue (MB), LY 83583, and N-acetyl-D-glucosamine (GlcNAc) on relaxations induced by wheat germ agglutinin (WGA) of the canine coronary artery. Endothelium-intact and denuded coronary arteries were contracted with 50 nM U46619, and the response to 900 nM WGA was measured. Representative isometric tension recordings of the inhibitory effects of 10 μM bovine haemoglobin, 3 μM methylene blue and 3 μM LY 83583 are shown in (a), (b) and (c) respectively. The effects of GlcNAc on the WGA-induced relaxation is shown in (d).

shown in Figure 4d. The sugar at 20 mM reversed the relaxation by 98.7 ± 2.8% ($n = 6$).

The EDRF/NO synthase inhibitor L-NMMA (1 mM) was preincubated with endothelium-intact canine coronary arteries for 5 min before responses to increasing concentrations of WGA were measured. Figure 5 shows that 1 mM L-NMMA decreased the sensitivity of the tissue to relaxations induced by WGA. The IC_{50} for the concentration-response curve to WGA in the presence of 1 mM L-NMMA was 341 ± 115 nM and is shown in Figure 5a. In the absence of L-NMMA, the IC_{50} for WGA was 112 ± 17 nM. There was a significant shift in the concentration-response curve in the presence of 1 mM L-NMMA illustrating that L-NMMA, by inhibiting nitric oxide synthase (known to be important for EDRF synthesis) is attenuating the WGA-induced relaxation (Figure 5a). Figure 5b demonstrates the effects of L-NMMA on responses of the canine coronary artery to acetylcholine. These experiments illustrate that the acetylcholine-induced relaxation of the dog coronary artery is also significantly attenuated by 1 mM L-NMMA.

The effects of WGA on canine carotid arteries in the cascade superfusion bioassay system using endothelium-denuded canine coronary artery as the bioassay tissue and canine carotid artery as the donor were investigated. Endothelium-denuded canine coronary artery was contracted with 10 nM U46619. After a sustained contraction was measured, basal EDRF was then measured after which the canine carotid artery was perfused with 900 nM WGA and a stimulated relaxation was measured. A representative trace is shown in Figure 6a. Comparison of basal EDRF release from canine carotid arteries with WGA stimulated EDRF release plus basal EDRF release is shown in Figure 6b. In 6 experiments, basal EDRF relaxations from carotid arteries measured 51.9 ± 8.7% when compared to the 10 nM U46619-induced contraction, while the WGA-stimulated release of EDRF plus basal EDRF release was measured at 82.1 ± 4.4% resulting in an overall WGA stimulation of relaxation of 28.6 ± 5.5% as compared to a 10 nM U46619 contraction. This further demonstrates the stimulated release of EDRF by WGA.

Atropine effectively inhibits vasodilatation induced by acetylcholine in rabbit aorta precontracted with noradrenaline (Furchgott & Zawadzki, 1980). The effects of atropine on the response of canine coronary arteries to both acetylcholine and WGA were investigated. Edothelium-intact and denuded coronary arteries were contracted with 50 nM of the thromboxane A_2-analogue, U46619. After a sustained contraction was obtained, 1 μM atropine was incubated with the tissue for 5 min after which 300 nM acetylcholine was added. After blockade of the acetylcholine response by 1 μM atropine, a response to 900 nM WGA was measured. There was no relaxation response to 300 nM acetycholine in the presence of 1 μM atropine (0 ± 0%, $n = 6$). The vasodilator response to 900 nM WGA in the presence of 1 μM atropine plus 300 nM acetylcholine was 97.6 ± 2.4% ($n = 6$) demonstrating that WGA does not cause vasodilatation by an effect

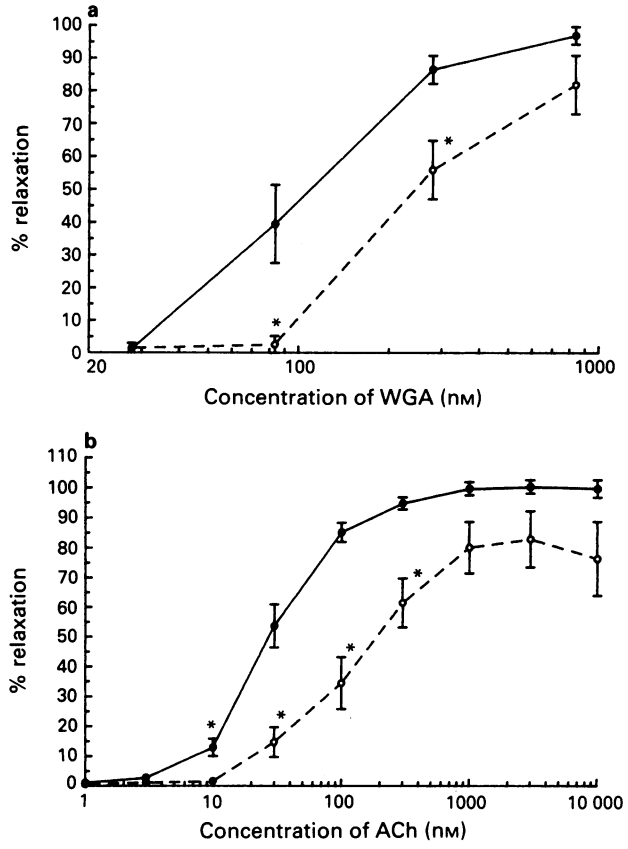


Figure 5 (a) The effects of N^G-monomethyl-L-arginine (L-NMMA) on the response of canine coronary artery to wheat germ agglutinin (WGA). Endothelium-intact canine coronary arteries were pretreated with 1 mM L-NMMA for 5 min before a contraction to 50 nM U46619 was measured, after which a concentration-response curve to WGA was obtained. Curves shown are endothelium-intact coronary artery in the presence (○) and absence (●) of 1 mM L-NMMA. Data are expressed as mean ± s.e.mean relaxations, (6 experiments). (b) The effects of L-NMMA (1 mM) on the response of the canine coronary artery to acetylcholine (ACh). Endothelium-intact canine coronary arteries were pretreated with 1 mM L-NMMA for 5 min before a concentration-response curve to acetylcholine was obtained. Curves shown are endothelium-intact coronary artery in the presence (○) and absence (●) of 1 mM L-NMMA. Data are expressed as mean ± s.e.mean relaxations (6 experiments). Significantly ($P < 0.05$) different points are indicated (*).

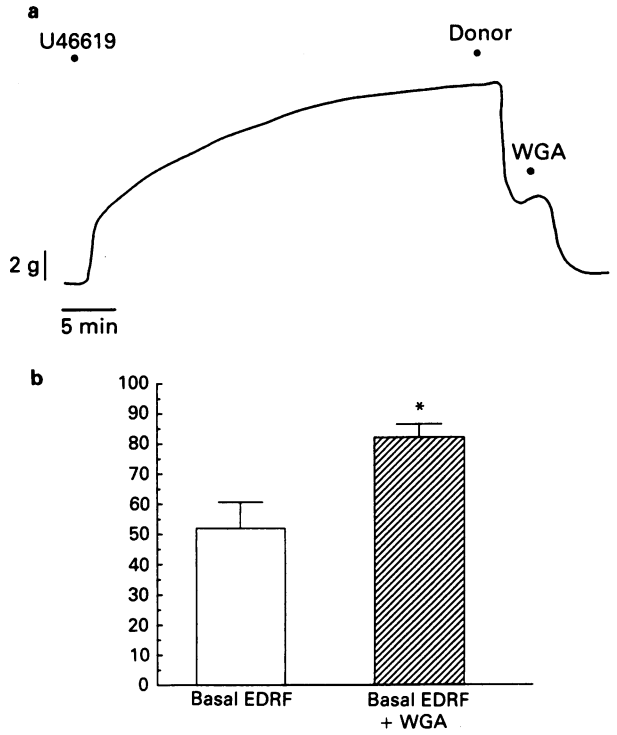


Figure 6 The stimulated release of EDRF from perfused canine carotid arteries by wheat germ agglutinin (WGA). Representative tracing of an endothelium-denuded canine coronary artery (bioassay tissue) superfused with effluent from a perfused canine carotid artery showing basal EDRF and WGA stimulated EDRF is shown in (a). Graph comparing basal release of EDRF with stimulated release by 900 nM WGA is shown in (b). The values shown are mean (with s.e.mean) relaxations of tissues contracted with 10 nM U46619 (6 experiments). Significantly ($P < 0.05$) different points are indicated (*).

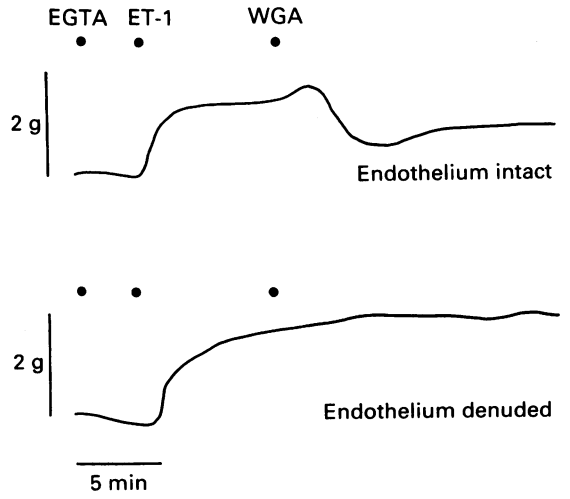


Figure 7 The relaxation effects of wheat germ agglutinin (WGA) on canine coronary artery in the absence of extracellular calcium. Endothelium-intact and endothelium-denuded arteries were contracted with 30 nM endothelin-1 in calcium-free, 2 mM EGTA PSS. A significant relaxation to 900 nM WGA was observed in endothelium-intact tissues with a representative tracing shown in (a). Only a slight relaxation was measured in endothelium-denuded tissues and is shown in (b).

on muscarinic acetylcholine receptors to stimulate EDRF release.

In order to test the possibility that WGA was acting on the thrombin receptor a series of 5 experiments was performed on tissues in which tachyphylaxis to thrombin had been induced by repetitive exposure. In tissues contracted with U46619, thrombin (2 iu ml^{-1}) was added three times which caused 100.9 ± 4.0%, 75.1 ± 10.0% and 45.2 ± 5.8% relaxations respectively. In matched tissues the first two relaxations to thrombin were not significantly different from the control tissue (105.4 ± 3.2% and 64.4 ± 14.3%) whereas the third response to 900 nM WGA (117.1 ± 18.4%) was significantly different from the thrombin response ($P > 0.05$).

Representative traces of the effects of WGA on canine coronary arteries with the endothelium-intact and endothelium-denuded in 2 mM EGTA calcium-free PSS are shown in Figure 7. In 5 experiments, 900 nM WGA caused an 80.6 ± 3.4% relaxation in endothelium-intact canine coronary arteries and a 17.2 ± 5.5% relaxation in endothelium-denuded arteries. In comparison, 900 nM WGA relaxed endothelium-intact canine coronary arteries 98.9 ± 2.7% in PSS containing 1.5 mM calcium chloride but there were no relaxations measured in endothelium-denuded canine coronary arteries.

Discussion

Wheat germ agglutinin is a pure protein with a molecular weight of 36,000 and is composed of two similar peptide chains with two polypeptide chains that contain sugar binding sites (Nagata & Burger, 1974). Most lectins have been used to characterize cell types and have been used extensively to isolate and partially purify many proteins including receptors for hormones, neurotransmitters, growth factors and immunoglobins (Hedo, 1984) along with adhesion molecules such as the endothelial leukocyte adhesion molecule-1 (ELAM-1) (Walz *et al.*, 1990). We have previously shown that WGA stimulates the relase of EDRF from rabbit aorta in a concentration-dependent manner (Kleha *et al.*, 1991).

The endothelium-dependent vasodilatation induced by WGA in canine coronary artery was inhibited by haemoglobin, methylene blue, LY 83583, and L-NMMA, all of which are known to inhibit pathways that lead to EDRF/NO-induced relaxation (Martin *et al.*, 1985; Mulsch *et al.*, 1988; Rees *et al.*, 1989). The endothelium-dependent relaxations were observed in the presence of ibuprofen suggesting that the relaxations are not due to the release of prostacyclin. The relaxations to WGA were specifically reversed by the sugar N-acetyl-D-glucosamine; however, this sugar had no effect on the partial endothelium-dependent relaxation of the dog coronary artery to Con A. The vasodilator effect of WGA was shown to be unaffected by atropine (known to inhibit acetylcholine-induced relaxation of rabbit aorta; Furchgott & Zawadzki, 1980) suggesting that WGA was not working on the ACh muscarinic receptor and probably causing the vasodilator response by acting on its own specific receptor.

The possibility that WGA may act as an agonist on the thrombin receptor was also tested. It was demonstrated that WGA was still able to produce full relaxation of tissues when the vasodilator response to thrombin had been reduced substantially by repeated exposure to thrombin.

The endothelium-dependent relaxations induced by WGA and LCA along with the partial endothelium-dependent relaxations induced by Con A on canine coronary artery demonstrate that there might be different lectin binding molecules present on the endothelial cell surface. However, it is possible that this result might be due to the different glycosylation states of the same receptor recognizing the different lectins on the endothelial cell surface.

In the absence of extracellular calcium and in the presence of 2 mM EGTA, endothelin has been shown to induce a maintained contraction (Pang *et al.*, 1990). This calcium-free contraction was utilized in order to determine if, in uncultured native endothelium, WGA could induce EDRF release. In cultured endothelial cells it has been demonstrated that EDRF release can occur in the absence of extracellular calcium via activation of phospholipase C (Freay *et al.*, 1989) and release of intracellular calcium. In calcium-free solutions we have demonstrated that WGA could release EDRF suggesting that it is working via a receptor-linked system rather than via a non-specific increase in cell permeability to extracellular calcium.

EDRF is released by endothelial cells and induces vasodilatation by activating guanylate cyclase in the underlying vascular smooth muscle (Ignarro *et al.*, 1986; 1987; Angus & Cocks, 1989; Murad & Ishii, 1990). Aggregating platelets cause a transient increase in tension on endothelium-denuded canine coronary arteries and a profound relaxation of endothelium-intact arteries (Cohen *et al.*, 1983; Houston *et al.*, 1986). Although this effect was attributed to factors released from the platelets, a direct interaction of the platelets with the endothelium was not ruled out and a direct interaction of blood elements, including platelets, with the endothelium has recently been described (Flier & Heistad, 1993). The effects of WGA on canine coronary artery endothelium would suggest a possible lectin recognition molecule on the surface of endothelial cells. The existence of lectin receptors linked to tyrosine kinase have been reported in human platelets (Inazu *et al.*, 1991) and a receptor linked to mobilization of intracellular calcium has been reported in human lymphoblastoma cells (Tordai *et al.*, 1989; Tregear *et al.*, 1991) and rat lymph node cells (Tregear *et al.*, 1991). The ability of WGA to aggregate human platelets is well documented with the capacity of this binding being dependent on the presence of platelet surface sialic acid (Ganguly & Fossett, 1984) and may involve the activation of a tyrosine kinase signal transduction system (Inazu *et al.*, 1991). Therefore, the lectins WGA, Con A and LCA could recognize a receptor (or receptors) on the endothelial cell surface which stimulate EDRF release by mechanisms already described in other cell types (Tordai *et al.*, 1989; Inazu *et al.*, 1991) and this receptor could be an adhesion molecule (or molecules) that has the ability to recognize different cell-specific, lectin-type molecules. Whether 'lectin receptors' are

linked to, or indistinguishable from, any of the many adhesion molecules has yet to be determined.

The authors would like to thank W.G. Lillis, D.W. Smith and F. Hoffman for their assistance in performing these experiments.

References

ANGUS, J.A. & COCKS, T.M. (1989). Endothelium-derived relaxing factor. *Pharmacol. Ther.*, **41**, 303–351.

COHEN, R.A., SHEPHERD, J.T. & VANHOUTTE, P.M. (1983). Inhibitory role of the endothelium in the response of isolated coronary arteries to platelets. *Science*, **221**, 273–274.

DEMEY, J.G. & VANHOUTTE, P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J. Physiol.*, **316**, 347–355.

FLIER, J.S. & HEISTAD, D.D. (1993). Platelet-endothelium interactions. *New. Engl. J. Med.*, **328**, 628–635.

FREAY, A.D., JOHNS, A., ADAMS, D.J., RYAN, U.S. & VAN BREEMEN, C. (1989). Bradykinin- and IP_3-stimulated calcium release from intracellular calcium stores in cultured bovine endothelial cells. *Plügers Arch.*, **414**, 377–384.

FURCHGOTT, R.F. (1984). Role of endothelium in responses of vascular smooth muscle to drugs. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 175–197.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GANGULY, P. & FOSSETT, N.G. (1984). The role of sialic acid in the activation of platelets by wheat germ agglutinin. *Blood*, **63**, 181–187.

GRULICH-HENN, J., WESKLER, B.B., WATANABE, K. & JAFFE, E.A. (1988). Wheat germ agglutinin inhibits thrombin-induced rises in cytosolic free calcium and prostacyclin synthesis by human umbilical vein endothelial cells. *J. Cell. Physiol.*, **137**, 533–538.

HOUSTON, D.S., SHEPARD, J.T. & VANHOUTTE, P.M. (1986). Aggregating human platelets cause direct contraction and endothelium-dependent relaxation of isolated canine coronary arteries. *J. Clin. Invest.*, **78**, 539–544.

HEDO, J.A. (1984). Lectins as tools for the purification of membrane receptors. *Recept. Biochem. Meth.*, **2**, 45–60.

IGNARRO, L.J., BYRNS, R.E. & WOOD, K.S. (1987). Endothelium-dependent modulation of cGMP levels and intrinsic smooth muscle tone in isolated bovine intrapulmonary artery and vein. *Circulation Res.*, **60**, 82–92.

IGNARRO, L.J., HARBISON, R.G., WOOD, K.S. & KADOWITZ, P.J. (1986). Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation by acetylcholine, bradykinin and archidonic acid. *J. Pharmacol. Exp. Ther.*, **237**, 893–900.

INAZU, T., TANIGUCHI, T., OHTA, S., MIYABO, S. & YAMAMURA, H. (1991). The lectin wheat germ agglutinin induces rapid protein tyrosine phosphorylation in human platelets. *Biochem. Biophys. Res. Commun.*, **174**, 1154–1158.

JOHNS, A., LATEGAN, T.W., LODGE, N.J., RYAN, U.S., VAN BREEMEN, C. & ADAMS, D.J. (1987). Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells. *Tissue Cell*, **19**, 733–745.

KLEHA, J.F., DEVESLY, P. & JOHNS, A. (1991). The effects of lectins on the release of EDRF from rabbit aorta. *Br. J. Pharmacol.*, **104**, 287–288.

LASKEY, R.E., ADAMS, D.J., JOHNS, A., RUBANYI, G.M. & VAN BREEMEN, C. (1990). Membrane potential and Na^+–K^+ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria. *J. Biol. Chem.*, **265**, 2613–2619.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endotheliun-dependent and glyceryl trinitrate induced relaxation by haemoglobin and methylene blue in rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MULSCH, A., BUSSE, R., LIEBAU, S. & FORSTERMANN, U. (1988). LY 83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.*, **247**, 283–288.

MURAD, F. & ISHII, K. (1990). Hormonal regulation of the different isoforms of guanylate cyclase: EDRF is a ubiquitous activator of soluble guanylate cyclase. In *Endothelium-Derived Relaxing Factors*. ed. Rubanyi, G.M. & Vanhoutte, P.M. pp. 151–165. Basel: Kager.

NAGATA, Y. & BURGER, M.M. (1974). Wheat germ agglutinin. *J. Biol. Chem.*, **249**, 3116–3122.

DE NUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988). Receptor-mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 2334–2338.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PANG, D.C., JOHNS, A., PATTERSON, K., PARKER BOTELHO, L.H. & RUBANYI, G.M. (1990). Cellular mechanisms of action of endothelin in isolated canine coronary arteries. In *Endothelium-Derived Contracting Factors*. ed. Rubanyi, G.M. & Vanhoutte, P.M. pp. 66–72. Basel: Kager.

RAUB, T.J., KOROLY, M.J. & ROBERTS, R.M. (1990). Endocytosis of wheat germ agglutinin binding sites from the cell surface into a tubular endosomal network. *J. Cell. Physiol.*, **143**, 1–12.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

SINETT-SMITH, J., ZACHARY, I. & ROZENGURT, E. (1988). Characterization of a bombesin receptor on swiss mouse 3T3 cells by affinity cross-linking. *J. Cell. Biochem.*, **38**, 237–249.

SHARON, N. & LIS, H. (1989). Lectins as cell recognition molecules. *Science*, **246**, 227–234.

TORDAI, A., SARKARDI, B., GOROG, G. & GARDOS, G. (1989). Inhibitors of the CD3-mediated calcium signal by protein kinase C activators in human T (Jurkat) lymphoblastoma cells. *Immunol. Lett.*, **20**, 47–52.

TREGEAR, R.T., HOYLAND, J. & MASON, W.T. (1991). Calcium signals in single T cells on activation by lectin. *Eur. J. Immunol.*, **21**, 1283–1288.

WALZ, G., ARUFFO, A., KOLANUS, W., BEVILACQUA, M. & SEED, B. (1990). Recognition by ELAM-1 of sialyl-Le^X determinant on myeloid and tumor cells. *Science*, **250**, 1132–1135.

WEKSLER, B.B., LEY, C.W. & JAFFE, E.A. (1978). Stimulation of endothelial prostacyclin production by thrombin, trypsin, and the ionophore A23187. *J. Clin. Invest.*, **62**, 923–930.

(Received June 14, 1993
Revised August 19, 1993
Accepted August 31, 1993)

Br. J. Pharmacol. (1993), **110**, 1479–1482 © Macmillan Press Ltd, 1993

Modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves

[1,2]M.J. Rand & [1]C.G. Li

Department of Pharmacology, University of Melbourne, Victoria 3052, Australia

1 Acetylcholine-induced contractions of the rat isolated anococcygeus muscle were blocked by atropine (0.1 μM), slightly enhanced by hexamethonium (0.1 mM) and tetrodotoxin (1 μM), but little affected by prazosin (0.1 μM).

2 In the presence of the α_2-adrenoceptor agonist, UK14304, which raised the tone of the muscle, acetylcholine had a biphasic effect consisting of an initial relaxation followed by a contraction.

3 Atropine (0.1 μM) enhanced the relaxant component and abolished the contractile component of the response, whereas tetrodotoxin, ω-conotoxin GVIA or hexamethonium abolished or greatly reduced the relaxant component.

4 The nitric oxide synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) increased acetylcholine-induced contractions in the absence of UK14304 and markedly reduced the relaxant component to acetylcholine in the presence of UK14304. The effects of L-NAME were annulled by L-arginine (300 μM).

5 The results suggest that acetylcholine acts concurrently on muscarinic receptors of the smooth muscle to cause contraction and nicotinic receptors of nitrergic nerves to cause relaxation. The observed response is the resultant of these two opposing effects and depends also on the prevailing tone.

Keywords: Acetylcholine; anococcygeus muscle; ω-conotoxin; hexamethonium; muscarinic actions; nicotinic actions; NANC transmission; N^G-nitro-L-arginine methyl ester (L-NAME); nitric oxide; tetrodotoxin

Introduction

The evidence that transmission at some neuroeffector junctions is mediated by nitric oxide (NO) has recently been reviewed (Rand, 1992a,b; Sanders & Ward, 1992; Sneddon & Graham, 1992). We have termed such transmission *nitrergic* (Rand & Li, 1990). Nitrergic transmission was first demonstrated for relaxant responses of rat and mouse anococcygeus muscle to stimulation of non-adrenergic non-cholinergic (NANC) nerves (Gillespie *et al.*, 1989; Li & Rand, 1989b; Ramagopal & Leighton, 1989; Gibson *et al.*, 1990).

The peripheral nitrergic nerves so far identified mainly belong to the parasympathetic system or, in the case of those in the enteric nervous system, have connections with the parasympathetic system. The anococcygeus muscles have a direct parasympathetic innervation (Gillespie & McGrath, 1973), and contain abundant acetylcholinesterase-positive fibres (Dail *et al.*, 1990), suggesting a cholinergic innervation. However, since acetylcholine does not mediate either motor or inhibitory transmission (Gillespie, 1972) the role of a cholinergic innervation is not clear.

Anococcygeus muscles respond to both muscarinic and nicotinic agonists. Muscarinic cholinoceptors of the smooth muscle cells subserve contraction (Gillespie, 1972), and they are also present on both noradrenergic and nitrergic nerve terminals where they subserve inhibition of transmission (Rajani & Gulati, 1988; Li & Rand, 1989a). Nicotinic cholinoceptors are present on both noradrenergic and nitrergic nerve terminals in the rat anococcygeus muscle, and nicotine acts on them to evoke release of the respective transmitters (Rand & Li, 1992).

The present study was carried out to determine whether responses to acetylcholine involve interactions with noradrenergic and/or nitrergic transmission in the rat anococcygeus muscle.

[1] Present address: Pharmacology Research Laboratory, Department of Medical Laboratory Science, Royal Melbourne Institute of Technology, Victoria 3001, Australia.
[2] Author for correspondence (at 'Present address').

Methods

Male Sprague-Dawley rats (200–400 g) were killed by decapitation and the anococcygeus muscles were removed as described by Gillespie (1972) and set up in a 8 ml organ bath in physiological salt solution (PSS) of the following composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid, 0.14. The PSS was gassed with 5% CO_2 and O_2 and maintained at 37°C. The tone of the muscle was measured isometrically at a resting tension of 1 g wt. Field stimulation (1 ms pulses, 17 V cm^{-1}, at 1 to 5 Hz, for 10 s) was delivered from a Grass S88 stimulator through platinum wire electrodes on either side of the muscle. The isolated muscles were allowed to equilibrate for 30 to 45 min before making experimental observations. NANC-mediated relaxations were revealed by raising the tone and inhibiting contractile responses to noradrenergic nerve stimulation with the α_2-adrenoceptor agonist, UK14304 (1 μM) or guanethidine (10–30 μM).

The effects of antagonists were studied after initial control responses to agonists had been obtained and the antagonists then remained in the PSS for the remainder of the experiment: corresponding agonist-induced responses without addition of an antagonist were elicited in the other anococcygeus muscle from the same donor rat to serve as a time control.

Differences between means were compared by Student's *t* test, and values of $P < 0.05$ were taken to indicate statistical significance.

Drugs

The drugs used were acetylcholine perchlorate (BDH, chemicals), L-arginine (Wellcome), atropine sulphate (Sigma), ω-conotoxin GVIA (Peninsula), guanethidine sulphate (Ciba), hexamethonium bromide (Sigma), N^G-nitro-L-arginine methyl ester (L-NAME, Wellcome), noradrenaline bitartrate (Sigma), prazosin hydrochloride (Pfizer), sodium nitroprusside (Sigma), tetrodotoxin (TTX, Sigma) and UK14304 (5-bromo-6-[2-imidazoline-2-Y1-amino]-quinoxaline; Pfizer).

Results

Contractile responses to acetylcholine

Acetylcholine (1–1000 μM) elicited concentration-dependent contractions which were sustained during the period of exposure. The muscle relaxed promptly after washing out the acetylcholine. Subsequent responses to the same concentration were reproducible in magnitude and time course.

Atropine (0.1 μM) abolished responses to 1–300 μM acetylcholine (as shown for 100 μM acetylcholine in Figure 1) and greatly reduced (>90%) the response to 1000 μM acetylcholine.

The typical contractile response to a single concentration of acetylcholine (100 μM) is shown in Figure 1: it consists of an initial rapidly developing phase, which is complete between 20–25 s after exposure to acetylcholine, and a plateau which is reached after 3–4 min of exposure to acetylcholine. Hexamethonium (100 μM) increased the initial contractile response to acetylcholine, which rose more rapidly to a greater peak and then gradually fell to a plateau which was slightly greater than in the absence of hexamethonium (Figure 1 and Table 1).

Contractions elicited by acetylcholine (100 μM) were also slightly enhanced by tetrodotoxin (1 μM), but the effect of tetrodotoxin was most conspicuous in increasing the initial contractile response to acetylcholine (Figure 1 and Table 1).

In the resting drug-free state, field stimulation for 10 s periods at 1–5 Hz produced frequency-dependent, reproducible contractions. These contractions, as well as those induced by exogenously applied noradrenaline (1 μM), were abolished by the selective α_1-adrenoceptor antagonist prazosin (0.1 μM) (results not shown). Prazosin (0.1 μM) had no significant effects on either the initial contraction or the plateau tension induced by acetylcholine (Figure 1 and Table 1).

Relaxant responses to acetylcholine

The adrenergic neurone blocking drug, guanethidine, raised the tone and reversed the response to field stimulation from a contraction to a relaxation, as first reported by Gillespie (1972). The α_2-adrenoceptor agonist, UK14304 (1 μM), also raised the tone and, like guanethidine, reversed responses to field stimulation from contractions to relaxations.

In the presence of UK14304, responses to acetylcholine (30–300 μM) contained contractile and relaxant components which were manifested in different proportions according to the concentration: with the lowest effective concentration (30 μM) the contraction predominanted; then, with increasing concentrations, there was usually an initial transient relaxation followed by a contraction, both phases of the response being concentration-dependent (Figure 2a). Atropine (0.1 μM) abolished the secondary contraction and greatly enhanced the relaxant component of the response (Figure 2a).

In the presence of guanethidine, acetylcholine (100–1000 μM) produced only contractions and no relaxant component was evident. However, when the contractions were reduced by atropine (0.1 μM), a biphasic response was revealed, consisting of an initial relaxation followed by a contraction (results not shown).

Effects of L-NAME on responses to acetylcholine

L-NAME (100 μM) significantly enhanced contractions elicited by field stimulation, and also enhanced contractions elicited by acetylcholine as shown in Figure 1. The effect of L-NAME (100 μM) was most evident in the initial contractile response to acetylcholine, which rose more rapidly to a greater peak and then fell to a plateau which was slightly greater than in the absence of L-NAME (Figure 1 and Table 1). The response to acetylcholine was restored substantially to its initial form by 300 μM L-arginine (Figure 1 and Table 1).

In the presence of UK14304, L-NAME (100 μM) generally produced a small increase in tone and relaxations elicited by field stimulation (1–5 Hz for 10 s) were significantly reduced, as shown previously (Rand & Li, 1992). Relaxations elicited by acetylcholine in the presence of UK14304 were greatly reduced by L-NAME (Figure 2b), and the inhibitory effect of L-NAME on these relaxations was annulled by L-arginine (300 μM) (Figure 2b).

Effects of hexamethonium, tetrodotoxin and ω-conotoxin GVIA on acetylcholine-induced relaxations

The relaxant action of acetylcholine in the presence of UK14304 and atropine was abolished by hexamethonium

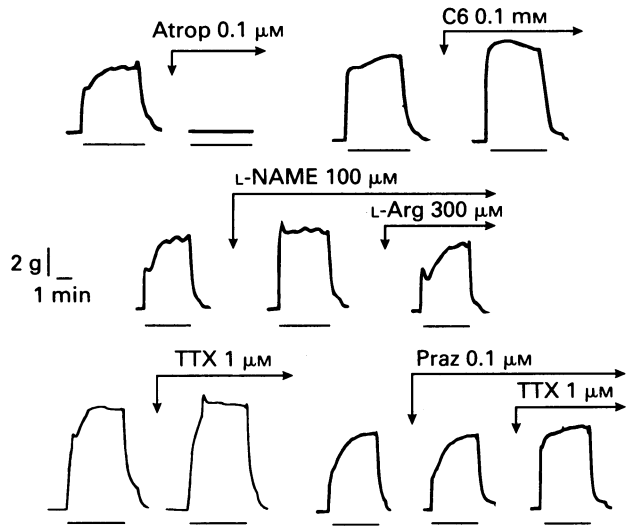


Figure 1 Responses elicited by exogenously applied acetylcholine (100 μM) in rat isolated anococcygeus muscles. Horizontal lines indicate the periods of exposure to acetylcholine. The contact time for atropine (Atrop, 0.1 μM), hexamethonium (C6, 0.1 mM), tetrodotoxin (TTX, 1 μM), N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), L-arginine (L-Arg, 300 μM) and prazosin (Praz, 0.1 μM) before eliciting responses to acetylcholine was 10 min. Similar results of each type were obtained in at least four separate experiments.

Table 1 Effects of various drugs on contractions of the rat anococcygeus to acetylcholine (100 μM)

Treatment	n	*Initial phase tension* (g)	*Plateau phase tension* (g)
Control	10	2.6 ± 0.3	3.7 ± 0.4
L-NAME (100 μM)	10	4.1 ± 0.6**	4.6 ± 0.6**
L-NAME + L-Arg (300 μM)	10	2.8 ± 0.4	3.8 ± 0.5
Control	5	2.6 ± 0.6	3.7 ± 0.6
Hexamethonium (100 μM)	5	3.9 ± 0.4**	4.6 ± 0.7**
Control	5	2.5 ± 0.5	3.5 ± 0.6
Prazosin (0.1 μM)	5	2.4 ± 0.4	3.4 ± 0.6
Control	9	2.4 ± 0.3	3.6 ± 0.4
Tetrodotoxin (1 μM)	9	2.8 ± 0.4*	3.7 ± 0.4
In the presence of prazosin (0.1 μM)			
Control	5	2.4 ± 0.3	3.6 ± 0.4
Tetrodotoxin (1 μM)	5	3.1 ± 0.5*	3.8 ± 0.7

The initial tension was calculated as the tension achieved in the first 20–25 s after the addition of acetylcholine (100 μM) and the plateau tension was that present after 4 min exposure of acetylcholine (see Figure 1).
*$P<0.05$; **$P<0.01$: compared with control (paired t test).

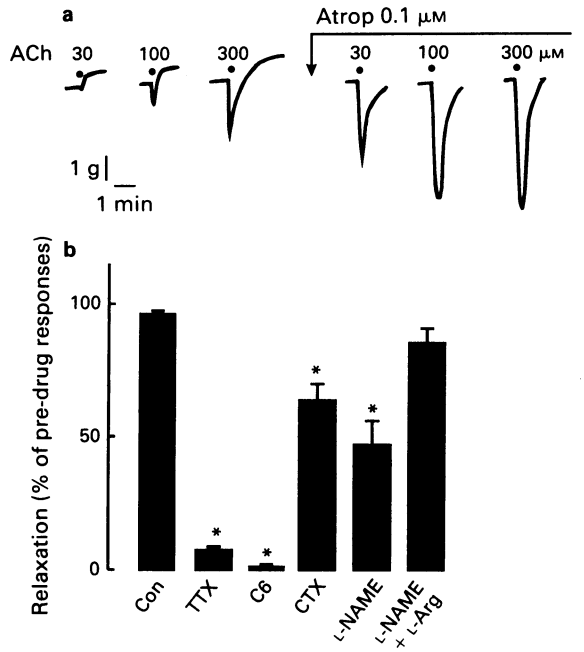


Figure 2 (a) Effect of atropine (Atrop, 0.1 μM, contact time 10 min) on responses elicited by acetylcholine (ACh, 30, 100 and 300 μM) in a rat anococcygeus muscle in which the tone was raised by UK14304 (1 μM). (b) Effects of hexamethonium (C6, 0.1 mM), tetrodotoxin (TTX, 1 μM), ω-conotoxin GVIA (CTX, 50 nM) and N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM), in the absence and presence of L-arginine (L-Arg, 300 μM) on the relaxations of rat anococcygeus muscles elicited by acetylcholine (100 μM). The contact time for C6, TTX, CTX, L-NAME and L-Arg was 10–20 min. The tone of the muscles was raised by UK14304 (1 μM) and all the experiments were conducted in the presence of atropine (0.1 μM). Columns represent mean values (with s.e.mean) ($n = 4-7$). $^*P < 0.05$ (t test) compared with the corresponding time control (Con).

(100 μM) and significantly reduced by tetrodotoxin (1 μM) and by ω-conotoxin (50 nM) (Figure 2b).

Discussion

The autonomic innervation of the rat anococcygeus muscle contains noradrenergic, cholinergic and NANC components (Gillespie, 1972). Stimulation of sympathetic nerves causes noradrenergically mediated contractions (Gillespie, 1972), while stimulation of the NANC nerves produces a NO-mediated relaxation (for references, see Introduction).

Contractions of the rat anococcygeus muscle elicited by acetylcholine can be attributed to activation of postjunctional muscarinic receptors (Gillespie, 1972). They were blocked by atropine, but field stimulation-induced contractions were not affected by atropine and therefore do not contain a cholinergic component.

In addition, to its contractile action, the results obtained in the present study indicate that acetylcholine can also relax the anococcygeus if there is pre-existing tone. The relaxant action of acetylcholine is increased in the presence of atropine. On the other hand, the relaxation induced by acetylcholine was blocked by the nicotinic receptor antagonist hexamethonium, suggesting that it resulted from activation of nicotinic receptors. The transient nature of the relaxant action by acetylcholine could be due to its desensitization of nicotinic receptor (Rand & Li, 1992). The findings that the relaxations elicited by acetylcholine were also blocked by tetrodotoxin, the neuronal N-type calcium channel blocker ω-conotoxin GVIA, and by L-NAME, and that the blocking effect of L-NAME was annulled by L-arginine, suggests that acetylcholine activates nicotinic cholinoceptors on nitrergic nerves resulting in release of NO or a NO-yielding substance, as we have previously demonstrated with nicotine (Rand & Li, 1992).

The effect of atropine in enhancing the relaxant action of acetylcholine is presumably largely due to blockade of the counteracting contractile action exerted directly on the smooth muscle. An additional factor that may contribute to the enhancement is blockade of prejunctional muscarinic receptors associated with the nitrergic nerve terminals that subserve inhibition of transmission (Li & Rand, 1989a). Similar dual effects of acetylcholine in activating nicotine receptors to produce transmitter release and muscarinic receptors to inhibit it have been noted at noradrenergic nerve terminals (Löffelholz *et al.*, 1967; Lindmar *et al.*, 1968).

Activation of nicotinic cholinoceptors on sympathetic nerve terminals in the rat anococcygeus also results in release of transmitter noradrenaline (Rand & Li, 1992); however, a nicotine-like effect of acetylcholine on sympathetic nerve terminals was not detected under the conditions used in the present study since acetylcholine-induced contractions were not affected by the α_1-adenoceptor antagonist, prazosin, which blocked responses to noradrenaline and stimulation of noradrenergic nerves. Alternatively, any change in the release of noradrenaline that acetylcholine may have produced was too small to contribute functionally to the contraction.

Contractions elicited by acetylcholine were enhanced by L-NAME, and this effect of L-NAME was annulled by L-arginine, which suggests that contractile responses to acetylcholine under resting conditions (without pre-existing tone) may actually contain a counteracting nitrergic component. In accord with this, acetylcholine-induced contractions were slightly enhanced by hexamethonium and tetrodotoxin, which indicates that the nicotine-like effect of acetylcholine is selective activation of nitrergic rather than noradrenergic transmission.

Recently, after completion of the present study, it was reported that L-NAME and other esters of arginine are muscarinic receptors antagonists and 100 μM of L-NAME was apparently effective in competitively inhibiting acetylcholine-induced contractions of endothelium-denuded rings of rabbit coronary artery and strips of circular smooth muscle from the dog colon (Buxton *et al.*, 1993). Although we have not investigated the possibility that such an action of L-NAME also occurred in the anococcygeus muscle, it is clear that even if it does occur it is far outweighed by the blockade of nitrergic mechanisms.

Activation of nitrergic transmission by acetylcholine may have a physiological significance in the control of the function of the muscle, since it has an abundant cholinergic innervation (Dail *et al.*, 1990). However, since the identification of a cholinergic innervation is based on the presence of nerve-associated cholinesterase, that enzyme might be present to prevent possible actions of acetylcholine.

Although a relaxant action of acetylcholine was clearly evident when UK14304 was used as a contracting agent, acetylcholine did not produce relaxations when guanethidine was used. We have shown previously that guanethidine inhibits nicotine-induced relaxation in the rat anococcygeus muscle (Rand & Li, 1992). Thus, the lack of a relaxant action of acetylcholine in the presence of guanethidine is due to attenuation of its nicotinic action; however, in the presence of both guanethidine and atropine, when the muscarinic contractile action of acetylcholine was blocked, the relaxant action of acetylcholine emerged.

The results obtained in the present study indicate that, in addition to its muscarinic smooth muscle-contracting action, acetylcholine also has a nicotinic relaxant action mediated by a nitrergic mechanism. There is a widespread distribution of nitrergic nerves (for references to reviews, see Introduction), and smooth muscle in most of the tissues innervated by them are responsive to muscarinic agonists. If the muscarinic

agonists are also nicotinic agonists, as is often the case, many of the actions of such agonists may need to be re-evaluated to determined whether the findings have been confounded by a contribution arising from activation of the nitrergic nerves.

This work was supported by an Australian Tobacco Research Foundation Programme Grant. We are indebted to Dr S. Moncada, Director, Wellcome Research Laboratories, Beckenham, U.K., for supplying samples of L-NAME.

References

BUXTON, I.L.O., CHEEK, D.J., ECKMAN, D., WESTALL, D.P., SANDERS, K.M. & KEEF, K.D. (1993). N^G-nitro-L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.*, **72,** 387–395.

DAIL, W.G., CAIRRILLO, Y. & WALTON, G., (1990). Innervation of the anococcygeus muscle of the rat. *Cell Tissues Res.*, **259,** 139–146.

GIBSON, A., MIRZAZADEH, S., HOBBS, A.J. & MOORE, P.K. (1990). L-N^G-monomethyl arginine and L-N^G-nitro arginine inhibit non-adrenergic, non-cholinergic relaxation of the mosue anococcygeus muscle. *Br. J. Pharmacol.*, **99,** 602–606.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmacol.*, **45,** 404–416.

GILLESPIE, J.S. & MCGRATH, J.C. (1973). The spinal origin of the motor and inhibitory innervation of the rat anococcygeus muscles. *J. Physiol.*, **230,** 659–672.

GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.*, **98,** 1080–1082.

LI, C.G. & RAND, M.J. (1989a). Prejunctional inhibition of non-adrenergic non-cholinergic transmission in the rat anococcygeus muscle. *Eur. J. Pharmacol.*, **168,** 107–110.

LI, C.G. & RAND, M.J. (1989b). Evidence for a role of nitric oxide in the NANC-mediated relaxations in rat anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.*, **16,** 933–938.

LINDMAR, R., LÖFFELHOLZ, K. & MUSCHOLL, E. (1968). A muscarinic mechanism inhibiting the release of noradrenaline from peripheral adrenergic nerve fibres by nicotinic agonists. *Br. J. Pharmacol.*, **32,** 280–294.

LÖFFELHOLZ, K., LINDMAR, R. & MUSCHOLL, E. (1967). Der Einfluss von Atropin auf die Noradrenalinfreisetzung durch Acetylcholin. *Naunyn-Schmied. Arch. Pharmacol.*, **257,** 308.

RAJANI, A.P. & GULATI, O.D. (1988). Evidence for the multiple prejunctional receptor sites in rat isolated anococcygeus muscle. *Arch. Int. Pharmacodyn.*, **292,** 86–100.

RAMAGOPAI, M.V. & LEIGHTON, H.J. (1989). Effects of N^G-monomethyl-L-arginine on field stimulation-induced decreases in cytosolic Ca^{2+} levels and relaxation in the rat anococcygeus muscle. *Eur. J. Pharmacol.*, **174,** 297–299.

RAND, M.J. (1992a). Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission. *Clin. Exp. Pharmacol. Physiol.*, **19,** 147–169.

RAND, M.J. (1992b). New perspectives in neurotransmission: nitric oxide, the mediator of nitrergic transmission. *Proc. Austral. Physiol. Pharmacol. Soc.*, **23,** 1–16.

RAND, M.J. & LI, C.G. (1990). Nitric oxide mediates non-adrenergic non-cholinergic relaxation in some neuroeffector systems: examples of nitrergic transmission. *Eur. J. Pharmacol.*, **183,** 1144.

RAND, M.J. & LI, C.G. (1992). Activation of noradrenergic and nitrergic mechanisms in the rat anococcygeus muscle by nicotine. *Clin. Exp. Pharmacol. Physiol.*, **19,** 103–111.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of non-adrenergic non-cholinergic transmission. *Am. J. Physiol.*, **262,** G379–G392.

SNEDDON, P. & GRAHAM, A. (1992). Role of nitric oxide in the autonomic innervation of smooth muscle. *J. Auton. Pharmacol.*, **12,** 445–456.

(Received June 14, 1993
Revised August 19, 1993
Accepted September 1, 1993)

Br. J. Pharmacol. (1993), **110**, 1483–1490 © Macmillan Press Ltd, 1993

Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabimimetic agents, CP 55,940, WIN 55,212-2 and anandamide

[1]R.G. Pertwee, L.A. Stevenson & G. Griffin

Department of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen AB9 1AS

1 Mice pretreated intraperitoneally for 2 days with delta-9-tetrahydrocannabinol (delta-9-THC) at a dose of 20 mg kg^{-1} day^{-1} and then challenged intravenously with this drug, 24 h after the second pretreatment, showed a 6 fold tolerance to the hypothermic effect of delta-9-THC. This pretreatment also induced tolerance to the hypothermic effects of the cannabimimetic agents, CP 55,940 (4.6 fold) and WIN 55,212-2 (4.9 fold), but not to the hypothermic effect of the putative endogenous cannabinoid, anandamide.

2 Vasa deferentia removed from mice pretreated intraperitoneally with delta-9-THC twice at a dose of 20 mg kg^{-1} day^{-1} were less sensitive to its inhibitory effect on electrically-evoked contractions than vasa deferentia obtained from control animals. The cannabinoid pretreatment induced a 30 fold parallel rightward shift in the lower part of the concentration-response curve of delta-9-THC and a marked reduction in the maximal inhibitory effect of the drug. It also induced tolerance to the inhibitory effects on the twitch response of CP 55,940 (8.7 fold), WIN 55,212-2 (9.6 fold) and anandamide (12.3 fold).

3 The results confirm that cannabinoid tolerance can be rapid in onset and support the hypothesis that it is mainly pharmacodynamic in nature. The finding that *in vivo* pretreatment with delta-9-THC can produce tolerance not only to its own inhibitory effect on the vas deferens but also to that of three other cannabimimetic agents, suggests that this tissue would be suitable as an experimental model for investigating the mechanisms responsible for cannabinoid tolerance.

4 Further experiments are required to establish why tolerance to anandamide-induced hypothermia was not produced by a pretreatment with delta-9-THC that did induce tolerance to the hypothermic effects of delta-9-THC, CP 55,940 and WIN 55,212-2 and to the inhibitory effects of delta-9-THC, CP 55,940, WIN 55,212-2 and anandamide on the twitch response of the vas deferens.

Keywords: Delta-9-tetrahydrocannabinol; CP 55,940; WIN 55,212-2; anandamide; cannabinoids; cannabinoid tolerance; mouse vas deferens; hypothermia

Introduction

Little is known about the mechanisms underlying the development of tolerance to psychotropic cannabinoids other than that they are largely pharmacodynamic in nature (Pertwee, 1991). As a first step aimed at identifying these mechanisms, the present investigation set out to establish whether *in vivo* pretreatment with delta-9-tetrahydrocannabinol (delta-9-THC) can induce tolerance to the inhibitory effect of this drug on electrically-evoked contractions of the mouse isolated vas deferens, our overall purpose being to explore the suitability of this preparation as a model with which to determine the basis of cannabinoid tolerance. The mouse vas deferens was used as there is already evidence that it is an appropriate tissue for investigating the mechanisms responsible for the acute effects of psychotropic cannabinoids. In particular, cannabimimetic agents are known to be highly potent and to show remarkable stereoselectivity as inhibitors of the electrically-evoked twitch response of this tissue (Pertwee *et al.*, 1992a; 1993). It is also known that the potency of cannabinoids as inhibitors of the twitch response correlates well with their potency as psychotropic agents (Pertwee, 1993).

The ability of an *in vivo* pretreatment with delta-9-THC to induce tolerance to the effects of other cannabimimetic agents on the twitch response of the mouse vas deferens was also investigated. These experiments were carried out with CP 55,940, WIN 55,212-2 and the putative endogenous cannabinoid, anandamide (Martin *et al.*, 1991; Devane *et al.*, 1992). The structures of these cannabimimetic agents are shown in Figure 1. It was decided that in the first experiments at least, the delta-9-THC pretreatment used should be one that produces tolerance to delta-9-THC-induced hypothermia, previous experiments in this laboratory having shown that such tolerance can develop quite rapidly (Fitton & Pertwee, 1982). Accordingly, our initial experiments were directed at establishing a reliable procedure for producing tolerance to the hypothermic effect of delta-9-THC in mice.

Some of the results described in this paper have been presented to the British Pharmacological Society (Griffin & Pertwee, 1993).

Methods

Animals

Experiments were performed with male albino MF1 mice weighing 23 to 30 g (body temperature experiments) or 36 to 59 g (vas deferens experiments). The animals received food and water *ad libitum*. Except when stated otherwise, *in vivo* experiments were carried out at ambient temperatures of 20 to 22°C.

Production of tolerance

Tolerance was induced by injecting delta-9-THC intraperitoneally at doses of up to 20 mg kg^{-1} either once or twice at an interval of 24 h. Control animals received intraperitoneal injections of Tween 80. The volume for intraperitoneal injection was 0.25 ml 25 g^{-1}. The degree of tolerance produced by delta-9-THC was determined 24 h after the final injection either by measuring the hypothermic effects of delta-9-THC, CP 55,940, WIN 55,212-2 or anandamide or by measuring

[1] Author for correspondence.

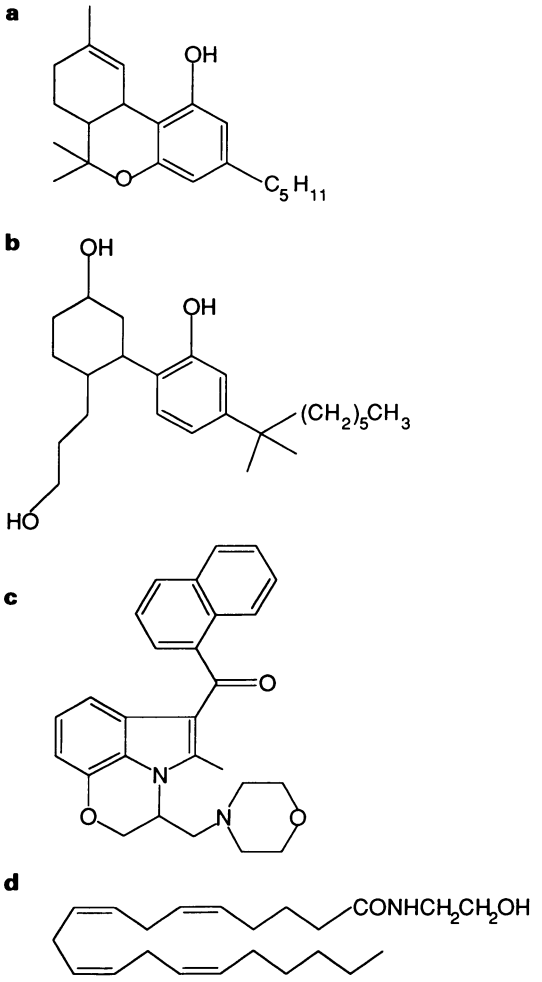


Figure 1 The chemical structures of (a) delta-9-tetrahydrocannabinol (delta-9-THC), (b) CP 55,940, (c) WIN 55,212-2 and (d) anandamide.

the inhibitory effects of these compounds on electrically-evoked contractions of the mouse vas deferens.

Body temperature experiments

Body temperature was measured with a thermistor probe (YSI 402) inserted 3 cm into the rectum. Except when they were being injected intravenously or being held in the hand for the measurement of body temperature, mice were kept unrestrained, each in a separate cage. Temperature readings were first taken 30 min and 5 min before drug administration which was at time zero. Readings were next taken 5 min after time zero, then at 5 min intervals until + 30 min and finally at + 45 and + 60 min. To produce hypothermia, drugs were injected into a lateral tail vein. The injection volume was 0.2 ml 25 g^{-1}. The injection procedure was facilitated by subjecting the mice to an ambient temperature of 34°C for the 5 min period immediately before injection so as to cause dilatation of the tail veins. Exposure of a group of six mice to this ambient temperature for 5 min did not produce any significant change in body temperature ($P > 0.05$; Student's t test for paired data), the group's mean rectal temperature being 37.05 ± 0.12°C just before the start of the exposure and 37.12 ± 0.17°C at the end.

Vas deferens experiments

Vasa deferentia were mounted in 4 ml organ baths, at an initial tension of 0.5 g. The baths contained Mg^{2+}-free Krebs solution kept at 37°C and bubbled with 95% O_2 and 5% CO_2. Isometric contractions were evoked by electrical field stimulation through a platinum electrode attached to the upper end of each bath and a stainless steel electrode attached to the lower end. The stimuli were generated by a Grass S48 stimulator, then amplified (Med-Lab channel attenuator) and finally divided to yield separate outputs to four organ baths (Med-Lab Stimusplitter). Contractions were registered on a computer (Apple Macintosh LC) using a data recording and analysis system (MacLab) that was linked via preamplifiers (Macbridge) to Pye Ether UF1 transducers. Tissues were stimulated with 0.5 s trains of three pulses of 110% maximal voltage (train frequency 0.1 Hz; pulse duration 0.5 ms). In most experiments each tissue was used to construct a single cumulative concentration-response curve and was subjected to five or six periods of stimulation. The first of these began after the tissue had equilibrated but before drug administration and continued for 11 min. Drug addition (10 µl) was made immediately after this first stimulation period. Subsequent stimulation periods lasted 5 min at the end of which the bath contents were washed out by overflow and another dose of drug added. Each 5 min stimulation period was started only after sufficient time had elapsed for the drug under investigation to achieve its full inhibitory effect. Results from pilot experiments indicated this to be 15 min for anandamide and CP 55,940, 20 min for WIN 55,212-2 and 25 min for delta-9-THC. In our first experiments (with delta-9-THC), tissues were exposed to just one concentration of drug (10 nM) and results from these experiments are also described below.

Drugs

Delta-9-THC was obtained from the National Institute on Drug Abuse, U.S.A., CP 55,940 from Dr L.S. Melvin (Pfizer) and WIN 55,212-2 from Dr S.J. Ward (Sterling Winthrop). Anandamide was synthesized and supplied by Professor R. Mechoulam (University of Jerusalem). Each compound was mixed with 2 parts of Tween 80 by weight and dispersed in 0.9% w/v NaCl solution (saline) as described previously for delta-9-THC (Pertwee *et al.*, 1992a).

Analysis of data

Values are expressed as means and limits of error as standard errors. The effect of delta-9-THC on body temperature was calculated by subtracting rectal temperature, measured 5 min before drug administration, from rectal temperature measured at the time of peak hypothermia. Inhibition of the twitch response is expressed in percentage terms and has been calculated by comparing the amplitude of the twitch response immediately before injection with its amplitude during each of the postinjection periods of stimulation. Degrees of tolerance to delta-9-THC, CP 55,940, WIN 55,212-2 and anandamide were determined by symmetrical (2 + 2) dose parallel line assays (Colquhoun, 1971). The significance of differences between means was evaluated by Dunnett's test (Dunnett, 1964) or by Student's t test (two-tail) for paired or unpaired data ($P >$ or < 0.05).

Results

Tolerance to the hypothermic effect of delta-9-THC

As shown in Figure 2, delta-9-THC induced dose-related decreases in rectal temperature. This ability of delta-9-THC to induce hypothermia was significantly attenuated by pretreating animals with the drug twice at a dose of 20 mg kg^{-1} i.p., this pretreatment producing a 6 fold parallel rightward shift in the log dose-hypothermic response curve of the drug (Figure 3 and Table 1).

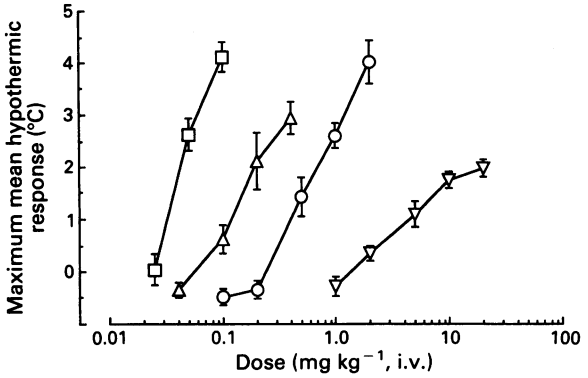


Figure 2 Mean dose-hypothermic response curves for CP 55,940 (□), WIN 55,212-2 (△), delta-9-tetrahydrocannabinol (○), and anandamide (▽) in mice. Each symbol represents the mean value ± s.e.mean of the maximum hypothermic response produced ($n = 6$).

The degree of tolerance that was observed following intraperitoneal pretreatment with delta-9-THC was influenced both by the size of the pretreatment dose used and by the number of pretreatments given. Whereas the hypothermic response to a submaximal dose of delta-9-THC (1.0 mg kg^{-1}, i.v.) was abolished in mice that had been subjected to two intraperitoneal injections of this drug at a dose of 20 mg kg^{-1} (Figure 4) it was only attenuated, albeit significantly, in mice that had been pretreated either with a single injection of the 20 mg kg^{-1} dose or with two injections of either 5 or 10 mg kg^{-1} i.p. (Table 2). Delta-9-THC pretreatment with a single injection of 10 mg kg^{-1} i.p. or with two injections of 1 or 2 mg kg^{-1} i.p. did not induce significant tolerance (Table 2). Maximum hypothermia usually occurred sooner after intravenous injection of delta-9-THC in drug pretreated mice than in animals that had been pretreated with Tween 80.

Some of the intraperitoneal doses of delta-9-THC used in the present experiments (10 and 20 mg kg^{-1}) are known to produce marked hypothermia in mice (Pertwee & Tavendale, 1977; Gray *et al.*, 1987). It was of interest, therefore, to establish whether tolerance to the hypothermic effect of

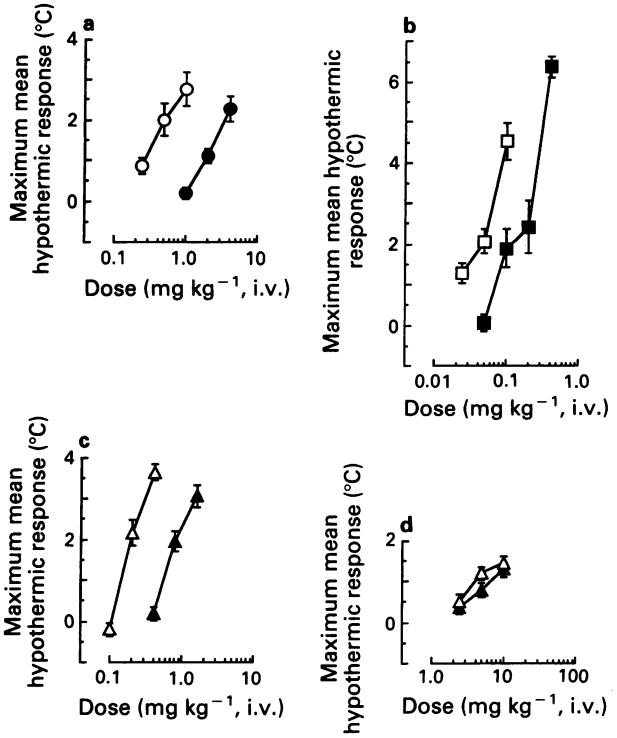


Figure 3 Mean dose-hypothermic response curves for (a) delta-9-tetrahydrocannabinol, (b) CP 55,940, (c) WIN 55,212-2 and (d) anandamide in mice pretreated once daily for 2 days with delta-9-THC at a dose of 20 mg kg^{-1}, i.p. (closed symbols) or with Tween 80 at a dose of 40 mg kg^{-1}, i.p. (open symbols). Each symbol represents the mean value ± s.e.mean of the maximum hypothermic response produced ($n = 6$).

delta-9-THC would still develop in animals that were prevented from experiencing any significant degree of delta-9-THC-induced hypothermia during the pretreatment period. Accordingly, one series of experiments was conducted in which mice were transferred to a chamber maintained at an

Table 1 Effect of *in vivo* pretreatment with delta-9-tetrahydrocannabinol (delta-9-THC) on the potencies of certain cannabimimetic drugs as hypothermic agents

Pretreatment drug	*Pretreatment dose* (mg kg^{-1}, i.p.)	*Treatment drug*	*Treatment doses* (mg kg^{-1}, i.v.)	*Potency ratio*	*95% Confidence limits*	n
Delta-9-THC	20	Delta-9-THC	1.0, 4.0	6.03	3.84 and 10.41	6
Tween	40	Delta-9-THC	0.25, 1.0			
Delta-9-THC	20	CP 55,940	0.05, 0.2	4.57	2.83 and 9.09	6
Tween	40	CP 55,940	0.025, 0.1			
Delta-9-THC	20	WIN 55,212-2	0.8, 1.6	4.94	3.75 and 7.04	6
Tween	40	WIN 55,212-2	0.2, 0.4			
Delta-9-THC	20	Anandamide	2.5, 10	1.24	0.77 and 2.14	6
Tween	40	Anandamide	2.5, 10			
None	–	Delta-9-THC	0.5, 2.0	1.14	0.59 and 1.89	6
Tween	40	Delta-9-THC	0.25, 1.0			

Mice were either unpretreated or pretreated intraperitoneally, once daily for 2 days, with delta-9-THC or with Tween 80. In the experiments in which mice were pretreated, the effects of treatment drugs, delta-9-THC, CP 55,940, WIN 55,212-2 and anandamide, on body temperature were measured 24 h after the second intraperitoneal injection. The final potency ratio listed in the Table indicates the extent by which the potency of delta-9-THC as a hypothermic agent was greater or less in unpretreated animals than in animals that had been pretreated with Tween 80. Pretreatment with Tween 80 did not lead to any significant change in the slope of the dose-response curve of delta-9-THC. Each of the other potency ratios listed above indicates the extent by which pretreatment with delta-9-THC reduced the potency of a treatment drug as a hypothermic agent.None of the delta-9-THC-induced rightward shifts in the dose-response curves of the treatment drugs deviated significantly from parallelism. Potency ratios, confidence limits and deviations of pairs of log dose-response curves from parallelism have been determined using symmetrical (2 + 2) dose parallel line assays and were calculated from data obtained with the treatment doses shown (Colquhoun, 1971).

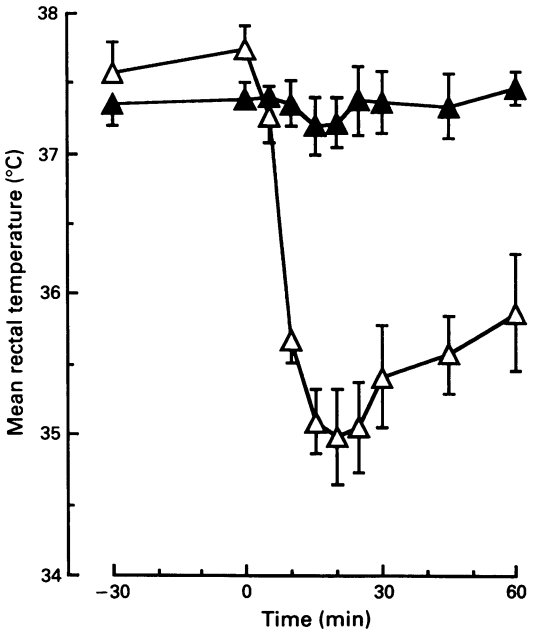


Figure 4 Effect of pretreatment with delta-9-tetrahydrocannabinol (delta-9-THC, ▲) or Tween 80 (△) on the ability of delta-9-THC to produce hypothermia. Mice received delta-9-THC (20 mg kg^{-1}, i.p.) or Tween 80 (40 mg kg^{-1}, i.p.) once daily for 2 days. All animals were challenged with an intravenous injection of delta-9-THC (1.0 mg kg^{-1}) at time zero, 24 h after the second pretreatment. Each symbol represents a mean value ± s.e.mean of rectal temperature ($n = 6$). It was assumed that rectal temperature at time zero was the same as rectal temperature at − 5 min (see Methods). In the Tween-pretreated group of animals, mean rectal temperatures were significantly less than the corresponding time zero value from + 10 min onwards ($P < 0.01$; Dunnett's test). In the delta-9-THC-pretreated group, no significant changes in mean rectal temperature occurred between time zero and + 60 min ($P > 0.05$; Dunnett's test).

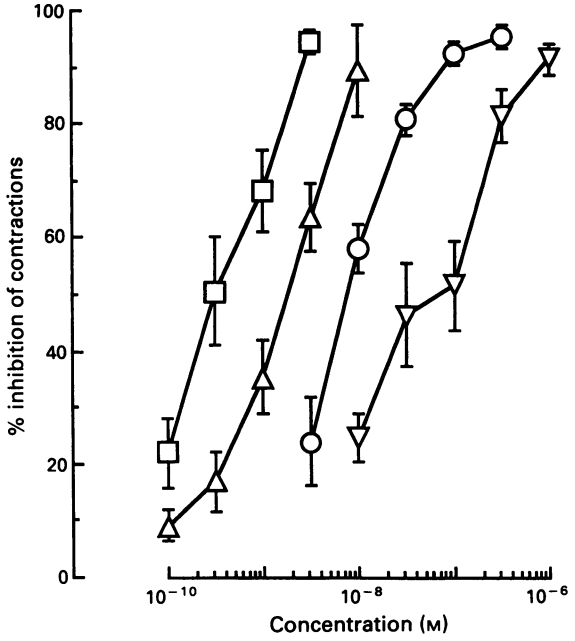


Figure 5 Mean cumulative concentration-response curves for CP 55,940 (□), WIN 55,212-2 (△), delta-9-tetrahydrocannabinol (delta-9-THC) (○), and anandamide (▽) in mouse isolated vasa deferentia. Each symbol represents the mean value ± s.e.mean of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of drug to the organ bath ($n = 7$ to 8 different vasa deferentia).

ambient temperature of 34°C immediately after each of two pretreatment injections with delta-9-THC (20 mg kg^{-1}, i.p.) or Tween 80. This ambient temperature is a 'thermoneutral' temperature for mice and was used as it can prevent delta-9-THC from causing mouse rectal temperature to fall below 37°C (Pertwee & Tavendale, 1977). The mice were kept at 34°C for 3 h. This was because, in mice kept at normal room temperature, the hypothermic effect of the pretreatment dose of delta-9-THC used (20 mg kg^{-1}, i.p.) is known to last for this length of time (Gray *et al.*, 1987). As shown in Table 2, the degree of tolerance to delta-9-THC-induced hypothermia was little different in mice that had been kept at 34°C during part of the pretreatment period than in animals kept at normal room temperature throughout this period.

Tolerance to the inhibitory effect of delta-9-THC on the electrically-evoked twitch response of the mouse vas deferens

Delta-9-THC induced a concentration-dependent inhibition of the twitch response (Figure 5). Vasa deferentia removed from mice that had been pretreated twice with delta-9-THC at doses of 10 or 20 mg kg^{-1} day^{-1} i.p. were significantly less sensitive to the inhibitory effects of a submaximal concentration of this drug on electrically-evoked contractions (10 nM) than tissues obtained from control animals that had been pretreated with Tween 80 (20 or 40 mg kg^{-1} day^{-1}, i.p.). The inhibitory effect of delta-9-THC was 5.36 ± 5.1% in tissue taken from mice pre-treated with the higher dose of drug and 42.44 ± 5.49% in tissue removed from animals pretreated with the higher dose of vehicle ($n = 8$). In tissues obtained from mice pretreated with the lower dose of drug or vehicle, the inhibitory effects of delta-9-THC were respectively 27.6 ± 6.53% ($n = 7$) and 53.87 ± 7.06% ($n = 8$).

Tolerance to the inhibitory effect of delta-9-THC on the twitch response could also be produced by single drug pretreatment. Thus 10 nM delta-9-THC produced significantly less inhibition of the twitch response in vasa deferentia obtained from mice pretreated once with this drug at a dose of 20 mg kg^{-1}, i.p. (26.77 ± 6.46%; $n = 7$) than in tissues obtained from animals pretreated once with Tween 80 at a dose of 40 mg kg^{-1}, i.p. (47.09 ± 6.07%; $n = 6$). The same concentration of delta-9-THC was also significantly less inhibitory in vasa deferentia obtained from mice pretreated once with the drug at a dose of 10 mg kg^{-1}, i.p. (32.75 ± 4.91%; $n = 8$) than in tissues obtained from animals that had received a single injection of Tween 80 at a dose of 20 mg kg^{-1}, i.p. (53.01 ± 5.22%; $n = 8$).

Experiments directed at establishing the degree of tolerance induced in the vas deferens by intraperitoneal pretreatment with delta-9-THC over 2 days, showed that pretreatment with a dose of 20 mg kg^{-1} day^{-1} produced a marked decrease in the maximum inhibitory effect of delta-9-THC (Figure 6). There was no significant change in the slope of the lower part of the log concentration-response curve of the drug which underwent a 30 fold shift to the right (Table 3). In tissue obtained from mice pretreated twice with delta-9-THC at doses of 5 or 10 mg kg^{-1}, i.p., the log concentration-response curve of the drug was similarly affected, albeit to a smaller extent (Figure 6 and Table 3). Pretreatment with delta-9-THC at a dose of 2 mg kg^{-1} day^{-1} also produced significant tolerance. The degree of the tolerance produced by this pretreatment was less than that produced by doses of 5 or 10 mg kg^{-1} day^{-1} and the size of the maximum response remained unaffected (Figure 6 and Table 3).

No significant difference was observed between the amplitudes of contractions produced by electrical stimulation in tissue obtained from mice pretreated with delta-9-THC and those of contractions evoked in tissues obtained from vehicle-

Table 3 Effect of *in vivo* pretreatment with delta-9-tetrahydrocannabinol (delta-9-THC) on the potencies of certain cannabimimetic drugs as inhibitors of the electrically-evoked twitch response of the mouse isolated vas deferens

Pretreatment drug	*Pretreatment dose* (mg kg^{-1}, i.p.)	*Treatment drug*	*Treatment concentrations* (nM)	*Potency ratio*	*95% Confidence limits*	n
Delta-9-THC	2	Delta-9-THC	10, 100	2.62	1.11 and 5.86	8
Tween	40	Delta-9-THC	3.16, 31.6			
Delta-9-THC	5	Delta-9-THC	31.6, 316	7.42	2.17 and 21.88	8
Tween	40	Delta-9-THC	3.16, 31.6			
Delta-9-THC	10	Delta-9-THC	31.6, 100	13.35	5.32 and 55.78	8
Tween	40	Delta-9-THC	3.16, 10			
Delta-9-THC	20	Delta-9-THC	31.6, 100	30.01	17.46 and 83.30	12
Tween	40	Delta-9-THC	3.16, 10			
Delta-9-THC	20	CP 55,940	3.16, 31.6	8.74	4.30 and 17.33	6
Tween	40	CP 55,940	0.316, 3.16			
Delta-9-THC	20	WIN 55,212-2	3.16, 31.6	9.62	5.78 and 15.95	10
Tween	40	WIN 55,212-2	0.316, 3.16			
Delta-9-THC	20	Anandamide	100, 1000	12.3	6.38 and 24.59	6
Tween	40	Anandamide	10, 100			
None	-	Delta-9-THC	3.16, 31.6	0.94	0.41 and 2.15	8
Tween	40	Delta-9-THC	3.16, 31.6			

Mice were either unpretreated or pretreated intraperitoneally, once daily for 2 days, with delta-9-THC or with Tween 80. In the experiments in which mice were pretreated, the effects of treatment drugs, delta-9-THC, CP 55,940, WIN 55,212-2 and anandamide, on the twitch response of the vas deferens were measured measured 24 h after the second intraperitoneal injection. The final potency ratio listed in the Table indicates the extent by which the potency of delta-9-THC as an inhibitor of the twitch response was greater or less in tissues from unpretreated animals than in tissues from animals that had been pretreated with Tween 80. Pretreatment with Tween 80 did not lead to any significant change in the slope of the concentration-response curve of delta-9-THC. Each of the other potency ratios listed above indicates the extent by which pretreatment with delta-9-THC reduced the potency of a treatment drug as an inhibitor of the twitch response. None of the delta-9-THC-induced rightward shifts in the concentration-response curves of the treatment drugs deviated significantly from parallelism. Potency ratios, confidence limits and deviations of pairs of log dose-response curves from parallelism have been determined from symmetrical (2 + 2) dose parallel line assays and were calculated from data obtained with the treatment concentrations shown (Colquhoun, 1971).

pretreated animals. For example, mean twitch amplitudes measured immediately before drug administration were 0.33 ± 0.04 g in tissues obtained from mice pretreated twice with delta-9-THC at a dose 20 mg kg^{-1} and 0.28 ± 0.05 g in tissue obtained from animals pretreated twice with Tween 80 at a dose of 40 mg kg^{-1} ($n = 12$). The ability of delta-9-THC to depress the twitch response was similar in vasa deferentia taken from Tween 80 pretreated mice to that in tissue taken from animals that had received no pretreatment at all (Table 3).

Cross-tolerance between delta-9-THC and CP 55,940, WIN 55,212-2 and anandamide

CP 55,940, WIN 55,212-2 and anandamide shared the ability of delta-9-THC to produce a dose-dependent decrease in rectal temperature (Figure 2) and a concentration-dependent inhibition of the twitch response of the vas deferens (Figure 5). Pretreatment with two intraperitoneal injections of delta-9-THC at a dose of 20 mg kg^{-1} gave rise to significant parallel rightward shifts both in the log dose-hypothermic response curves of CP 55,940 and WIN 55,212-2 (Figure 3 and Table 1) and in the log concentration-response curves of these drugs for inhibition of the twitch response (Figure 6 and Table 3). The same pretreatment with delta-9-THC also significantly attenuated the ability of anandamide to inhibit the twitch response (Figure 6 and Table 3). However, this delta-9-THC pretreatment did not produce any detectable tolerance to anandamide-induced hypothermia (Figure 3 and Table 1).

Discussion

The results obtained in this investigation confirm previous reports that delta-9-THC, CP 55,940, WIN 55,212-2 and anandamide can produce marked decreases in mouse deep body temperature (Pertwee, 1985; Little *et al.*, 1988; Martin *et al.*, 1991; Fride & Mechoulam, 1993). In addition, the present results confirm that delta-9-THC, CP 55,940 and WIN 55,212-2 can induce a concentration-related inhibition of the electrically-evoked twitch response of the mouse vas deferens (Pacheco *et al.*, 1991; Pertwee *et al.*, 1992a,b; 1993). The ability of anandamide to inhibit the twitch response has also been reported previously (Devane *et al.*, 1992). However the earlier experiments were carried out with material extracted from pig brain whereas the samples of anandamide used in the present experiments were synthetic. The concentration-response curve of the synthetic compound (Figure 3) has proved to be essentially the same as that of the naturally occurring material, a finding that provides additional support for the conclusion drawn by Devane *et al.* (1992) that the substance extracted from the brain was indeed anandamide. The potencies of the drugs used in the present experiments exhibited the same rank order for inhibition of the twitch response of the vas deferens (Figure 5) as for the production both of hypothermia (Figure 2) and of certain other cannabimimetic effects *in vivo* (Martin *et al.*, 1991; Fride & Mechoulam, 1993): CP 55,940 > WIN 55,212-2 > delta-9-THC > anandamide. Consequently, the present data provide further support for the idea that the mouse isolated vas deferens is an appropriate preparation for investigating the central pharmacology of cannabinoids (Pertwee, 1993). It

Table 3 Effect of *in vivo* pretreatment with delta-9-tetrahydrocannabinol (delta-9-THC) on the potencies of certain cannabimimetic drugs as inhibitors of the electrically-evoked twitch response of the mouse isolated vas deferens

Pretreatment drug	*Pretreatment dose* (mg kg^{-1}, i.p.)	*Treatment drug*	*Treatment concentrations* (nM)	*Potency ratio*	*95% Confidence limits*	n
Delta-9-THC	2	Delta-9-THC	10, 100	2.62	1.11 and 5.86	8
Tween	40	Delta-9-THC	3.16, 31.6			
Delta-9-THC	5	Delta-9-THC	31.6, 316	7.42	2.17 and 21.88	8
Tween	40	Delta-9-THC	3.16, 31.6			
Delta-9-THC	10	Delta-9-THC	31.6, 100	13.35	5.32 and 55.78	8
Tween	40	Delta-9-THC	3.16, 10			
Delta-9-THC	20	Delta-9-THC	31.6, 100	30.01	17.46 and 83.30	12
Tween	40	Delta-9-THC	3.16, 10			
Delta-9-THC	20	CP 55,940	3.16, 31.6	8.74	4.30 and 17.33	6
Tween	40	CP 55,940	0.316, 3.16			
Delta-9-THC	20	WIN 55,212-2	3.16, 31.6	9.62	5.78 and 15.95	10
Tween	40	WIN 55,212-2	0.316, 3.16			
Delta-9-THC	20	Anandamide	100, 1000	12.3	6.38 and 24.59	6
Tween	40	Anandamide	10, 100			
				0.94	0.41 and 2.15	8
None	–	Delta-9-THC	3.16, 31.6			
Tween	40	Delta-9-THC	3.16, 31.6			

Mice were either unpretreated or pretreated intraperitoneally, once daily for 2 days, with delta-9-THC or with Tween 80. In the experiments in which mice were pretreated, the effects of treatment drugs, delta-9-THC, CP 55,940, WIN 55,212-2 and anandamide, on the twitch response of the vas deferens were measured measured 24 h after the second intraperitoneal injection. The final potency ratio listed in the Table indicates the extent by which the potency of delta-9-THC as an inhibitor of the twitch response was greater or less in tissues from unpretreated animals than in tissues from animals that had been pretreated with Tween 80. Pretreatment with Tween 80 did not lead to any significant change in the slope of the concentration-response curve of delta-9-THC. Each of the other potency ratios listed above indicates the extent by which pretreatment with delta-9-THC reduced the potency of a treatment drug as an inhibitor of the twitch response. None of the delta-9-THC-induced rightward shifts in the concentration-response curves of the treatment drugs deviated significantly from parallelism. Potency ratios, confidence limits and deviations of pairs of log dose-response curves from parallelism have been determined from symmetrical (2 + 2) dose parallel line assays and were calculated from data obtained with the treatment concentrations shown (Colquhoun, 1971).

should be noted that the potency of anandamide *in vivo* has previously only been compared in the same laboratory with that of delta-8-THC (Fride & Mechoulam, 1993). However, since delta-8-THC is known to be less potent than delta-9-THC as a psychotropic agent (Compton *et al.*, 1991), the finding by Fride & Mechoulam (1993) that anandamide is less potent than delta-8-THC constitutes evidence that it is also less potent than delta-9-THC.

The present results indicate that significant tolerance to the hypothermic effect of delta-9-THC can develop within 24 h of a single intraperitoneal injection of this drug. This rate of onset of tolerance to delta-9-THC is even greater than has been reported previously for the development of tolerance not only to delta-9-THC-induced hypothermia (Fitton & Pertwee, 1982) but also to other effects produced by this drug in mammalian species *in vivo* (Pertwee, 1991). It was found that tolerance could be induced both by pretreating mice with doses of delta-9-THC (10 and 20 mg kg^{-1}, i.p.) that are known to be hypothermic and by pretreating them with a dose (5 mg kg^{-1}, i.p.) that is essentially non-hypothermic at normal room temperature (Gray *et al.*, 1987). It was also found that tolerance to the hypothermic effect of delta-9-THC still develops when significant drug-induced decreases in deep body temperature are prevented during the pretreatment period by raising the ambient temperature. It seems unlikely, therefore, that mice need to experience delta-9-THC-induced hypothermia in order to become tolerant to this effect.

Tolerance to the inhibitory effect of delta-9-THC on the electrically-evoked twitch response of the mouse vas deferens was found to develop no less rapidly than tolerance to the drug's hypothermic effect. For both effects, the degree of tolerance produced was related to the size of the pretreatment dose of delta-9-THC used and to the number of pretreatments given, supporting the idea that the extent to which the tissues of the body are exposed to cannabinoids is an important factor in the development of cannabinoid tolerance (Pertwee, 1991). The mouse vas deferens is one of only two *in vitro* preparations in which it has been found possible to induce cannabinoid tolerance by prior *in vivo* cannabinoid administration, the other being the myenteric plexus-longitudinal muscle preparation of the mouse small intestine (Pertwee *et al.*, 1992b). The finding that the sensitivity of the mouse vas deferens to delta-9-THC can be attenuated by *in vivo* pretreatment with delta-9-THC suggests that this tissue is suitable as a model with which to explore the basis of cannabinoid tolerance. This idea is reinforced by the observation in the present investigation that *in vivo* pretreatment with delta-9-THC can produce tolerance not only to its own inhibitory effect on the twitch response of the vas deferens but also to that of three other drugs, CP 55,940, WIN 55,212-2 and anandamide (Figure 6), each of which is known to bind avidly to cannabinoid binding sites and to possess cannabimimetic pharmacological properties *in vivo* (Little *et al.*, 1988; Martin *et al.*, 1991; Pacheco *et al.*, 1991; Devane *et al.*, 1992; Howlett *et al.*, 1992; Jansen *et al.*, 1992; Fride & Mechoulam, 1993; Pertwee, 1993).

The demonstration that an isolated tissue can be made tolerant to psychotropic cannabinoids strengthens the hypothesis that cannabinoid tolerance is primarily pharmacodynamic in nature rather than dispositional or metabolic (Pertwee, 1991). The observation that cross tolerance can develop between delta-9-THC and CP 55,940, WIN 55,212-2 and anandamide in the vas deferens may constitute further evidence for this hypothesis if, as seems likely, these chemically quite unrelated compounds are not all inactivated by the same

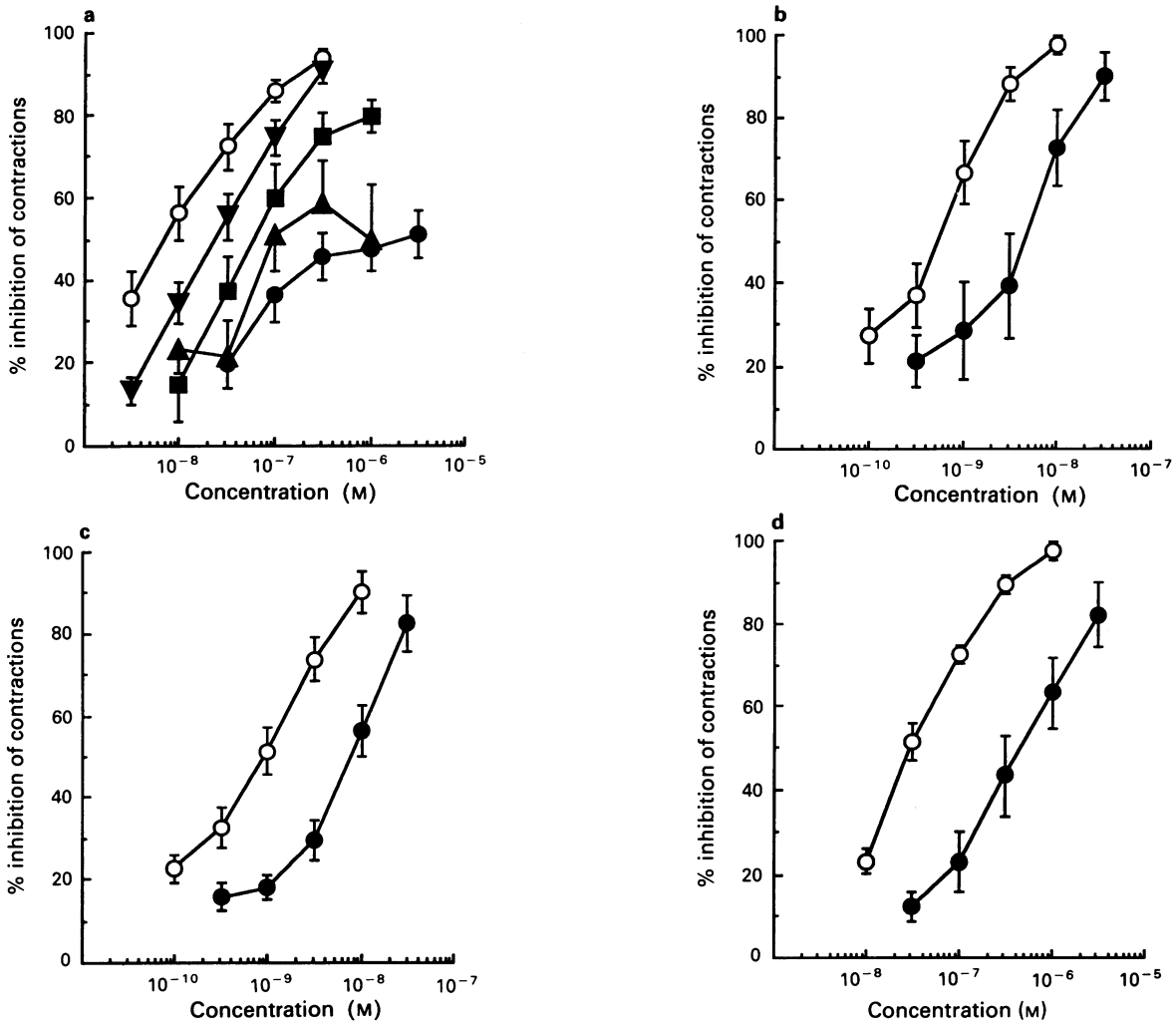


Figure 6 Mean cumulative concentration-response curves for (a) delta-9-tetrahydrocannabinol (delta-9-THC), (b) CP 55,940, (c) WIN 55,212-2, and (d) anandamide in isolated vasa deferentia obtained from mice pretreated once daily for 2 days with Tween 80 at a dose of 40 mg kg^{-1}, i.p. (○) or with delta-9-THC at doses of 2 mg kg^{-1}, i.p. (▼), 5 mg kg^{-1}, i.p. (■), 10 mg kg^{-1}, i.p. (▲) or 20 mg kg^{-1}, i.p. (●). Each symbol represents the mean value ± s.e.mean of inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of drug to the organ bath ($n = 6$ to 12 different vasa deferentia). The amplitudes of contractions evoked immediately before drug administration in tissues obtained from mice pretreated twice with delta-9-THC were not significantly different from those evoked in tissues obtained from vehicle-pretreated animals.

metabolic pathways. The same observation may also support the idea that delta-9-THC, CP 55,940, WIN 55,212-2 and anandamide share a common mode of action, the strength of this support depending on whether or not it transpires that cross tolerance also develops in the mouse vas deferens between delta-9-THC and inhibitors of the twitch response that do not possess cannabimimetic properties.

It was found that *in vivo* pretreatment with delta-9-THC induced significantly greater tolerance to its own inhibitory effect on the twitch response of the vas deferens than to that of CP 55,940 or WIN 55,212-2. It was also found that the degree of tolerance to delta-9-THC induced by such pretreatment was significantly greater for inhibition of the twitch response than for the production of hypothermia. The reasons for these differences remain to be established. It is worth noting, however, that there are already reports in the literature that tolerance develops more readily to some effects of cannabinoids than to others (Pertwee, 1991). Whilst the pretreatment with delta-9-THC that produced tolerance to the effects of delta-9-THC, CP 55,940 and WIN 55,212-2 on body temperature and on the twitch response of the vas deferens also produced tolerance to anandamide-induced inhibition of the twitch response, it did not produce any detectable tolerance to the effect of anandamide on body temperature. Given the hypothesis that anandamide is an endogenous cannabinoid it is important that the explanation for this apparent anomaly is found. One possibility is that the mode of action of anandamide for the production of hypothermia is different from that of the other cannabimimetic agents used in the present investigation, the compound perhaps achieving its hypothermic effect not only by interacting with cannabinoid receptors but also by acting through less specific mechanisms that do not develop tolerance to cannabimimetic agents. Consistent with this hypothesis are the present findings that the effective hypothermic dose-range of anandamide is rather high and that the log dose-hypothermic response curve of anandamide is somewhat shallower than that of delta-9-THC, CP 55,940 or WIN 55,212-2 (Figure 1).

We thank the Wellcome Trust for financial support, the National Institute on Drug Abuse, U.S.A., for delta-9-THC, Dr L.S. Melvin (Pfizer) for CP 55,940, Dr S.J. Ward (Sterling Winthrop) for WIN 55,212-2 and Professor R. Mechoulam (University of Jerusalem) for anandamide.

References

COLQUHOUN, D. (1971). *Lectures on Biostatistics*. Oxford: Oxford University Press.

COMPTON, D.R., PRESCOTT, W.R., MARTIN, B.R., SIEGEL, C., GORDON, P.M. & RAZDAN, R.K. (1991). Synthesis and pharmacological evaluation of ether and related analogues of delta-8, delta-9, and delta-9,11-tetrahydrocannabinol. *J. Med. Chem.*, **34**, 3310–3316.

DEVANE, W.A., HANUS, L., BREUER, A., PERTWEE, R.G., STEVENSON, L.A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*, **258**, 1946–1949.

DUNNETT, C.W. (1964). New tables for multiple comparisons with a control. *Biometrics*, **20**, 482–491.

FITTON, A.G. & PERTWEE, R.G. (1982). Changes in body temperature and oxygen consumption rate of conscious mice produced by intrahypothalamic and intracerebroventricular injections of delta-9-tetrahydrocannabinol. *Br. J. Pharmacol.*, **75**, 409–414.

FRIDE, E. & MECHOULAM, R. (1993). Pharmacological activity of the cannabinoid receptor agonist, anandamide, a brain constituent. *Eur. J. Pharmacol.*, **231**, 313–314.

GRAY, G.A., HEDLEY, D. & PERTWEE, R.G. (1987). Enhancement of the hypothermic response of mice to delta-9-tetrahydrocannabinol by subhypothermic doses of chlorpromazine and phentolamine. *Neuropharmacology*, **26**, 229–235.

GRIFFIN, G. & PERTWEE, R.G. (1993). Vasa deferentia obtained from mice pretreated with delta-9-tetrahydrocannabinol (THC) show tolerance to delta-9-THC, WIN 55,212-2 and CP 55,490. *Br. J. Pharmacol.*, **109**, 105P.

HOWLETT, A.C., EVANS, D.M. & HOUSTON, D.B. (1992). The cannabinoid receptor. In *Marijuana/Cannabinoids. Neurobiology and Neurophysiology*. ed. Murphy, L. & Bartke, A. pp. 35–72. Boca Raton: CRC Press.

JANSEN, E.M., HAYCOCK, D.A., WARD, S.J. & SEYBOLD, V.S. (1992). Distribution of cannabinoid receptors in rat brain determined with aminoalkylindoles. *Brain Research*, **575**, 93–102.

LITTLE, P.J., COMPTON, D.R., JOHNSON, M.R., MELVIN, L.S. & MARTIN, B.R. (1988). Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. *J. Pharmacol. Exp. Ther.*, **247**, 1046–1051.

MARTIN, B.R., COMPTON, D.R., THOMAS, B.F., PRESCOTT, W.R., LITTLE, P.J., RAZDAN, R.K., JOHNSON, M.R., MELVIN, L.S., MECHOULAM, R. & WARD, S.J. (1991). Behavioural, biochemical, and molecular modeling evaluations of cannabinoid analogs. *Pharmacol. Biochem. Behav.*, **40**, 471–478.

PACHECO, M., CHILDERS, S.R., ARNOLD, R., CASIANO, F. & WARD, S.J. (1991). Aminoalkylindoles: actions on specific G-protein-linked receptors. *J. Pharmacol. Exp. Ther.*, **257**, 170–183.

PERTWEE, R.G. (1985). Effects of cannabinoids on thermoregulation: a brief review. In *Marihuana '84*. ed. Harvey, D.J. pp. 263–277. Oxford: IRL Press.

PERTWEE, R.G. (1991). Tolerance to and dependence on psychotropic cannabinoids. In *The Biological Bases of Drug Tolerance and Dependence*. ed. Pratt, J.A. pp. 231–263. London: Academic Press.

PERTWEE, R.G. (1993). The evidence for the existence of cannabinoid receptors. *Gen. Pharmacol.*, **24**, 811–824.

PERTWEE, R.G., STEVENSON, L.A., ELRICK, D.B., MECHOULAM, R. & CORBETT, A.D. (1992a). Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br. J. Pharmacol.*, **105**, 980–984.

PERTWEE, R.G., STEVENSON, L.A., FERNANDO, S.R. & CORBETT, A.D. (1992b). The production of cannabinoid tolerance in preparations of mouse vas deferens and of myenteric plexus-longitudinal muscle of mouse small intestine. *Br. J. Pharmacol.*, **107**, 365P.

PERTWEE, R.G., STEVENSON, L.A., FERNANDO, S.R. & CORBETT, A.D. (1993). *In vitro* effects of the cannabinoid, CP 55,940, and of its (+)-enantiomer, CP 56,667. In *Problems of Drug Dependence, 1992: Proceeding of the 54th Annual Scientific Meeting of the College on Problems of Drug Dependence, Inc.: National Institute on Drug Abuse Research Monograph Series, Research Monograph*, 132. ed. Harris, L. p. 374. Rockville: National Institute on Drug Abuse.

PERTWEE, R.G. & TAVENDALE, R. (1977). Effects of delta-9-tetrahydrocannabinol on the rates of oxygen consumption of mice. *Br. J. Pharmacol.*, **60**, 559–568.

(Received August 4, 1993)
Accepted September 1, 1993)

Br. J. Pharmacol. (1993), **110**, 1491–1495 © Macmillan Press Ltd, 1993

PPADS selectively antagonizes P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder

*†Airat U. Ziganshin, *Charles H.V. Hoyle, *Xuenong Bo, **Günter Lambrecht, **Ernst Mutschler, ††Hans G. Bäumert & [1,]*Geoffrey Burnstock

*Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower Street, London, WC1E 6BT; †Kazan Medical Institute, 49 Butlerov Street, Kazan, 420012, Russia; Departments of **Pharmacology and ††Biochemistry, University of Frankfurt, Theodor-Stern-Kai 7, Geb. 75A, D-6000 Frankfurt/M, Germany

1 Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), an inhibitor of P_{2X}-purinoceptor-mediated responses in rabbit vas deferens, was investigated for its ability to antagonize contractions evoked by α,β-methylene ATP (α,β-MeATP), carbachol and electrical field stimulation in the rabbit urinary bladder detrusor muscle.

2 PPADS (1–30 μM) caused concentration-dependent inhibition of contractions to the stable P_{2X}-purinoceptor agonist, α,β-MeATP, decreasing the maximum response to α,β-MeATP (30 μM) at concentrations of 3–30 μM. The pD_2 value for α,β-MeATP in the absence of PPADS was 6.52 ± 0.10 (8). In the presence of PPADS at concentrations of 1, 3, 10 and 30 μM the negative log concentrations of α,β-MeATP that cause the same contractile response as the pD_2 value were significantly different from control, being respectively 6.17 ± 0.09 (8), 5.64 ± 0.12 (7), 5.15 ± 0.23 (7) and 4.78 ± 0.22 (5).

3 PPADS (1–30 μM) caused concentration-dependent inhibition of contractions to stimulation of intramural purinergic nerves (1–32 Hz). There was a greater inhibition at lower frequencies (1–8 Hz) than at higher frequencies (16–32 Hz). PPADS, 30 μM, did not produce significantly greater antagonism than 10 μM.

4 PPADS (30 μM) had no significant influence on the contractile potency of carbachol: the pD_2 values of carbachol in the absence and presence of PPADS were not significantly different being 6.42 ± 0.16 (5) and 6.33 ± 0.18 (5), respectively. However, PPADS caused a small, but significant, suppression of the maximal response of carbachol, reducing it by approximately 9%.

5 Radioligand binding studies carried out on rabbit bladder membranes with [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) showed that PPADS concentration-dependently inhibited the binding of [^{3}H]-α,β-MeATP to P_{2X}-purinoceptors, while the binding of [^{3}H]-quinuclidinyl benzilate to muscarinic cholinoceptors was not affected.

6 Thus, PPADS (1–30 μM) antagonized responses mediated via P_{2X}-purinoceptors in the rabbit urinary bladder. It was selective for P_2-purinoceptor-mediated contractions rather than those mediated via muscarinic receptors. Binding studies demonstrated that the antagonistic effect of PPADS is via a direct interaction with P_{2X}-purinoceptors.

Keywords: PPADS; α,β-methylene ATP; [^{3}H]-α,β-methylene ATP; P_{2X}-purinoceptors; carbachol; [^{3}H]-quinuclidinyl benzilate; electrical field stimulation; rabbit urinary bladder

Introduction

It is widely accepted that there are two major types of purinoceptors, P_1 (mainly responsive to adenosine) and P_2 (mainly responsive to ATP) (Burnstock, 1978; 1990; Hoyle, 1992). They are primarily distinguished by different agonist and antagonist profiles, and by different transduction mechanisms (Burnstock & Buckley, 1985). P_2-purinoceptors have been further subdivided into P_{2X}- and P_{2Y}-receptors based largely on the rank order of agonist potency of adenosine triphosphate (ATP) analogues (Burnstock & Kennedy, 1985); in addition, several other subtypes of P_2-purinoceptors have been defined, including P_{2T}- and P_{2Z}-receptors (see Gordon, 1986; Hoyle, 1992). There are potent and selective antagonists of P_1-purinoceptors such as 8-phenyltheophylline and related compounds (Cooper & Londos, 1988; Williams, 1991), whereas a more definitive characterization of P_2-purinoceptor subtypes continues to be hindered by the lack of potent, selective and competitive antagonists. Suramin displays a fairly specific antagonistic activity at P_2-purinoceptors but only at concentrations higher than 10 μM (Hoyle *et al.*, 1990). It does not discriminate between P_{2X}- and P_{2Y}-purinoceptors. Reactive blue 2 is only useful within a narrow range of concentrations for blocking selectively P_{2Y}-purinoceptors, above which it has non-specific effects (Burnstock & Warland, 1987). Desensitization by α,β-methylene ATP (α,β-MeATP) is generally specific for P_{2X}-purinoceptors (Kasakov & Burnstock, 1983), and although it has been used successfully in many preparations, it is not a competitive antagonist. Arylazidoaminopropionyl ATP ($ANAPP_3$) forms a covalent attachment to the P_{2X}-purinoceptor following irradiation with ultraviolet light (Hogaboom *et al.*, 1980) and is also not competitive; further, the irreversible nature of its action limits its use in studying subtypes of the P_2-purinoceptor.

It has long been observed that at least two transmitters are involved in excitatory neuromuscular transmission in mammalian urinary bladder, and that acetylcholine and ATP are the predominant excitatory neurotransmitters mediating cholinergic, possibly via muscarinic M_3-receptors (Noronha-Blob *et al.*, 1991) and purinergic responses via P_{2X}-purinoceptors, respectively (Burnstock & Kennedy, 1985; Fujii, 1988; Hoyle *et al.*, 1990; Bo & Burnstock, 1992; Kishii *et al.*, 1992; Sneddon & McLees, 1992). The purinergic component of neurogenic contractions in mammalian urinary bladder strips can be blocked by the trypanocidal agent, suramin, albeit with low potency (Hoyle *et al.*, 1990), the

[1] Author for correspondence.

photoaffinity ATP analogue, $ANAPP_3$ (Theobald, 1986) or by desensitization of P_{2X}-purinoceptors with α,β-MeATP (Kasakov & Burnstock, 1983; Hoyle & Burnstock, 1985; Moss & Burnstock, 1985; Hoyle et al., 1989; Sneddon & McLees, 1992).

In the search for more potent and more selective antagonists of P_2-purinoceptors, Lambrecht et al. (1992) examined the activity of pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on contractile responses of the rabbit vas deferens to α,β-MeATP and to electrical field stimulation. They found PPADS (1–10 μM) to be an apparently selective inhibitor of P_{2X}-purinoceptor-mediated responses. With these observations in mind, the current study was undertaken in order to evaluate the antagonistic properties of PPADS in another tissue of the same species with excitatory P_{2X}-purinoceptors, the rabbit urinary bladder (Fujii, 1988; Bo & Burnstock, 1992; Kishii et al., 1992; Sneddon & McLees, 1992). PPADS was examined for interactions with exogenous α,β-MeATP and for effects on purinergic neuromuscular transmission. Radioligand binding assays were also carried out to study the interaction of PPADS with P_{2X}-purinoceptors as well as its selectivity for this type of receptor.

Methods

Organ bath experiments

Male New Zealand White rabbits (2–3 kg) were killed by an overdose of sodium pentobarbitone (Sagatal), which was injected via the marginal ear vein, and exsanguination. The urinary bladder was removed and longitudinal strips of the detrusor muscle, approximately 2 by 15 mm, were prepared. The strips were suspended vertically in 10 ml organ baths for isometric recording of mechanical activity. An initial load of 1 g was applied to the strips which were then allowed to equilibrate for at least 60 min. Electrical field stimulation was applied via two platinum-wire rings 2.5 mm in diameter, 10 mm apart, through which the strip was threaded. The modified Krebs solution for these experiments had the following composition (mM): NaCl 133, KCl 4.7, $NaHCO_3$ 16.4, $MgSO_4$ 0.6, NaH_2PO_4 0.8, $CaCl_2$ 2.5 and glucose 7.7, and was gassed with 95% O_2/5% CO_2 (pH 7.3–7.4) and was maintained at 37 ± 1°C. In experiments with electrical field stimulation, atropine (0.3 μM) was also included in the Krebs solution. Contractions were recorded by either a Dynamometer UF1 or Grass FTOC3 force-displacement transducer, and displayed on a Grass 79D ink-writing oscillograph. Electrical field stimulation was provided by a Grass S9 stimulator and applied at a given frequency (1–32 Hz) with a pulse width of 0.5 ms and supramaximal voltage, until a maximum contraction had been reached and the tone had declined by about a third. In the experiments with exogenous agonists, single doses of α,β-MeATP (0.1–30 μM) or carbachol were added directly to the organ bath and were washed out after a maximum contraction had been reached. Intervals of at least 30 min, with a wash every 10 min, were allowed between consecutive applications of α,β-MeATP in order to avoid desensitization, otherwise at least 10 min were allowed for washout. Frequency-response and concentration-response relationships for α,β-MeATP were examined in preparations before and after incubation with PPADS (1–30 μM, minimum 20 min). Carbachol (0.03–100 μM) was tested in parallel on two preparations from each animal. After the first concentration-response curves were constructed on both preparations, one of them was incubated with 30 μM PPADS (at least 20 min), and then the second concentration-response curves were constructed. Thus, the preparation to which PPADS had not been added, was used as a time-control for carbachol.

Binding experiments

The rabbits were killed as described above. Urinary bladders were removed immediately and placed in modified Krebs solution. They were trimmed free from surrounding adipose and connective tissues, minced and homogenized in 10 volumes of ice-cold 50 mM Tris/HCl buffer including various protease inhibitors (1 mM ethylene glycol-*bis*(β-aminoethyl ether) (EGTA), 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.01% bacitracin, and 0.002% soybean trypsin inhibitors, pH 7.4) with a Polytron PT-3000 homogenizer (Kinematica, Switzerland) twice in 15 s bursts at 30,000 r.p.m. The homogenate was centrifuged at 170 *g* for 5 min at 4°C. The supernatant was passed through double layers of nylon mesh and centrifuged at 105,000 *g* for 50 min at 4°C. The pellets were washed and suspended in the buffer for binding assay. The protein content in the membrane preparations was measured with the method described by Peterson (1977).

The displacement experiments on P_{2X}-purinoceptors were performed in a final volume of 0.5 ml including 10 nM [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP), 20 μg membrane proteins, and PPADS in various concentrations. The incubation was carried out at 4°C for 2 h. The reaction was terminated by rapid filtration of the mixture through Whatman GF/B glass fibre filters presoaked in 20 mM disodium pyrophosphate under vacuum. The filters were washed with two aliquots of 5 ml ice-cold Tris/HCl buffer, dried under an infrared lamp, and then put into a mini-scintillation vial. To each vial, 4 ml OptiPhase 'Hi-Safe' II was added, and the radioactivity trapped in the filters was measured in a Beckman SC6100I scintillation counter with an efficiency of about 56 to 61%. Non-specific binding was determined with 100 μM β,γ-methylene ATP. Each assay was carried out in triplicate and five assays were performed.

The basic procedure for the displacement experiment on muscarinic cholinoceptors was the same as that for P_{2X}-purinoceptors. Final [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) concentration was 0.5 nM, the filters were presoaked in Tris/HCl buffer, and the non-specific binding was determined with 10 μM atropine.

Drugs used

Atropine sulphate was obtained from Phoenix Pharmaceuticals Ltd. α,β-Methylene ATP lithium salt, carbamyl-β-methyl choline chloride (carbachol), β,γ-methylene ATP sodium salt, EGTA, benzamidine, PMSF, bacitracin, soybean trypsin inhibitors and Folin reagents were obtained from Sigma Chemical Co. Ltd. Sagatal was supplied by May and Baker. [^{3}H]-α,β-MeATP was custom-synthesized by Amersham International plc. (Amersham, U.K.), with a specific activity of 19.2 Ci mmol^{-1} and chemical purity of 98–99%. [^{3}H]-QNB with a specific activity of 44 Ci mmol^{-1} was purchased from the same company. PPADS was synthesized in one of our laboratories.

Analysis of results

Means were compared by Student's paired and unpaired *t* tests. A probability of less than or equal to 0.05 was considered as significant. The data from binding studies were analysed with a computer programme EBDA-LIGAND (Munson & Rodbard, 1980). The models for one or two binding sites were chosen based on the results of the *F*-test on the weighted sum of squares. Data are presented as mean ± s.e.mean (*n*).

In the experiments with carbachol, responses were calculated as the percentage of the maximal carbachol contraction. The mean response of the preparations from each animal was calculated at each concentration of carbachol used. These mean responses for each animal were then subjected to a probit transformation (Bliss, 1935; Finney, 1971)

which converts the sigmoidal log-concentration curve to a straight line. Linear regression of the probit values against log concentration was then carried out in order to interpolate the log-concentration yielding a 50% response i.e. the pD_2 (Hoyle & Greenberg, 1988).

Results

In isolated strips of detrusor muscle, α,β-MeATP (0.1–30 μM) caused concentration-dependent contractions with a maximum response of 55.1 ± 3.2% (9) relative to the maximum contraction evoked by KCl at a concentration of 240 mM (Figure 1). The pD_2 value (negative log EC_{50}) was 6.52 ± 0.10 (9). PPADS at 1–30 μM caused concentration-dependent shifts to the right of the α,β-MeATP concentration-response curve. The negative log[α,β-MeATP], producing a contractile response in the presence of PPADS equivalent to that produced by the control pD_2 value was 6.17 ± 0.09 (8), 5.64 ± 0.12 (7) and 5.15 ± 0.23 (7) at PPADS concentrations of 1, 3 and 10 μM, respectively. All these values were significantly different both from control and from each other ($P<0.05$, paired and unpaired t tests). However, the − log[α,β-MeATP] value of 4.78 ± 0.22 (5) obtained at 30 μM PPADS was not significantly different from that at 10 μM.

In the presence of atropine (0.3 μM), electrical field stimulation produced frequency-dependent contractions of the rabbit isolated detrusor muscle with maximum at a frequency of 8 Hz (Figure 2). PPADS (1–30 μM) caused concentration-dependent inhibition of contractions evoked by electrical field stimulation. At a concentration of 1 μM, PPADS inhibited contractile responses only at lower frequencies (2–8 Hz). At a concentration of 3 μM, the significant inhibition of contractile responses to electrical field stimulation by PPADS extended up to 16 Hz. PPADS at 10 and 30 μM significantly inhibited neurogenic contractions of detrusor muscle at all frequencies (1–32 Hz). However, there was no significant difference between the inhibitions produced by PPADS at these two concentrations. PPADS at all applied concentrations (1–30 μM) caused a greater inhibition at lower stimulation frequencies (1–8 Hz) than at higher stimulation frequencies (16 and 32 Hz),

Carbachol (0.03–100 μM) caused concentration-dependent contractions of the detrusor muscle with a maximum contraction of 130.8 ± 5.2% (5) relative to that evoked by KCl

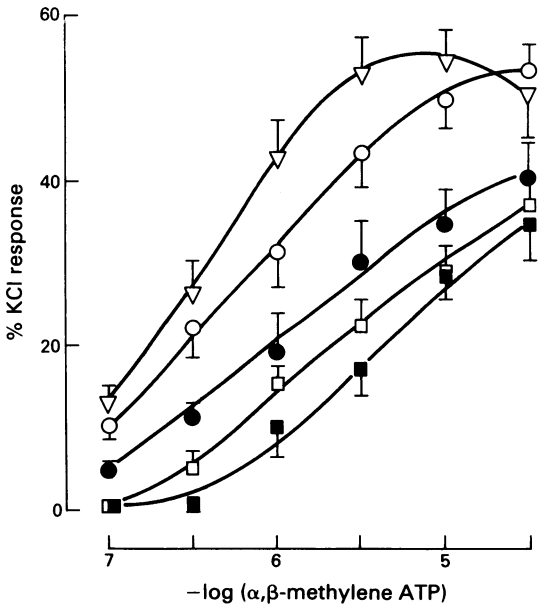


Figure 1 Rabbit urinary bladder, isolated detrusor muscle: concentration-response relationships for α,β-methylene ATP (α,β-MeATP) in the presence and absence of PPADS. Responses (ordinate axis) are expressed as percentage relative to the maximum contraction to 240 mM KCl. Concentration-response curves are shown for α,β-MeATP in the absence (▽, $n=9$) and presence of PPADS at concentrations of 1 μM (○, $n=8$), 3 μM (●, $n=8$), 10 μM (□, $n=8$) and 30 μM (■, $n=5$). The points represent the mean ± s.e.mean.

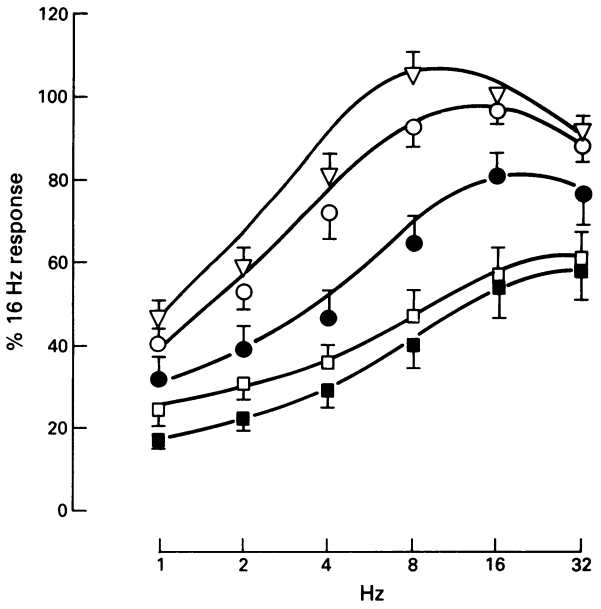


Figure 2 Rabbit urinary bladder, isolated detrusor muscle. Frequency-response relationships. Responses (ordinate axis) are expressed as a percentage of contraction evoked by electrical field stimulation at a frequency of 16 Hz in controls. Frequency-response curves are shown in the absence (▽, $n=9$) and presence of PPADS at concentrations of 1 μM (○, $n=8$), 3 μM (●, $n=8$), 10 μM (□, $n=7$) and 30 μM (■, $n=7$). Atropine (0.3 μM) was present throughout. Points show mean ± s.e.mean. The abscissae are on a logarithmic scale.

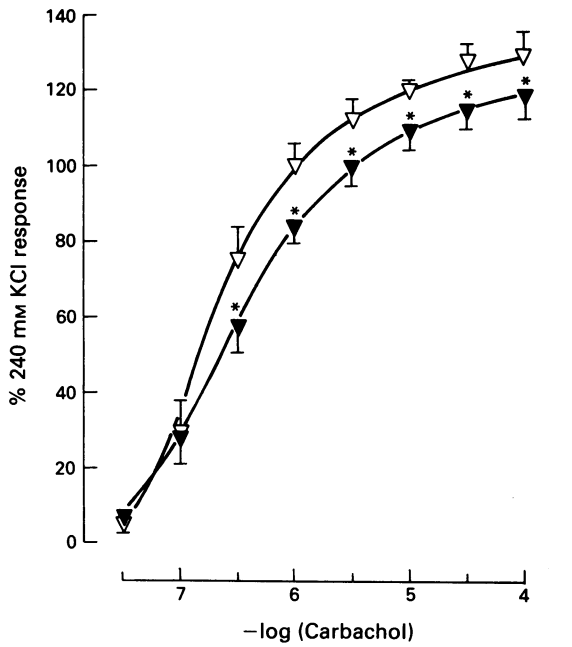


Figure 3 Rabbit urinary bladder, isolated detrusor muscle: concentration-response curves for carbachol in the absence (▽, $n=5$) and in the presence of PPADS at 30 μM (▼, $n=5$). Points show the mean ± s.e.mean. Responses are expressed relative to the contracture produced by a standard concentration of KCl (240 mM). *$P<0.05$.

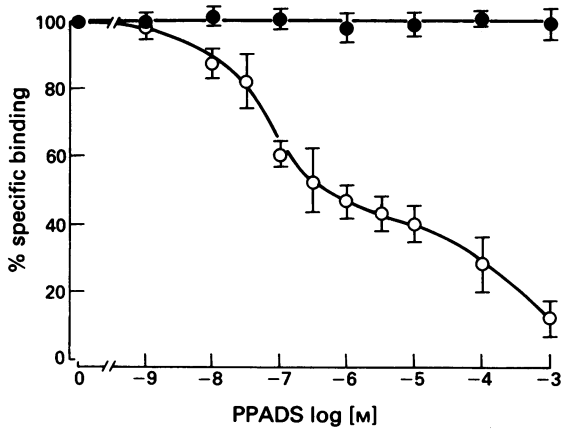


Figure 4 Displacement of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) (○) and [^{3}H]-quinuclidinylbenzilate ([^{3}H]-QNB) (●) binding to rabbit urinary bladder membranes by PPADS. Membrane preparations of rabbit urinary bladder were incubated with 10 nM [^{3}H]-α,β-MeATP or 0.5 nM [^{3}H]-QNB in the presence of various concentrations of PPADS. Non-specific binding was determined with 100 μM β,γ-methylene ATP or 10 μM atropine respectively. Data shown are mean ± s.e.mean from five triplicate experiments.

at a concentration of 240 mM. In the time-control preparations, there was no significant difference between the first and the second pD_2 values for carbachol being 6.38 ± 0.13 (5) and 6.36 ± 0.11 (5), respectively. PPADS (30 μM) did not significantly affect the pD_2 values of carbachol which were 6.42 ± 0.14 (5) and 6.37 ± 0.18 (5) in the absence and presence of PPADS, respectively. However, there was a small but significant decrease of the maximum carbachol contraction in the presence of PPADS which was 119.6 ± 5.6% (5) ($P<0.05$, paired t test) relative to that evoked by 240 mM KCl (Figure 3).

PPADS at any concentration did not significantly change the basal tone or spontaneous activity of the rabbit urinary bladder. The effect of PPADS (30 μM) did not appear to be readily reversible. After prolonged wash-out (> 2 h) the responses to electrical field stimulation and α,β-MeATP recovered only by 15–20%.

The displacement of [^{3}H]-α,β-MeATP binding to rabbit bladder membrane preparations by PPADS was concentration-dependent (Figure 4). Curvilinear fitting of the data revealed that PPADS interacted with the two affinity states of the P_{2X}-purinoceptor for [^{3}H]-α,β-MeATP with K_i values of $2.2 \pm 0.3 \times 10^{-8}$ M (5) and $4.8 \pm 1.1 \times 10^{-5}$ M (5), respectively. PPADS showed no obvious effect on the binding of [^{3}H]-QNB to muscarinic cholinoceptors in the rabbit bladder membranes (Figure 4).

Discussion

The results show that PPADS had an antagonistic effect on P_{2X}-purinoceptors in the rabbit urinary bladder detrusor muscle. The action of PPADS seemed to be selective for the P_{2X}-purinoceptors, since there was no change in the potency of carbachol for contracting the bladder detrusor muscle via muscarinic receptors, and no displacement of specific [^{3}H]-QNB binding to muscarinic receptors, although there was a small decrease in the efficacy of carbachol to contract the detrusor muscle. These results are consistent with those described for the action of PPADS in rabbit vas deferens (Lambrecht *et al.*, 1992).

PPADS at a concentration of 1 μM, significantly affected the concentration-response relationship to α,β-MeATP, but caused significant inhibition of the purinergic contractions evoked by electrical field stimulation only at 2–8 Hz. At concentrations of 3 and 10 μM, PPADS caused a greater antagonism of P_{2X}-purinoceptor-mediated responses evoked either by exogenous α,β-MeATP or by purinergic nerve stimulation. It seems that PPADS had a maximum antagonistic effect on P_{2X}-purinoceptors in bladder detrusor muscle at a concentration around 10 μM, since there was no significant difference between the inhibition caused by PPADS at 10 and 30 μM in experiments with either α,β-MeATP or electrical field stimulation.

Mammalian urinary bladder detrusor muscle is provided with parasympathetic excitatory innervation and acetylcholine is a neurotransmitter in these nerves. However, it is well known, that the muscarinic antagonist atropine is unable to abolish the excitatory response induced by nerve stimulation (Langley & Anderson, 1895; Ursillo, 1961; Longhurst *et al.*, 1984). It has been suggested that the acetylcholine-induced contraction of bladder muscle is mediated via muscarinic M_3-receptors (Noronha-Blob *et al.*, 1991) and that the atropine-resistant component is purinergic with ATP as the principle neurotransmitter (Burnstock *et al.*, 1978; Hoyle & Burnstock, 1985; Fujii, 1988). The purinoceptors that mediate the excitatory mechanical responses of rabbit (and other mammalian species) bladder to field stimulation and to exogenous ATP and related agonists were classified as of the P_{2X}-subtype, based on the relative potencies of ATP and its analogues (α,β-MeATP > β,γ-methylene ATP > ATP) (Burnstock & Kennedy, 1985; Kishii *et al.*, 1992; Sneddon & McLees, 1992), on the selective binding of [^{3}H]-α,β-MeATP to rabbit bladder smooth muscle (Bo & Burnstock, 1992), on selective desensitization by α,β-MeATP of ATP-mediated responses (Chen, 1990; Sneddon & McLees, 1992), and on the inability of the putative P_{2Y}-purinoceptor antagonist, reactive blue 2, to antagonize ATP-induced contraction (Kishii *et al.*, 1992).

In our experiments with electrical field stimulation, atropine (0.3 μM) was present in the Krebs solution to exclude cholinergic transmission. Thus, inhibition by PPADS of neurogenic contractions was due to antagonism of the purinergic component. Lambrecht *et al.* (1992) have reported that PPADS is an antagonist of P_{2X}-purinoceptor-mediated responses in the rabbit vas deferens. At concentrations of 30 and 10 μM, it abolished contractions of the rabbit vas deferens evoked by electrical field stimulation in the presence of prazosin (1 μM) and to exogenous α,β-MeATP (up to 100 μM), respectively. In the rabbit urinary bladder, PPADS (up to 30 μM) did not abolish the neurogenic contractions or the contractile responses to α,β-MeATP. This implies that there may be some differences between the P_{2X}-purinoceptors in rabbit vas deferens and urinary bladder, as has already been noted amongst different species (Hoyle & Burnstock, 1991; Bo & Burnstock, 1992; Hoyle, 1992; Theobald, 1992). In addition, the resistance of a significant level of the contraction to α,β-MeATP and electrical field stimulation, shown by PPADS, in rabbit bladder, could possibly be due to the presence of multiple excitatory P_2-purinoceptor subtypes (Theobald, 1992), at least one of which is resistant to PPADS. Thus, further experiments with PPADS on other tissues exhibiting clear-cut P_2-purinoceptor subtype-mediated responses could be useful for a more complete characterization of the P_2-purinoceptors in rabbit bladder.

[^{3}H]-α,β-MeATP has been proved to bind preferentially to P_{2X}-purinoceptors in radioligand binding assays and autoradiographic studies (Bo & Burnstock, 1989; 1990). In this study, PPADS was shown to displace competitively the binding of [^{3}H]-α,β-MeATP to rabbit bladder membranes, which indicates that PPADS acts directly on P_{2X}-purinoceptors. In the rabbit bladder membrane preparations it has been demonstrated that [^{3}H]-α,β-MeATP binds to two affinity states of P_{2X}-purinoceptors (Bo & Burnstock, 1992). Computer-assisted curve-fitting of the data revealed that PPADS displaced the [^{3}H]-α,β-MeATP binding to rabbit bladder membranes also from two affinity states, thus confirming the observation from the saturation assays. PPADS showed no

effects on [^{3}H]-QNB binding to muscarinic cholinoceptors in rabbit bladder membranes in the range of concentrations tested. Therefore, the observed slight inhibition of cholinergic responses by PPADS was via mechanisms other than a direct action on muscarinic cholinoceptors.

In conclusion, PPADS (1–30 μM) blocks P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder. Such an action of PPADS is due to its direct antagonism of P_{2X}-purinoceptors. If further studies clarify the subtype-selectivity, this compound might become the starting point for the development of a new generation of selective purinoceptor antagonists.

The authors thank the Wellcome Trust (A.U.Z.), the British Heart Foundation (X.B.), the Fonds der Chemischen Industrie (Germany) (G.L., E.M.) and the Deutsche Forschungsgemeinschaft (H.G.B., SFB 169) for financial support.

References

BLISS, C.I. (1935). The calculation of the dosage-mortality curve. *Ann. Appl. Biol.*, **22**, 134–167.

BO, X. & BURNSTOCK, G. (1989). [^{3}H]α,β-Methylene ATP: a radioligand for P_2-purinoceptors. *J. Auton. Nerv. Syst.*, **28**, 85–88.

BO, X. & BURNSTOCK, G. (1990). High- and low-affinity binding sites for [^{3}H]-α,β-methylene ATP in rat urinary bladder membranes. *Br. J. Pharmacol.*, **101**, 291–296.

BO, X. & BURNSTOCK, G. (1992). Species differences in characteristics and distribution of [^{3}H]α,β-methylene ATP binding sites in urinary bladder and urethra of rat, guinea-pig and rabbit. *Eur. J. Pharmacol.*, **216**, 59–66.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach.* ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. (1990). Classification and characterization of purinoceptors. In *Purines in Cellular Signalling – Targets for New Drugs.* ed. Jacobson, K.A., Daly, J.W. & Manganiello, V. pp. 241–253. New York: Springer-Verlag.

BURNSTOCK, G. & BUCKLEY, N.J. (1985). The classification of receptors for adenosine and adenine nucleotides. In *Methods Used in Adenosine Research (Methods in Pharmacology Series).* ed. Paton, D.M. pp.193–212. New York: Raven Press.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & WARLAND, J.J.I. (1987). P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P_{2Y}- but not P_{2X}-purinoceptor. *Br. J. Pharmacol.*, **90**, 383–391.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **63**, 125–138.

CHEN, H.I. (1990). Evidence for the presynaptic action of 5-hydroxytryptamine and the involvement of purinergic innervation in the rabbit lower urinary tract. *Br. J. Pharmacol.*, **101**, 212–216.

COOPER, D.M.F. & LONDOS, C. (1988). *Receptor Biochemistry and Methodology*, Vol. II: *Adenosine Receptors.* New York: Alan R. Liss Inc.

FINNEY, D.J. (1971). *Probit Analysis.* 3rd edition. Cambridge: Cambridge University Press.

FUJII, K. (1988). Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig, rabbit and pig urinary bladder. *J. Physiol.*, **404**, 39–52.

GORDON, J.L. (1986). Extracellular ATP: effects, sources and fate. *Biochem. J.*, **233**, 309–319.

HOGABOOM, G.K., O'DONNELL, J.P. & FEDAN, J.S. (1980). Purinergic receptors: a photoaffinity analog of adenosine triphosphate is a specific adenosine triphosphate antagonist. *Science*, **208**, 1273–1276.

HOYLE, C.H.V. (1992). Transmission: purines. In *Autonomic Neuroeffector Mechanisms.* ed. Burnstock, G. & Hoyle, C.H.V. pp. 367–407. Chur: Harwood Academic Publishers.

HOYLE, C.H.V. & BURNSTOCK, G. (1985). Atropine-resistant excitatory junction potentials in rabbit bladder are blocked by α,β-methylene ATP. *Eur. J. Pharmacol.*, **114**, 239–240.

HOYLE, C.H.V. & BURNSTOCK, G. (1991). ATP receptors and their physiological roles. In *Adenosine in the Nervous System.* ed. Stone, T.W. pp. 43–76. London: Academic Press.

HOYLE, C.H.V., CHAPPLE, C. & BURNSTOCK, G. (1989). Isolated human bladder: evidence for an adenine dinucleotide acting on $P_{2\alpha}$-purinoceptors and for purinergic transmission. *Eur. J. Pharmacol.*, **174**, 115–119.

HOYLE, C.H.V. & GREENBERG, M. (1988). Actions of adenyl compounds in invertebrates from several phyla: evidence for internal purinoceptors. *Comp. Biochem. Physiol.*, **90C**, 113–122.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

KASAKOV, L. & BURNSTOCK, G. (1983). The use of the slowly degradable analog, α,β-methylene ATP, to produce desensitization of the P_2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea pig urinary bladder. *Eur. J. Pharmacol.*, **86**, 291–294.

KISHII, K.-I., HISAYAMA, T. & TAKAYANAGI, I. (1992). Comparison of contractile mechanisms by carbachol and ATP in detrusor strips of rabbit urinary bladder. *Jpn. J. Pharmacol.*, **58**, 219–229.

LAMBRECHT, G., FRIEBE, T., GRIMM, U., WINDSCHEIF, U., BUNGARDT, E., HILDEBRANDT, C., BÄUMERT, H.G., SPATZ-KÜMBEL, G. & MUTSCHLER, E. (1992). PPADS, a novel functionally selective antagonist of P_2 purinoceptor-mediated responses. *Eur. J. Pharmacol.*, **217**, 217–219.

LANGLEY, J.N. & ANDERSON, H.K. (1895). The innervation of the pelvic and adjoining viscera. Part 2. The bladder. *J. Physiol.*, **19**, 71–84.

LONGHURST, P.A., BELIS, J.A., O'DONNELL, J.P., GALIE, J.R. & WESTFALL, D.P. (1984). A study of the atropine-resistant component of the rabbit urinary bladder. *Eur. J. Pharmacol.*, **99**, 295–302.

MOSS, H.E. & BURNSTOCK, G. (1985). A comparative study of electrical field stimulation of the guinea-pig, ferret and marmoset urinary bladder. *Eur. J. Pharmacol.*, **114**, 311–316.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerised approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

NORONHA-BLOB, L., PROSSER, J.C., STURM, B.L., LOWE, V.C. & ENNA, S.J. (1991). (±)-Terodiline: an M_1-selective muscarinic receptor antagonist. In vivo effects at muscarinic receptors mediating urinary bladder contraction, mydriasis and salivary secretion. *Eur. J. Pharmacol.*, **201**, 135–142.

PETERSON, G.L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.*, **83**, 346–356.

SNEDDON, P. & McLEES, A. (1992). Purinergic and cholinergic contractions in adult and neonatal rabbit bladder. *Eur. J. Pharmacol.*, **214**, 7–12.

THEOBALD, R.J. (1986). The effect of arylazido aminopropionyl ATP ($ANAPP_3$) on inhibition of pelvic nerve evoked contractions of the cat urinary bladder. *Eur. J. Pharmacol.*, **130**, 351–354.

THEOBALD, R.J. (1992). Subclasses of purinoceptors in feline bladder. *Eur. J. Pharmacol.*, **229**, 125–130.

URSILLO, R.C. (1961). Investigation of certain aspects of atropine-resistant nerve effects. *J. Pharmacol. Exp. Ther.*, **131**, 231–236.

WILLIAMS, M. (1991). Adenosine receptor agonists and antagonists. In *Adenosine in the Nervous System.* ed. Stone, T.W. pp. 137–171. London: Academic Press.

(Received April 28, 1993
Revised July 8, 1993
Accepted July 26, 1993)

Br. J. Pharmacol. (1993), **110**, 1496–1500 © Macmillan Press Ltd, 1993

Suppression by intradermal administration of heparin of eosinophil accumulation but not oedema formation in inflammatory reactions in guinea-pig skin

[1]M.M. Teixeira & P.G. Hellewell

Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 Heparin is widely used in the treatment of thrombotic disorders and as an aid in surgery. Anti-inflammatory effects of heparin have also been described. In this study, we have investigated the effects of locally-injected heparin on the oedema formation and eosinophil accumulation induced by various inflammatory stimuli in guinea-pig skin.

2 Heparin dose-dependently suppressed the accumulation of ^{111}In-labelled eosinosphils induced by i.d. injection of zymosan-activated plasma (ZAP). The ^{111}In-eosinophil accumulation induced by other inflammatory stimuli (compound 48/80, platelet activating factor, interleukin-8 and in a passive cutaneous anaphylaxis reaction) was also suppressed by locally-injected heparin.

3 Oedema formation in response to these same stimuli was not altered by the local injection of heparin.

4 Fucoidin, a negatively-charged sulphated algal polymer, had no effect on the ^{111}In-eosinophil accumulation or oedema formation induced by ZAP. Nevertheless, fucoidin significantly suppressed the oedema formation induced by i.d. injection of cationic protein-containing extracts of *Schistosoma mansoni* larvae. Heparin also inhibited oedema induced by the extracts, suggesting that both fucoidin and heparin were effectively neutralizing the cationic protein of the extracts to inhibit their oedema-inducing activity.

5 Thus, heparin significantly inhibited the accumulation of ^{111}In-eosinophils, but not oedema formation, induced by various inflammatory stimuli. This, taken together with the lack of effect of fucoidin, suggests that heparin interferes with the process of eosinophil trafficking by a mechanism that does not depend on neutralisation of the charge of the stimulatory molecules.

Keywords: Heparin; eosinophil; oedema formation; inflammation; fucoidin; passive cutaneous anaphylaxis reaction

Introduction

Heparin is a linear anionic polyelectrolyte drug obtained commercially from beef lung or porcine intestinal mucosa and used mainly in the prevention and treatment of thrombotic disorders (Jaques, 1980; Hirsh, 1991; Freedman, 1992). Anti-inflammatory actions of heparin have also been described. For example, it has been shown to suppress neutrophil function and chemotaxis *in vitro* (Matzner *et al.*, 1984; Bazzoni *et al.*, 1993), reduce neutrophil migration into the inflamed rat paw (McGovern, 1957) and inhibit increases in vascular permeability in rabbit skin (Lecomte & Hugues, 1954; Carr, 1979). Heparin also appears to diminish the bronchoconstrictor response of sheep to allergic and non-allergic stimuli (Ahmed *et al.*, 1992) and the immediate response to antigen in the skin and lungs of allergic human subjects (Bowler *et al.*, 1993).

Heparin is found in many tissues, especially in association with mast cells granules where it binds to histamine and cationic proteins (Jaques, 1980). Mast cells appear to play an important role in allergic diseases such as asthma and rhinitis (Wasserman, 1987; Holgate & Church, 1992). Thus, mast cells are directly responsible for the acute clinical manifestations of allergic diseases (Holgate & Church, 1992) but may also influence the chronic states through the release of cytokines (Plaut *et al.*, 1989; Bradding *et al.*, 1992; Kulmburg *et al.*, 1992). Another feature of allergic inflammation is the presence of eosinophils which are potent effector cells capable of causing tissue destruction and dysfunction (Djukanovic *et al.*, 1990; Kay, 1991). Mast cells are thought to contribute to the recruitment of eosinophils through the release of mediators including phospholipids and chemotactic cytokines (Kulmburg *et al.*, 1992) and this may be relevant to allergic disorders such as asthma.

In preliminary studies, we observed that heparin effectively inhibited experimental inflammation in guinea-pig skin induced by cationic protein-containing extracts of *Schistosoma mansoni* larvae (Teixeira *et al.*, unpublished observations). Interestingly, eosinophil accumulation was inhibited much more effectively than the formation of oedema which suggested an effect other than merely neutralization of cercarial cationic proteins. In the present study, we investigated whether local administration of heparin could also inhibit the accumulation of eosinophils and oedema formation induced by known mediators of inflammation in guinea-pig skin, and whether another highly anionic molecule, the sulphated algal polymer fucoidin, could mimic the effects of heparin.

Methods

Preparation of zymosan-activated plasma

Zymosan-activated plasma (ZAP) was used as a source of guinea-pig C5a des-Arg. Guinea-pig heparinized (10 iu ml^{-1}) plasma was incubated with zymosan (5 mg ml^{-1}) for 30 min at 37°C. Zymosan was removed by centrifugation (2 × 10 min at 3000 *g*). The activated plasma was desalted in a PD-10 Sephadex G-25M column and stored in aliquots at − 20°C. The maximum concentration of heparin in ZAP was approximately 7 iu ml^{-1} (i.e. 0.7 iu per skin site).

Preparation of passive cutaneous anaphylaxis sera and reactions

Details of the preparation of IgG_1-rich sera are given elsewhere (Weg *et al.*, 1991). Briefly, male guinea-pigs (Harlan Porcellus, 350–400 g) were immunized with bovine gamma-

[1] Author for correspondence.

globulin (BGG) in Freund's complete adjuvant (0.2 mg BGG $0.2\,ml^{-1}$ of adjuvant s.c.). A boost of antigen in Freund's incomplete adjuvant was given on day 21 and the serum prepared on day 30. For the skin assays, recipient animals received an injection of 50 µl of a 1/50 dilution of the antisera i.d., followed 16–20 h later by the i.d. injection of antigen (BGG, 1 µg per site).

Induction, purification and radiolabelling of guinea-pig eosinophils

The method is described in detail elsewhere (Faccioli *et al.*, 1991). Briefly, eosinophils obtained from the peritoneal cavity of horse serum-treated ex-breeder female guinea-pigs (Harlan Porcellus, Oxon) were purified on a discontinuous percoll gradient. The cells obtained were washed twice in HBSS (calcium- and magnesium-free) and labelled with $^{111}InCl_3$ (100 µCi in 10 µl) chelated to 2-mercaptopyridine-N-oxide. The purity of the eosinophils obtained was always greater than 93%. The main contaminating cells were monocytes with a few neutrophils (<3%).

Measurement of local oedema formation and eosinophil accumulation in guinea-pig skin

Radiolabelled eosinophil infiltration and oedema formation were measured simultaneously. ^{125}I-labelled human serum albumin (~5 µCi) was added to the ^{111}In-labelled eosinophils and these were injected intravenously (5×10^6 cells per animal) into recipient guinea-pigs (350–400 g, Harlan Porcellus, Oxon) anaesthetized with Hypnorm (0.15 ml, i.m.). After 5 min inflammatory stimuli or antigen (in the sites pretreated with antiserum) were injected i.d. in 0.1 ml volumes into the dorsal skin of shaved animals with or without heparin. All drugs were mixed before the injection. Each animal received a duplicate of each treatment following a randomized injection plan and the inflammatory response (^{111}In-labelled eosinophil accumulation and oedema formation) was assessed after 2 h. At this time, a blood sample was obtained by cardiac puncture, the animals were killed by an overdose of sodium pentobarbitone, the dorsal skin was removed, cleaned free of excess of blood and the skin sites punched out with a 17 mm punch. The samples were counted in an automatic 5-head gamma-counter (Canberra Packard Ltd, Pangbourne, Berks) and the counts were cross-channel corrected for the two isotopes.

Eosinophil accumulation is expressed as the number of ^{111}In-labelled eosinophils per skin site and oedema formation as µl of plasma obtained by dividing the ^{125}I counts of the skin sample by the ^{125}I counts in 1 µl of plasma.

Materials

Reagents The following compounds were purchased from Sigma Chemical Company (Poole, Dorset): bradykinin, bovine gamma globulin, compound 48/80, zymosan, fucoidin. Hanks solutions, HEPES and horse serum were purchased from Gibco Limited (Paisley, Penfrewshire). Percoll was purchased from Pharmacia (Milton Keynes, Bucks), PAF from Bachem (Saffron Walden, Essex) and porcine intestinal mucosa heparin (Monoparin, 5000 iu ml^{-1}) from C.P. Pharmaceuticals (Wrexham, Wales). $^{111}InCl_3$ and ^{125}I-labelled human serum albumin were purchased from Amersham International (Amersham, Hertfordshire). Cercarial extracts were kindly generated and donated by Dr M.J. Doenhoff and C. McNiece (School of Biological Sciences, Bangor, Wales). Human recombinant interleukin-8 (IL-8) was a gift from Dr I. Lindley (Sandoz, Vienna, Austria).

Statistics

Data are presented as mean ± s.e.mean for the number of experiments indicated and were analyzed by using two-way analysis of variance (ANOVA) on normally distributed data. P values were assigned by the Neuman-Keuls procedure. Values of $P<0.05$ were considered significant.

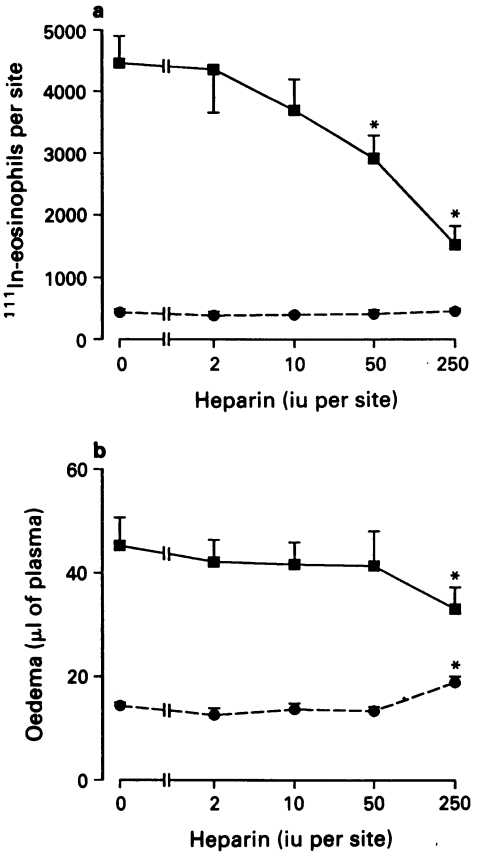


Figure 1 Effect of increasing concentrations of heparin on the accumulation of ^{111}In-labelled eosinophils (a) and oedema formation (b) induced by zymosan-activated plasma (ZAP) in guinea-pig skin. ^{111}In-eosinophil accumulation and oedema formation were assessed 2 h after the i.d. injections. Heparin was injected alone (●) or mixed with ZAP (30% in saline, ■). Results are mean ± s.e.mean of $n = 4$ guinea-pigs. $^*P<0.05$ when compared to values obtained in the absence of heparin.

Results

Two hours after the injection of the ^{111}In-labelled eosinophils, 9.6 ± 1.3% ($n = 22$) of the labelled cells were circulating and over 94% of the total blood ^{111}In was bound to these cells. Although histology was not performed in this study, previous work from our laboratory has shown that the eosinophils do accumulate within 2 h in sites of inflammation (Faccioli *et al.*, 1991; Collins *et al.*, 1993). Autoradiographic data confirmed that radiolabelled cells migrated into the tissue (Weg *et al.*, unpublished observations).

Effect of heparin on ^{111}In-eosinophil accumulation

The effect of increasing doses of heparin on ZAP (10% solution in saline)-induced inflammation in guinea-pig skin is shown in Figure 1. Local administration of heparin dose-dependently inhibited ZAP-induced eosinophil accumulation (Figure 1a). At a dose of 50 iu per site, heparin also effectively inhibited the eosinophil accumulation induced by increasing doses of ZAP (Figure 2a) although the effectiveness of heparin appeared to diminish at higher doses of ZAP. Thus, eosinophil accumulation induced by 10%,

Table 1 Effect of i.d. heparin on eosinophil accumulation and oedema formation in guinea-pig skin

	^{111}*In-eosinophils* *Heparin* (iu/site)			*Oedema* (μl of plasma) *Heparin* (iu/site)		
	Control	10	50	*Control*	10	50
ZAP	4224 ± 357	3170 ± 304	2462 ± 288*	27.8 ± 2.0	27.6 ± 2.6	24.2 ± 2.2
PAF	6748 ± 548	3814 ± 604*	3079 ± 511*	69.2 ± 2.6	62.6 ± 5.0	61.9 ± 5.2
PCA	13067 ± 2723	8619 ± 1908*	6876 ± 2367*	47.5 ± 2.4	41.6 ± 2.5	49.5 ± 4.7
48/80	4170 ± 1027	2028 ± 113*	1166 ± 93*	46.6 ± 4.1	47.8 ± 2.8	39.6 ± 2.0
IL-8	1036 ± 140	902 ± 113	711 ± 100*	14.4 ± 1.7	15.8 ± 1.6	16.2 ± 2.1
Sal	750 ± 56	544 ± 48	570 ± 58	14.2 ± 0.6	14.4 ± 1.1	13.8 ± 0.8

All stimuli and the antigen were mixed with heparin prior to i.d. injection. ^{111}In-eosinophil accumulation and oedema formation were assessed after 2 h. The following stimuli were used: zymosan-activated plasma (ZAP, 10% in saline), PAF (5×10^{-9} mol per site), compound 48/80 (48/80, 10 μg per site), interleukin-8 (IL-8, 10^{-10} mol per site), antigen (PCA reaction, 1 μg of antigen per site) and saline injected sites (Sal). Results are expressed as the mean ± s.e.mean for 4–6 guinea-pigs. *$P<0.05$ when compared to control values.

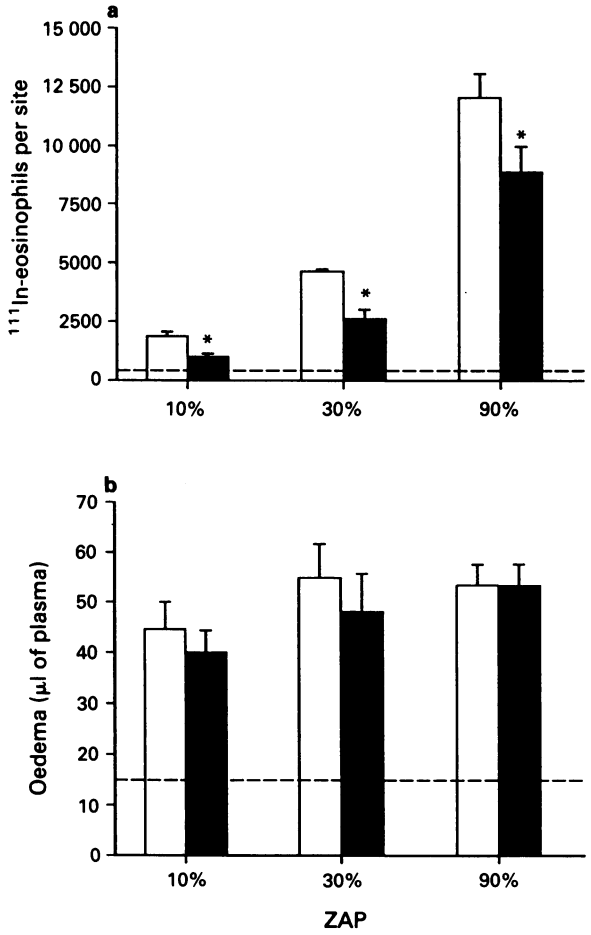


Figure 2 Effect of heparin on the accumulation of ^{111}In-eosinophils (a) and oedema formation (b) induced by increasing concentrations of zymosan-activated plasma (ZAP) in guinea-pig skin. ^{111}In-eosinophil accumulation and oedema formation were assessed 2 h after the i.d. injections. ZAP was injected alone (open columns) or mixed with heparin (50 iu per site, solid columns). The line across the graph represents background values obtained in response to i.d. injection of saline. Results are mean ± s.e.mean of $n = 4$ guinea-pigs. *$P<0.05$ when compared to values obtained in the absence of heparin.

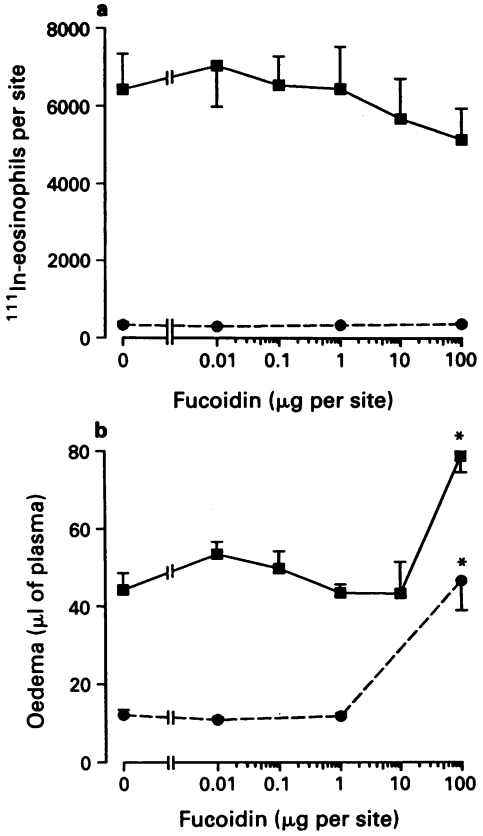


Figure 3 Effect of increasing concentrations of fucoidin on the accumulation of ^{111}In-labelled eosinophils (a) and oedema formation (b) induced by zymosan-activated plasma (ZAP) in guinea-pig skin. ^{111}In-eosinophil accumulation and oedema formation were assessed 2 h after the i.d. injections. Fucoidin was injected alone (●) or mixed with ZAP (30% in saline, ■). Results are mean ± s.e.mean of $n = 4$ guinea-pigs. *$P<0.05$ when compared to values obtained in the absence of fucoidin.

30% and 90% ZAP was suppressed by 64, 49 and 27%, respectively.

The effects of heparin on the inflammatory response induced by different stimuli is depicted in Table 1. In these experiments, local administration of heparin effectively inhibited the accumulation of eosinophils induced by various stimuli and in a PCA reaction (Table 1). Whereas heparin did not significantly inhibit ZAP-induced eosinophil accumulation at the dose of 10 iu per site (Figure 1a), it was effective against most mediators studied at this dose.

Effect of heparin on oedema formation

Local administration of heparin had no significant effects on local oedema formation induced by all mediators tested and in a PCA reaction (Figure 1b, Figure 2b and Table 1). Oedema formation induced by ZAP (10% in saline) was inhibited only at the top dose of heparin (250 iu per site) but it also induced significant oedema formation when injected alone (Figure 1b). In contrast, as presented above, heparin significantly reduced eosinophil accumulation in the same

Table 2 Effect of i.d. fucoidin and heparin on the oedema formation induced by extracts of *S. mansoni* larvae in guinea-pig skin

	% inhibition
Fucoidin (10 μg per site)	67.3 ± 7.9*
Heparin (50 iu per site)	27.5 ± 5.6*

Cercarial extracts and fucoidin or heparin were mixed prior to the i.d. injections and oedema formation was assessed after 2 h. Intradermal injection of cercarial extract alone (5 μg of protein per site) induced a leakage of 57.0 ± 9.5 μl compared with 13.3 ± 2.1 μl in saline-injected sites. Results are mean ± s.e.mean of $n = 4-6$ guinea pigs. *$P < 0.05$ when compared to values obtained in the absence of heparin or fucoidin.

sites. Bradykinin-induced oedema formation was also not significantly affected by heparin (bradykinin 10^{-10} mol per site, 52.1 ± 5.8 μl; bradykinin + heparin 10 iu per site, 47.5 ± 3.6 μl; bradykinin + heparin 50 iu per site, 44.0 ± 4.2 μl).

Effect of fucoidin on local inflammation in guinea-pig skin

The effects of fucoidin, a fungal polysaccharide with many electronegative charges, is shown in Figure 3. Fucoidin had no effect on ZAP-induced eosinophil accumulation when used up to 100 μg per site (Figure 3a). When injected alone or with ZAP, fucoidin, at the dose of 100 μg per site, caused an increased formation of oedema, but it had no effect when injected in smaller doses (Figure 3b). Nevertheless, fucoidin at a dose of 10 μg per site significantly inhibited the oedema formation induced by cationic protein-containing extracts of *Schistosoma mansoni* larvae (Table 2).

Discussion

The discovery of heparin some 70 years ago opened new and exciting possibilities in the treatment of human disease. With the use of this drug, it has been possible to perform cardiac and orthopaedic surgeries effectively, to anticoagulate properly high risk patients and, more recently, it is being used in the prevention of thrombotic complications following surgery, long term hospitalization and cancer (Hirsh, 1991; Freedman, 1992). Heparin has also received some attention previously as an anti-inflammatory drug but the results of different trials are conflicting (Boyle *et al.*, 1964; Fine *et al.*, 1968). Recent reports have again stressed the anti-inflammatory actions of heparin in man (Page, 1991; Volkl *et al.*, 1991).

We have found that heparin, when injected i.d. with several different known mediators of inflammation, antigen or compound 48/80, effective inhibited the accumulation of ^{111}In-labelled eosinophils. ZAP-induced neutrophil accumulation was assessed in some experiments and it was also found to be inhibited (data not shown). For example, ^{111}In-neutrophil accumulation induced by ZAP (10% in saline) was inhibited by 42% ($n = 5$, $P < 0.05$) by local administration of heparin (50 iu per site). Interestingly, heparin had no significant effect on local oedema formation induced by the same mediators or in a PCA reaction.

Because of its electronegative charge, heparin can bind to many different substances and this may lead to their inactivation or enhancement of their function (Jaques, 1980; Yamashita *et al.*, 1992). For example, heparin may interact with eosinophil cationic protein leading to inactivation of this highly toxic protein (Fredens *et al.*, 1991). If this happens *in vivo*, it may prove to be an important mechanism by which heparin, and possibly mast cells, may control tissue damage by cationic proteins (Fredens *et al.*, 1991). Heparin has also been shown to interact with and inhibit the action of compound 48/80, antigen and bradykinin (Jaques, 1980). We cannot exclude this interaction in our system since the stimuli were mixed with heparin prior to injection. However, the observation that oedema formation induced by the various stimuli was unchanged in the same sites that eosinophil (and neutrophil) accumulation was suppressed suggests that heparin inhibits eosinophil accumulation by a mechanism other than neutralization of the mediators injected or generated endogenously. This suggestion is supported by the results of the experiments carried out with cationic protein-containing extracts of *S. mansoni* larvae. Fucoidin significantly inhibited, by approximately 70%, extract-induced oedema formation, presumably as a result of charge mediated-neutralization. Heparin (50 iu per site) was less effective (approximately 30% inhibition). However, at this same 'charge-neutralizing' dose, fucoidin had no effect on ZAP-induced inflammation while heparin was inhibitory for eosinophil accumulation.

Leucocytes express different adhesion molecules which play an important role in their interaction with endothelial cells and migration into the tissue (reviewed in Williams & Hellewell, 1992). The first step in the leucocyte-endothelial cell adhesion is the loose attachment and rolling which occurs via a selectin-dependent mechanism (Ley *et al.*, 1991b; Williams & Hellwell, 1992). Heparin and also fucoidin, when given systemically, have been shown to inhibit the process of rolling and this appears to be related to the capacity of these drugs to interfere with L-selectin-mediated adhesion (Ley *et al.*, 1991a; 1993; Arfors & Ley, 1993). The observation that local administration of fucoidin did not inhibit eosinophil accumulation in guinea-pig skin suggests that interference by heparin with leucocyte rolling *in vivo* is unlikely to be the mechanism of its inhibitory action in the present study. However, heparin is actively taken up by endothelial cells (Hiebert & Jaques, 1976; Hiebert & Liu, 1991) and this leaves the possibility that it may be re-expressed on the lumenal side of the circulation where it could inhibit rolling of eosinophils. Furthermore, the ability of heparin to displace the binding of chemokines to proteoglycans on the endothelial surface (Rot, 1992; Tanaka *et al.*, 1993) may also represent a further mechanism by which it could interfere with leucocyte adhesion to endothelial cells, at least for IL-8-induced eosinophil accumulation.

Heparin and related polysaccharides may interact with extracellular matrix components and this may be important for the control of local matrix metabolism (Claman, 1969; Yamashita *et al.*, 1992). After attaching to endothelial cells, leucocytes have to interact with the extracellular matrix to move into the tissue (Nathan & Sporn, 1991; Williams & Hellewell, 1992). Heparins are capable of inhibiting tumour metastasis (Saiki *et al.*, 1991), an effect which is related to their ability to modulate the interaction of tumour cells with the extracellular matrix (McCarthy *et al.*, 1990; Asch *et al.*, 1991). Thus, this inhibitory effect of heparin on cell-extracellular matrix interaction may represent an additional mechanism by which heparin could inhibit tissue accumulation of eosinophils.

Eosinophils are potent cells associated with various allergic processes (Djukanovic *et al.*, 1990; Kay, 1991; Thorne & Mazza, 1991). In these diseases, eosinophil numbers and state of activation are related to worsening of symptoms and to the degree of lesion to the target organ (Djukanovic *et al.*, 1990). The safety of heparin (Jaques, 1980) and its capacity to inhibit eosinophil accumulation (this study) and function (Fredens *et al.*, 1991) may represent a potential therapeutic target for the control of diseases in which the oesinophils play an important pathogenetic role, such as asthma. The effects of heparin on the acute symptoms of allergen challenge in man may also be relevant to this situation (Bowler *et al.*, 1993).

In summary, we have shown in this study that local

administration of heparin dose-dependently inhibits the eosinophil accumulation in skin, induced by mediators of inflammation whereas it had little effect on oedema formation. This suggests that an interference with eosinophil traffic rather than just neutralization of the mediators is responsible for the inhibitory effects of heparin. The lack of effect of fucoidin, a highly electronegative molecule, on the accumulation of eosinophils further supports this idea.

M.M.T. was supported by Sandoz, Basel and P.G.H. by The National Asthma Campaign. We thank Dr M.J. Doenhof and Mr C. McNiece for the gift of cercarial extracts.

References

AHMED, T., ABRAHAM, W.M. & D'BROT, J. (1992). Effects of inhaled heparin on immunologic and nonimmunologic bronchoconstrictor responses in sheep. *Am. Rev. Respir. Dis.*, **145**, 566–570.

ARFORS, K.-E. & LEY, K. (1993). Sulfated polysaccharides in inflammation. *J. Lab. Clin. Med.*, **121**, 201–202.

ASCH, A.S., TEPLER, J., SILBIGER, S. & NACHMAN, R.L. (1991). Cellular attachment to thrombospondin. Cooperative interactions between receptor systems. *J. Biol. Chem.*, **266**, 1740–1745.

BAZZONI, G., NUNEZ, A.B., MASCELLANI, G., BIANCHINI, P., DEJANA, E. & DEL MASCHIO, A. (1993). Effect of heparin, dermatan sulfate, and related oligo-derivatives on human polymorphonuclear leukocyte function. *J. Lab. Clin. Med.*, **121**, 268–275.

BOWLER, S.D., SMITH, S.M. & LAVERCOMBE, P.S. (1993). Heparin inhibits the immediate response to antigen in the skin and lungs of allergic subjects. *Am. Rev. Respir. Dis.*, **147**, 160–163.

BOYLE, J.P., SMART, R.H. & SHIREY, J.K. (1964). Heparin in the treatment of chronic obstructive bronchopulmonary disease. *Am. J. Cardiol.*, **14**, 25–28.

BRADDING, P., FEATHER, I.H., HOWARTH, P.H., MUELLER, R., ROBERTS, J.A., BRITTEN, K., BEWS, J.P.A., HUNT, C.A., OKAYAMA, Y., HEUSSER, C.H., BULLOCK, G.R., CHURCH, M.K. & HOLGATE, S.T. (1992). Interleukin 4 is localized to and released by humans mast cells. *J. Exp. Med.*, **176**, 1381–1386.

CARR, J. (1979). The anti-inflammatory action of heparin: heparin as an antagonist to histamine, bradykinin and prostaglandin E_1. *Thromb. Res.*, **16**, 507–516.

CLAMAN, H.N. (1969). On Scleroderma. Mast cells, endothelial cells, and fibroblasts. *J. Am. Med. Ass.*, **262**, 1206–1209.

COLLINS, P.D., WEG, V.B., FACCIOLI, L.H., WATSON, M.L., MOQBEL, R. & WILLIAMS, T.J. (1993). Eosinophil accumulation induced by human interleukin-8 in the guinea-pig in vivo. *Immunology*, **79**, 312–318.

DJUKANOVIC, R., ROCHE, W.R., WILSON, J.W., BEASLEY, C.R.W., TWENTYMAN, O.P., HOWARTH, P.H. & HOLGATE, S.T. (1990). Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.*, **142**, 434–457.

FACCIOLI, L.H., NOURSHARGH, S., MOQBEL, R., WILLIAMS, F.M., SEHMI, R., KAY, A.B. & WILLIAMS, T.J. (1991). The accumulation of ^{111}In-eosinophils induced by inflammatory mediators in vivo. *Immunology*, **73**, 222–227.

FINE, N.L., SHIM, C. & WILLIAMS, M.H. (1968). Objective evaluation of heparin in the treatment of asthma. *Am. Rev. Respir. Dis.*, **98**, 886–887.

FREDENS, K., DAHL, R. & VENGE, P. (1991). In vitro studies of the interaction between heparin and eosinophil cationic protein. *Allergy*, **46**, 27–29.

FREEDMAN, M.D. (1992). Pharmacodynamics, clinical indications, and adverse effects of heparin. *J. Clin. Pharmacol.*, **32**, 584–596.

HIEBERT, L. & LIU, J.M. (1991). Protective action of polyelectrolytes on endothelium. *Sem. Thromb. Haemost.*, **17**, 42–46.

HIBERT, L.M. & JAQUES, L.B. (1976). Heparin uptake on endothelium. *Artery*, **2**, 26–37.

HIRSH, J. (1991). Heparin. *N. Engl. J. Med.*, **324**, 1565–1573.

HOLGATE, S.T. & CHURCH, M.K. (1992). The mast cell. *Br. Med. Bull.*, **48**, 40–50.

JAQUES, L.B. (1980). Heparins – anionic polyelectrolyte drugs. *Pharmacol. Rev.*, **31**, 99–166.

KAY, A.B. (1991). Biological properties of eosinophils. *Clin. Exp. Allergy*, **21**, 23–29.

KULMBURG, P.A., HUBER, N.E., SCHEER, B.J., WRANN, M. & BAUMRUKER, T. (1992). Immunoglobulin E plus antigen challenge induces a novel intercrine/chemokine in mouse mast cells. *J. Exp. Med.*, **176**, 1773–1778.

LECOMTE, J. & HUGUES, J. (1954). Action inhibitrice de l'heparine sur le phenomene d'Arthus. *Int. Arch. Allergy*, **5**, 367–373.

LEY, K., CERRITO, M. & ARFORS, K.-E. (1991a). Sulfated polysaccharides inhibit leukocyte rolling in rabbit mesentery venules. *Am. J. Physiol.*, **260**, H1667–H1673.

LEY, K., GAEHTGENS, P., FENNIE, C., SINGER, M.S., LASKY, L.A. & ROSEN, S.D. (1991b). Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood*, **77**, 2553–2555.

LEY, K., LINNERMANN, G., MEINEN, M., STOOLMAN, L.M. & GAEHTGENS, P. (1993). Fucoidin, but not yeast polyphosphomannan PPME, inhibits leukocyte rolling in venules of the rat mesentery. *Blood*, **81**, 177–185.

MATZNER, Y., MARX, G., DREXLER, R. & ELDOR, A. (1984). The inhibitory effect of heparin and related glycosaminoglycans on neutrophil chemotaxis. *Thromb. Haemost.*, **52**, 134–137.

MCCARTHY, J.B., SKUBITZ, A.P.N., YI, X.-Y., MICKELSON, D.J., KLEIN, D.J. & FURCHT, L.T. (1990). RGD-independent cell adhesion to the carboxy-terminal heparin-binding fragment of fibronectin involves heparin-dependent and -independent activities. *J. Cell Biol.*, **110**, 777–787.

MCGOVERN, V.J. (1957). The mechanism of inflammation. *J. Path. Bact.*, **73**, 99–106.

NATHAN, C. & SPORN, M. (1991). Cytokines in context. *J. Cell Biol.*, **113**, 981–986.

PAGE, C.P. (1991). One explanation of the asthma paradox: inhibition of natural anti-inflammatory mechanism by β_2-agonists. *Lancet*, **337**, 717–720.

PLAUT, M., PIERCE, J.H., WATSON, C.J., HANLEY-HYDE, J., NORDAN, R.P. & PAUL, W.E. (1989). Mast cell lines produce lymphokines in response to cross-linkage of FcεRI or to calcium ionophores. *Nature*, **339**, 64–67.

ROT, A. (1992). Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol. Today*, **31**, 291–294.

SAIKI, I., MAKABE, T., YONEDA, J., MURATA, J., ISHIZAKI, Y., KIMIZAKI, F., KATO, I. & AZUMA, I. (1991). Inhibitory effect of fibronectin and its recombinant polypeptides on the adhesion of metastatic melanoma to laminin. *Jpn. J. Cancer Res.*, **82**, 1112–1119.

TANAKA, Y., ADAMS, D.H. & SHAW, S. (1993). Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol. Today*, **14**, 111–115.

THORNE, K.J.I. & MAZZA, G. (1991). Eosinophilia activated eosinophils and human schistosomiasis. *J. Cell Sci.*, **98**, 265–270.

VOLKL, K.P., KONERDING, M.A. & KROLL, M. (1991). The effect of topical heparin on histamine-induced wheals. A double-blind study. *Forts. Med.*, **109**, 462–464.

WASSERMAN, S.I. (1987). The regulation of inflammatory mediator production by mast cell products. *Am. Rev. Respir. Dis.*, **135**, S46–S48.

WEG, V.B., WATSON, M.L., CORDEIRO, R.S.B. & WILLIAMS, T.J. (1991). Histamine, leukotriene D_4 and platelet activating factor in guinea pig passive cutaneous anaphylaxis. *Eur. J. Pharmacol.*, **204**, 157–163.

WILLIAMS, T.J. & HELLEWELL, P.G. (1992). Adhesion molecules involved in the microvascular inflammatory response. *Am. Rev. Respir. Dis.*, **146**, S45–S50.

YAMASHITA, Y., NAKAGOMI, K., TAKEDA, T., HASEGAWA, S. & MITSUI, Y. (1992). Effect of heparin on pulmonary fibroblasts and vascular cells. *Thorax*, **47**, 634–639.

(Received May 17, 1993
Revised July 16, 1993
Accepted July 29, 1993)

Br. J. Pharmacol. (1993), **110**, 1501–1507 © Macmillan Press Ltd, 1993

Effect of the platelet-activating factor antagonist, TCV-309, and the cyclo-oxygenase inhibitor, ibuprofen, on the haemodynamic changes in canine experimental endotoxic shock

Shinya Yamanaka, Hiroshi Iwao, Tokihito Yukimura, Shokei Kim & [1]Katsuyuki Miura

Department of Pharmacology, Osaka City University Medical School, Osaka 545, Japan

1 The present study was conducted in order to examine the effects of the platelet-activating factor (PAF) antagonist, TCV-309, and the cyclo-oxygenase inhibitor, ibuprofen, on the acute haemodynamic responses to endotoxin in anaesthetized dogs.

2 Endotoxin (2 mg kg^{-1}, i.v.) induced a severe hypotension by decreasing both total peripheral resistance (TPR) and cardiac output. Endotoxin also decreased central venous pressure and increased effective vascular compliance (EVC), indicating a blood pooling in the capacitance vessels.

3 The endotoxin-induced hypotension but not the fall in cardiac output, was markedly attenuated by ibuprofen. Ibuprofen abolished the decrease in TPR and even caused a systemic vasoconstriction. Ibuprofen abolished the increase in EVC.

4 The hypotension caused by endotoxin was attenuated by TCV-309 to a lesser extent than ibuprofen. However, the reduction in cardiac output produced by endotoxin was markedly attenuated by the PAF antagonist. TCV-309 also abolished the increase in EVC. In contrast to ibuprofen, TCV-309 did not affect the decrease in TPR caused by endotoxin.

5 Combined treatment with ibuprofen and TCV-309 markedly attenuated the endotoxin-induced hypotension, but not the fall in cardiac output. Nevertheless, when compared with animals treated with ibuprofen alone, treatment with ibuprofen and TCV-309 partly attenuated the endotoxin-induced reduction in cardiac output and systemic vasoconstriction.

6 These data indicate that dilatation of both resistance vessels and capacitance vessels contributes to the endotoxin-induced hypotension. It is suggested that (i) both prostanoids and PAF are involved in dilatation of capacitance vessels, (ii) prostanoids, but not PAF cause dilatation of resistance vessels and (iii) PAF may partly contribute to prostanoid-independent reduction in cardiac output in acute canine experimental endotoxin shock.

Keywords: Endotoxic shock; platelet-activating factor; prostaglandins; blood pooling; TCV-309; ibuprofen

Introduction

Septic shock is caused by endotoxin (lipopolysaccharide), the toxic moiety of the outer cell membrane of gram negative bacteria. Endotoxic shock is characterized by severe hypotension, respiratory dysfunction and disseminated intravascular coagulation (Morrison & Ryan, 1987). Agents which protect against the deleterious effects of endotoxic shock have been extensively studied (Bone, 1992). For instance, cyclo-oxygenase inhibitors improve hypotension and mortality in animal models of endotoxic shock (Jacobs *et al.*, 1982; Almqvist *et al.*, 1984), indicating that cyclo-oxygenase metabolites are mediators of endotoxin shock. Recently, antagonists of platelet-activating factor (PAF) were also shown to block the fatal effects of endotoxin and improve haemodynamic parameters (Terashita *et al.*, 1985; Sanchez Crespo & Fernandez Gallardo, 1991). As plasma concentrations of PAF are elevated in endotoxin shock (Mozes *et al.*, 1991), it is likely that PAF is another important mediator in endotoxin shock. Cyclo-oxygenase products and PAF have a close relationship in their production and biological activities. For example, we have previously reported that the PAF-induced systemic hypotension in dogs is mediated by prostaglandins (Yamanaka *et al.*, 1992). It is conceivable that these two lipid mediators have synergistic actions in endotoxin shock. However, the role of these autacoids and their interaction for the pathophysiology of endotoxic shock is still unclear.

The purpose of this study was to examine the effect of ibuprofen, a cyclo-oxygenase inhibitor, and TCV-309, a PAF antagonist (Terashita *et al.*, 1992), on the haemodynamic changes associated with endotoxic shock and to investigate how these lipids mediate the actions of endotoxin. This study particularly focuses on the endotoxin-induced alterations in vascular tone in both resistance vessels and capacitance vessels, for a loss of vascular tone plays a crucial role in the pathophysiology of endotoxic shock in many species including man (Gilbert, 1960; Siegel *et al.*, 1967; D'Orio *et al.*, 1989). This is the first study to investigate the role of PAF in venous blood pooling in endotoxic shock.

Methods

Animal preparation

Mongrel dogs of either sex weighing 9 to 15 kg were anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.). During the subsequent experimental period, pentobarbitone was continuously infused at a rate of 2.5 mg kg^{-1} h^{-1} to maintain surgical anaesthesia. Dogs were intubated and ventilated mechanically with a respirator (model 607, Harvard Apparatus, MA), at a respiration rate of 18 min^{-1} and a tidal volume of 15 ml kg^{-1}. The left brachial artery and vein were cannulated for monitoring blood pressure (BP) and heart rate and for drug infusion, respectively. A Swan-Ganz catheter was inserted via the left femoral vein into the pulmonary artery to monitor pulmonary arterial pressure (PAP), pulmonary capillary wedge pressure (PCWP) and central venous pressure (CVP). Cardiac output (CO) was measured by a thermal dilution method (Ganz & Swan, 1972). Total peri-

[1] Author for correspondence.

pheral resistance (TPR) and pulmonary vascular resistance (PVR) were calculated as follows:

$$TPR = (BP - CVP)/CO$$

$$PVR = (PAP - PCWP)/CO$$

Blood pressure, pulmonary arterial pressure, pulmonary capillary wedge pressure and central venous pressure were measured with pressure transducers (model TP-400T, Nihon Kohden, Tokyo, Japan) and amplifiers (model AP-601, Nihon Kohden). Heart rate was measured with a tachygraph (model AT-601G, Nihon Kohden) and cardiac output was measured with a cardiac output computer (model MTC-6210, Nihon Kohden).

Reflexes were minimized by administration of hexamethonium chloride (10 mg kg^{-1} loading dose followed by 10 mg kg^{-1} h^{-1} continuous infusion). Heparin was given intravenously (500 iu kg^{-1} i.v. bolus and 250 iu kg^{-1} h^{-1} infusion). Saline and dextran 40 (mean molecular weight 40 kD, 10% w/v) were infused continuously, (10 ml kg^{-1} loading dose followed by an infusion of 2.5 ml kg^{-1} h^{-1}). Dextran (4 mg kg^{-1}) was replaced for the same volume of blood, which was stored in a water bath at 37°C for the measurement of effective vascular compliance (EVC).

Measurement of effective vascular compliance

EVC was measured according to the method of Münzel *et al.* (1988). The stored blood (4 ml kg^{-1}) was infused into the abdominal aorta through the femoral artery catheter at a rate of 2 ml kg^{-1} min^{-1} for 2 min with a Masterflex pump equipped with quick load head (model 7520-25 and 7021-20, Cole-Parmer Instrument Co., IL, U.S.A.). After 1 min, the same volume was withdrawn at the same rate for 2 min. After another minute, the withdrawal procedure was repeated, followed by reinfusion in an identical manner. Thus, the total cycle time was 11 min and the respective blood volume was changed four times (Figure 2a). The change in central venous pressure for each blood volume change was recorded, and the ratio of volume change to pressure change was calculated. EVC was taken as the mean value of these ratios and expressed as ml $mmHg^{-1}$ kg^{-1}.

Determination of plasma concentration of 6-keto $PGF_{1\alpha}$ and PGE_2

The detailed method for the determination of plasma concentrations of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto$PGF_{1\alpha}$) and PGE_2 is described elsewhere (Miura *et al.*, 1991; Yamanaka *et al.*, 1992). Mixed venous blood was collected from the pulmonary artery via the Swan-Ganz catheter. [^{3}H]-6-keto $PGF_{1\alpha}$ and [^{3}H]-PGE_2 (final concentration 5000 d.p.m. ml^{-1}) were added to 1 ml of plasma. This plasma was applied to BondElut C_{18} extraction cartridge columns (200 mg, Analytichem Intl., Inc., CA). The fraction containing prostaglandins was further purified by reverse phase h.p.l.c. The plasma concentrations of 6-keto $PGF_{1\alpha}$ and PGE_2 were determined with [^{125}I]-6-keto $PGF_{1\alpha}$ and PGE_2 radioimmunoassay (RIA) kits (New England Nuclear, MI, U.S.A.). The recoveries of [^{3}H]-6-keto $PGF_{1\alpha}$ and [^{3}H]-PGE_2 were 56.9 ± 2.0 and 79.1 ± 1.7%, respectively ($n = 45$). The plasma concentrations of prostaglandins were corrected according to these recovery rates.

Protocol

After 1 h of equilibration, dogs were given endotoxin (*E. coli* 0127:B8, 2 mg kg^{-1}, i.v.). EVC was measured during the control period and at 60 and 120 min after endotoxin injection and blood samples were collected at the same time points. Cardiac output and haematocrit were determined every 30 min. All other parameters were recorded continuously.

Dogs were divided into four groups to examine the roles of PAF and prostaglandins in the haemodynamic changes caused by endotoxin. In the first group ($n = 4$), dogs were given endotoxin alone. Dogs in the second group ($n = 4$) were pretreated with ibuprofen (cyclo-oxygenase inhibitor, 12.5 mg kg^{-1}, i.v. bolus and 12.5 mg kg^{-1} h^{-1} continuous infusion) at 35 min prior to receiving endotoxin. In the third group ($n = 5$), dogs were pretreated with TCV-309 (3-bromo-5-[N-phenyl-*N*-[2-[[2-(1,2,3,4-tetrahydro-2-isoquinolycarbonyloxy) ethyl]carbamoyl]ethyl]carbamoyl]-1-propylpyridinium nitrate) (100 μg kg^{-1}, i.v. bolus and 500 μg kg^{-1} h^{-1} continuous infusion) at 35 min prior to receiving endotoxin. The fourth group ($n = 4$) received both ibuprofen (12.5 mg kg^{-1} and 12.5 mg kg^{-1} h^{-1}) and TCV-309 (100 μg kg^{-1} and 500 μg kg^{-1} h^{-1}) at 35 min prior to receiving endotoxin to examine the interaction between cyclo-oxygenase metabolites and PAF. In this group, plasma levels for 6-keto $PGF_{1\alpha}$ and PGE_2 were not determined. The effectiveness of TCV-309 was assessed in additional experiments in which PAF (0.5 μg kg^{-1}, i.v.) was given alone ($n = 3$) and with TCV-309 (100 μg kg^{-1} and 500 μg kg^{-1} h^{-1}) treatment ($n = 3$). PAF alone decreased blood pressure by 45 ± 2 mmHg, while this hypotensive action of PAF was abolished by TCV-309. This dose of TCV-309 also abolished the hypotensive action of PAF (0.5 μg kg^{-1}, i.v.) given at the end of the experimental period.

Statistics

Data are expressed as mean ± s.e.mean. One-way analysis of variance was used to compare the data among four groups of dogs, and randomized block analysis of variance was used to analyze data for time course effects (Zar, 1984). Individual comparisons were made by the Duncan new multiple range

Table 1 Baseline values for haemodynamic parameters in each group

Variable	*Group 1* (endotoxin alone)	*Group 2* (ibuprofen)	*Group 3* (TCV-309)	*Group 4* (ibuprofen + TCV)
n	4	4	5	4
BP (mmHg)	103 ± 3	108 ± 12	120 ± 9	129 ± 3
HR (beat min^{-1})	133 ± 7	145 ± 8	144 ± 6	122 ± 7
CO (l min^{-1})	2.02 ± 0.12	2.72 ± 0.25	1.98 ± 0.25	2.17 ± 0.27
PAP (mmHg)	12.5 ± 0.9	17.3 ± 2.0	13.8 ± 1.4	14.3 ± 1.0
PCWP (mmHg)	6.8 ± 0.6	7.8 ± 2.9	6.0 ± 1.2	7.5 ± 0.3
CVP (mmHg)	2.9 ± 0.4	3.9 ± 0.2	4.1 ± 0.53	2.9 ± 0.2
Ht (%)	26.3 ± 3.2	30.6 ± 2.7	32.7 ± 2.3	29.7 ± 1.0
SVR (mmHg min l^{-1})	47.4 ± 3.2	38.0 ± 2.8	59.5 ± 7.9	60.3 ± 5.6
PVR (mmHg min l^{-1})	2.84 ± 0.07	3.57 ± 0.44	5.66 ± 1.34	3.26 ± 0.65
EVC (ml $mmHg^{-1}$ kg^{-1})	6.46 ± 0.40	5.15 ± 0.60	4.45 ± 0.54	3.29 ± 0.4*

Values are given as means ± s.e.mean. BP, systemic blood pressure; HR, heart rate; CO, cardiac output; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; CVP, central venous pressure; Ht, hematocrit; SVR, systemic vascular resistance; PVR, pulmonary vascular resistance; EVC, effective vascular compliance. *$P < 0.05$ compared to control.

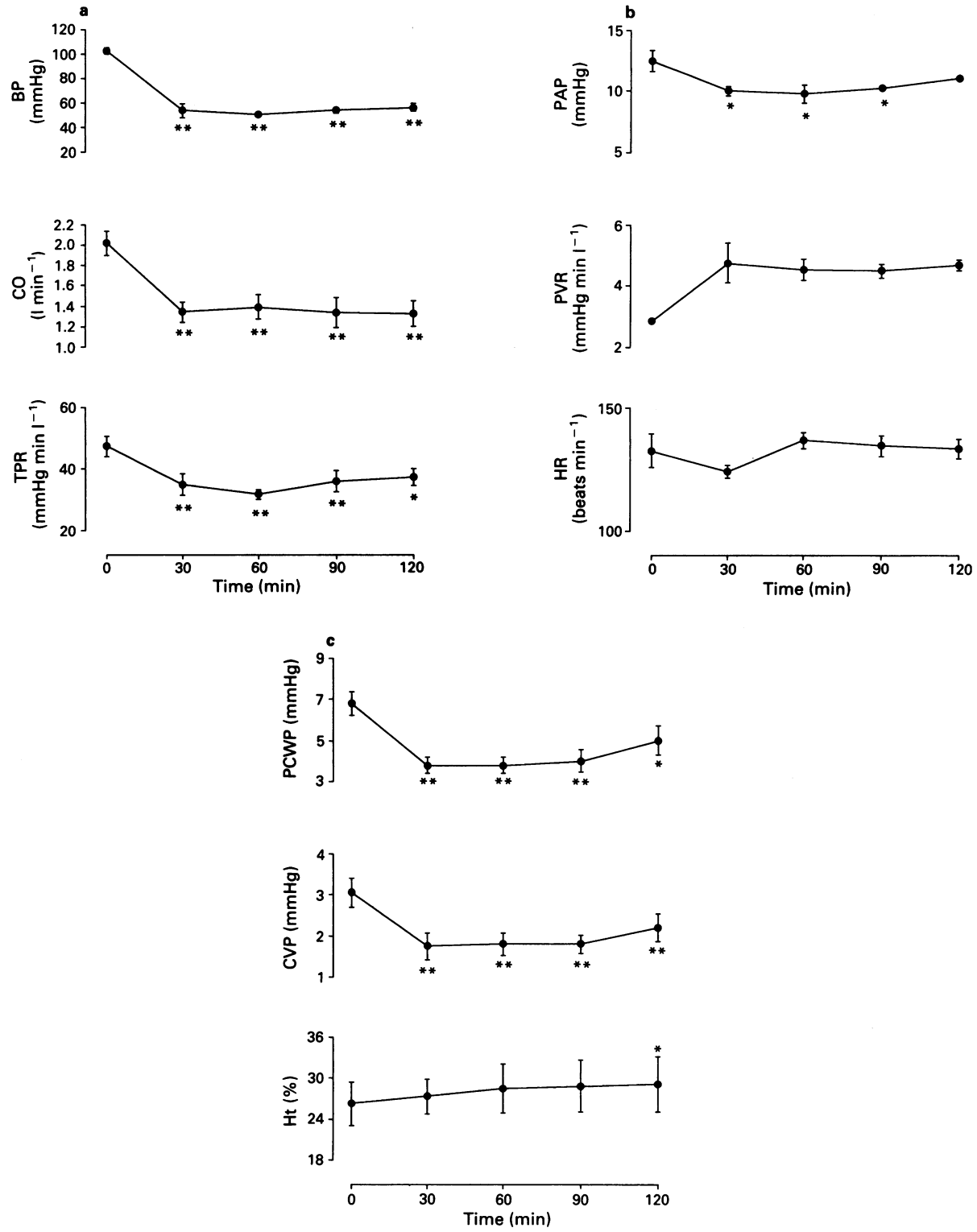


Figure 1 Time course of the haemodynamic changes induced by endotoxin. (a) BP, blood pressure; CO, cardiac output; TPR, total peripheral vascular resistance; (b) PAP, pulmonary arterial pressure; PVR, pulmonary vascular resistance; HR, heart rate; (c) PCWP, pulmonary capillary wedge pressure; CVP, central venous pressure; Ht, haematocrit. $*P<0.05$ and $**P<0.01$ when compared to the baseline value.

test. As the values of plasma concentrations of prostanoids sometimes showed heteroscedasticity, logarithmic transformation was performed before data analysis (Zar, 1984). A P value of <0.05 was considered statistically significant.

Drugs

The following drugs were dissolved in 0.9% saline and administered intravenously: endotoxin (Difco laboratories, MI, U.S.A.), pentobarbitone sodium (Tokyo Chemical Industry, Tokyo, Japan), heparin sodium and hexamethonium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), ibuprofen (donated by Kaken Pharmaceutical Co., Tokyo, Japan) and TCV-309 (donated by Takeda Chemical Industry, Osaka, Japan). Dextran 40 was donated by Green Cross (Osaka, Japan). PAF (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in ethanol and stored at − 20°C. Just before use, the ethanol was evaporated with nitrogen

gas, and PAF was dissolved in 0.9% saline containing 0.25% bovine serum albumin.

Results

There were no statistically significant differences in the control haemodynamic values among the four groups of animals except for the EVC in group 4 (Table 1). The baseline plasma concentration of PGE_2 in group 2 was significantly lower than those in group 1 and 3 (Table 2).

Endotoxin-induced haemodynamic changes (Group 1)

Endotoxin induced a pronounced and long lasting hypotension (from 103 ± 3 to 51 ± 2 mmHg at 60 min, $P < 0.01$), as well as a decrease in cardiac output (from 2.02 ± 0.12 to 1.32 ± 0.13 l min^{-1} at 120 min, $P < 0.01$) and TPR (from 47 ± 3 to 32 ± 2 mmHg min l^{-1} at 60 min, $P < 0.01$) (Figure 1). In response to endotoxin, pulmonary arterial pressure decreased from 12.5 ± 0.9 (control) to 9.8 ± 0.8 mmHg at 60 min ($P < 0.05$), while pulmonary vascular resistance showed no significant change. Endotoxin did not affect heart rate at any time period during the experimental period. Both pulmonary capillary wedge pressure and central venous pressure were decreased by endotoxin from 6.8 ± 0.6 (control) to 3.8 ± 0.4 mmHg ($P < 0.01$) and from 3.1 ± 0.3 (control) to 1.8 ± 0.3 mmHg at 60 min ($P < 0.01$), respectively. Haematocrit increased gradually, but the change was small and only reached statistical significance at 120 min after endotoxin administration (from 26.3 ± 3.2 to $29.2 \pm 4.1\%$, $P < 0.05$). Figure 2a shows representative recordings of central venous pressure during a cycle of volume changes. The change in central venous pressure at 60 min after endotoxin administration was smaller than that at baseline conditions, indicating an increase in EVC. The mean value for EVC (shown in Figure 2b) was increased by endotoxin from 6.5 ± 0.4 to 12.0 ± 1.4 ml mmHg^{-1} kg^{-1} ($P < 0.01$). The plasma concentrations of 6-keto $PGF_{1\alpha}$ were markedly increased by endotoxin from 110 ± 42 (control) to 1567 ± 200 pg ml^{-1} at 60 min ($P < 0.01$) and 1698 ± 366 pg ml^{-1} at 120 min ($P < 0.01$). The plasma concentrations of PGE_2 were also increased from 24 ± 12 (control) to 203 ± 47 pg ml^{-1} at 60 min ($P < 0.01$) and 345 ± 127 pg ml^{-1} at 120 min ($P < 0.01$).

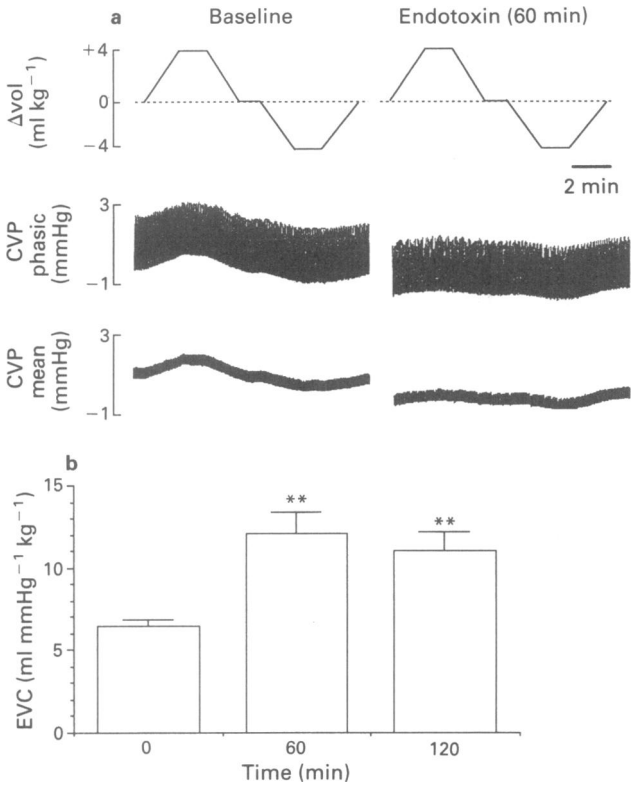


Figure 2 (a) Typical recordings of central venous pressure (CVP) during a cycle of volume changes under control conditions and 60 min after administration of endotoxin. (b) Effect of endotoxin on effective vascular compliance (EVC). $^{**}P < 0.01$ when compared with the baseline value.

Effect of cyclo-oxygenase inhibition on experimental endotoxin shock (Group 2)

The endotoxin-induced hypotension was significantly blocked by ibuprofen (Figure 3). The decrease in TPR was abolished by ibuprofen and even a systemic vasoconstriction was observed in this group. The increases in EVC and the plasma concentrations of 6-keto $PGF_{1\alpha}$ and PGE_2 were abolished by ibuprofen (Table 2). Ibuprofen, however, did not affect the reduction in cardiac output induced by endotoxin.

Effect of PAF receptor antagonist on experimental endotoxin shock (Group 3)

TCV-309 significantly attenuated the endotoxin-induced hypotension, but to a lesser degree than ibuprofen. TCV-309 inhibited only the early, but not the delayed hypotension after endotoxin (Figure 3). The increase in EVC was abolished by TCV-309. In contrast to ibuprofen, TCV-309 markedly attenuated the reduction in cardiac output and had no effect on the decrease in TPR. TCV-309 did not significantly affect the increases in plasma concentrations of 6-keto $PGF_{1\alpha}$ and PGE_2 (Table 2).

Table 2 Endotoxin-induced changes in plasma concentrations of prostanoids

	Group 1 (endotoxin alone)	*Group 2* (ibuprofen)	*Group 3* (TCV-309)
6-keto Prostaglandin $F_{1\alpha}$ (pg ml^{-1})			
Baseline	110 ± 42	24 ± 6	53 ± 14
60 min	1457 ± 189	− 15 ± 6**	1150 ± 503
120 min	1588 ± 349	− 10 ± 4**	1309 ± 488
Prostaglandin E_2 (pg ml^{-1})			
Baseline	24 ± 12	8 ± 1*	17 ± 3
60 min	179 ± 49	− 1 ± 2**	314 ± 65
120 min	320 ± 128	− 1 ± 2**	312 ± 74

Values are means ± s.e.mean. Blood samples were collected before endotoxin administration (baseline) and 60 and 120 min after its administration. The data for 60 and 120 min are expressed as increments from the corresponding baseline value. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the other two groups. Since the increments of 6-keto prostaglandin $F_{1\alpha}$ in Group 3 were smaller than the ones observed in Group 1, the number of experiments in this group was increased to 7 in order to investigate whether this tendency was significant.

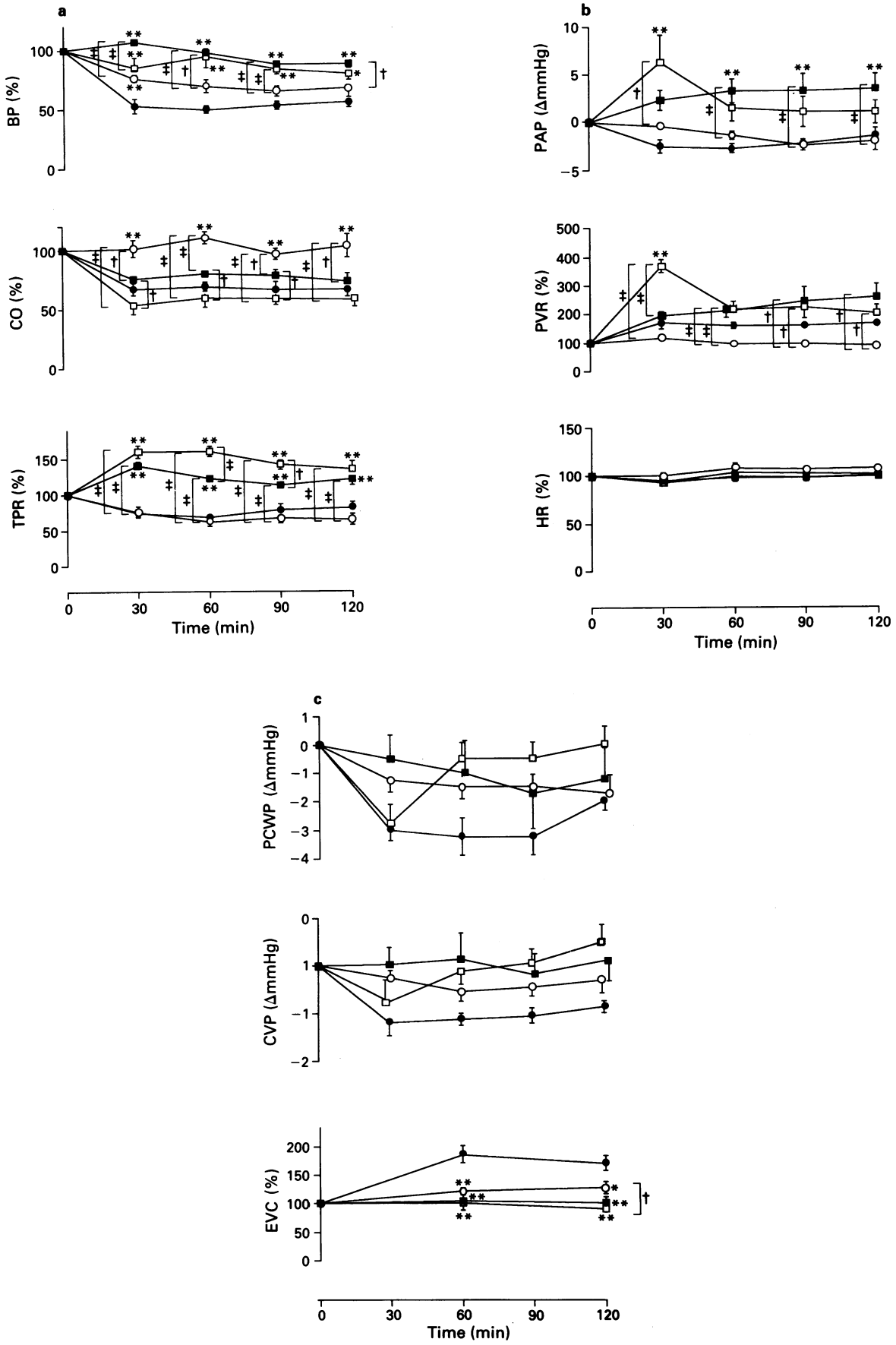


Figure 3 Time course of the effect of ibuprofen and TCV-309 on haemodynamic changes induced by endotoxin. Symbols represent the following: (●) endotoxin alone; (○) endotoxin plus TCV-309; (□) endotoxin plus ibuprofen; (■) endotoxin plus combined treatment with ibuprofen and TCV-309. (a) BP, blood pressure; CO, cardiac output; TPR, total peripheral vascular resistance; (b) PAP, pulmonary arterial pressure; PVR, pulmonary vascular resistance; HR, heart rate; (c) PCWP, pulmonary capillary wedge pressure; CVP, central venous pressure; EVC, effective vascular complicance. $*P<0.05$ and $**P<0.01$ when compared to endotoxin alone. †$P<0.05$ and ‡$P<0.01$ when values were compared between groups at the same time point.

Effect of combined treatment with ibuprofen and TCV-309 on experimental endotoxin shock (Group 4)

The effects of combined treatment with ibuprofen and TCV-309 on the endotoxin-induced haemodynamic changes were similar to the ones observed with ibuprofen alone. The combined treatment protocol markedly blocked the endotoxin-induced hypotension, had no effect on the reduction in cardiac output, caused a systemic vasoconstriction, and abolished the increase in EVC. However, when compared to animals subjected to endotoxin in the presence of ibuprofen alone, combined treatment with TCV-309 and ibuprofen attenuated the reduction in cardiac output and the systemic vasoconstriction elicited by endotoxin (Figure 3).

Discussion

We examined the effect of TCV-309 and ibuprofen on endotoxin-induced haemodynamic changes in the anesthetized dogs in order to elucidate the role of PAF and prostanoids and the interaction of these lipid autacoids in endotoxin shock. Intravenous administration of endotoxin caused a severe hypotension that was attributable to a decrease in both TPR and cardiac output. Both TCV-309 and ibuprofen significantly blocked the endotoxin-induced hypotension, but to a different extent and at different time points after endotoxin challenge. Data concerning effects of these blockers on other haemodynamic changes suggest that the roles of PAF and prostanoids in endotoxin shock are partly common and partly different.

It has been reported that loss of vascular tone in both resistance vessels and capacitance vessels plays an important role in endotoxin-induced hypotension (Gilbert, 1960; Siegel *et al.*, 1967; D'Orio *et al.*, 1989). Our present study also showed that these two vascular effects contributed to the hypotension in endotoxaemia. The observed decrease in TPR indicates that the dilatation of resistance vessels contributes to the endotoxin-induced hypotension. The loss of vascular tone in capacitance vessels (blood pooling) is indicated by our findings that cardiac output, central venous pressure and pulmonary capillary wedge pressure were decreased by endotoxin, while the increase in haematocrit was only small and gradual. This result suggests that an endotoxin-induced reduction in cardiac output is due to a decrease in venous return, which is caused by blood pooling rather than extravasation of plasma (Yamanaka *et al.*, 1992). The hypothesis that endotoxin caused blood pooling is also supported by our findings that endotoxin increased EVC. The term, EVC was first described by Echt *et al.* (1974) and later modified by Münzel *et al.* (1988). Changes in mean arterial pressure and cardiac output do not significantly affect EVC and the changes in EVC mainly reflect the tone in capacitance vessels (Elsner *et al.*, 1986; Münzel *et al.*, 1988). Thus, the endotoxin-induced increase in EVC indicates a loss of vascular tone in capacitance vessels (blood pooling).

Ibuprofen markedly attenuated the endotoxin-induced hypotension and abolished the reduction in TPR and the increase in EVC. These data suggest that ibuprofen blocked the endotoxin-induced loss of vascular tone in both resistance and capacitance vessels. The inhibition of the dilatation of resistance vessels by ibuprofen is supported by previous findings of Cryer *et al.* (1990) demonstrating that *E. coli*-induced dilatation of small arterioles of skeletal muscle in rats was attenuated by topical application of the cyclo-oxygenase inhibitor, meclofenamate. The plasma concentrations of 6-keto $PGF_{1\alpha}$ and PGE_2 were significantly increased by endotoxin, an effect that was abolished by ibuprofen. Abel & Beck (1991) reported that the increase in portal venous complicance caused by endotoxin was blocked by ibuprofen. Taken together, these data indiate that the endotoxin-induced vasodilatation in both resistance and capacitance vessels is due to an enhanced formation of endogenous vasodilator prostaglandins.

TCV-309 blocked the endotoxin-induced hypotension to a lesser degree than ibuprofen. It also attenuated the endotoxin-induced increase in EVC, suggesting that the blood pooling caused by endotoxin is blocked by TCV-309 as well as ibuprofen. However, TCV-309 did not affect the decrease in TPR, suggesting that the vasodilatation in resistance vessels induced by endotoxin is independent of PAF. Thus, in contrast to ibuprofen which attenuated the loss of vascular tone in both resistance and capacitance vessels, TCV-309 only blocked the blood pooling. Thus, the decrease in cardiac output resulting from blood pooling was markedly attenuated by TCV-309, while TCV-309 reduced the hypotension caused by endotoxin to a lesser degree than ibuprofen. The notion that PAF is involved in venodilatation, but not in the systemic vasodilatation associated with endotoxin shock does not necessarily mean that PAF is only produced in veins, for exogenous PAF also induced venodilatation, but not systemic vasodilatation (Yamanaka *et al.*, 1992). The mechanism by which PAF selectively dilates capacitance vessels is unclear.

We previously reported that exogenous PAF stimulates the release of vasodilator prostaglandins, which cause blood pooling and hypotension (Yamanaka *et al.*, 1992). It seems likely therefore that endotoxin first causes the release of PAF, which in turn induces prostaglandin-dependent blood pooling. This notion is supported by our finding here that the treatment with ibuprofen alone blocked the endotoxin-induced increase in EVC to the same extent as the combined treatment with both ibuprofen and TCV-309. However, the increases in the plasma concentrations of 6-keto $PGF_{1\alpha}$ and PGE_2 were not significantly affected by TCV-309. Moore *et al.* (1991) also reported that BN 52021, another PAF receptor antagonist, did not significantly affect the release of prostaglandins in canine endotoxaemia. In our previous study, exogenously administered PAF ($0.5\,\mu g\,kg^{-1}$) elicited a profound reduction in systemic blood pressure due to prostaglandin-dependent blood pooling (from 108 ± 7 to 66 ± 6 mmHg), although the increases in the plasma concentrations of prostaglandins were only $328 \pm 77\,pg\,ml^{-1}$ (6-keto $PGF_{1\alpha}$) and $18 \pm 8\,pg\,ml^{-1}$ (PGE_2) (Yamanaka *et al.*, 1992). These increases in plasma prostaglandin concentrations are only about one-fifth and one-tenth of the corresponding values caused by endotoxin in the present study. Thus, we speculate that the amounts of prostaglandins released by PAF in our study are relatively small and that we are unable to distinguish them from those which are independent of PAF and responsible for the vasodilatation in resistance vessels.

Despite an attenuation of hypotension, cardiac output remained low in the ibuprofen-treated group, suggesting that other mechanisms independent of vasodilatation also contribute to the reduction in cardiac output. Cardiac depression, as reported by Papadakis *et al.* (1988) and others (see Raymond, 1990), may be one of those mechanisms. In the ibuprofen-treated group, endotoxin induced an increase in TPR (50%), even when reflexes were blocked with ganglionic blockade. This increase in afterload presumably also contributes to the decrease in cardiac output. This prostaglandin-independent reduction of cardiac output and systemic vasoconstriction was partly blocked by the combined treatment with TCV-309 (Figure 3). We previously reported that exogenous PAF induced a decrease in cardiac output and an increase in TPR even after the cyclo-oxygenase inhibition (Yamanaka *et al.*, 1992). In addition, PAF has direct cardiodepressant actions (Kenzora *et al.*, 1984). These data indicate that PAF partly contributes to the prostaglandin-independent reduction of cardiac output and systemic vasoconstriction. However, it is obvious that other mechanisms are also involved, which require further investigation.

In contrast to systemic resistance vessels, endotoxin alone did not induce a significant change in pulmonary vascular resistance. Although endotoxin caused a significant decrease in pulmonary arterial pressure, this change might well be due

to the reduction in cardiac output. However, in the ibuprofen-treated group, endotoxin induced a significant pulmonary vasoconstriction. Thus, it is suggested that endotoxin may induce a prostaglandin-dependent pulmonary vasodilatation and a prostaglandin-independent pulmonary vasoconstriction simultaneously.

Recent studies revealed that an enhanced formation of nitric oxide (NO), a potent vasodilator, is a key feature in circulatory shock (Julou-Schaeffer *et al.*, 1990; Thiemermann & Vane, 1990). NO causes not only the loss of vascular tone, but also the loss of the responsiveness to pressor drugs such as noradrenaline (Julou-Schaeffer *et al.*, 1990). Although the role of NO was not tested in the present study, blockade of NO synthesis reduced the endotoxin-induced hypotension in rats (Thiemermann & Vane, 1990) and dogs (Kalbunde & Ritger, 1991). The role of NO in this model and/or on possible interactions between NO and these mediators requires further study.

In conclusion, the present study demonstrates that (1) both prostanoids and PAF are involved in dilatation of capacitance vessels, (2) prostanoids, but not PAF cause dilatation of resistance vessels, and (3) PAF may partly contribute to prostanoids-independent reduction in cardiac output and systemic vasoconstriction in acute canine experimental endotoxic shock.

We are grateful to Kaken Pharmaceutical Co. (Tokyo, Japan) for providing ibuprofen, Takeda Chemical Industry (Osaka, Japan) for providing TCV-309, and The Green Cross Corp. (Osaka, Japan) for providing Dextran 40.

This work was supported in part by a grant from Chichibu Cement.

References

ABEL, F.L. & BECK, R.R. (1991). Portal venous compliance in canine endotoxin shock. *Circ. Shock*, **35**, 96–101.

ALMQVIST, P.M., KUENZIG, M. & SCHWARTZ, S.I. (1984). Treatment of experimental canine endotoxin shock with ibuprofen, a cyclooxygenase inhibitor. *Circ. Shock*, **13**, 227–232.

BONE, R.C. (1992). Phospholipids and their inhibitors: a critical evaluation of their role in the treatment of sepsis. *Crit. Care Med.*, **20**, 884–890.

CRYER, H.G., GARRISON, R.N., HARRIS, P.D., GREENWALD, B.H. & ALSIP, N.L. (1990). Prostaglandins mediate skeletal muscle arteriole dilatation in hyperdynamic bacteremia. *Am. J. Physiol.*, **259**, H728–H734.

D'ORIO, V., WAHLEN, C., NALDI, M., FOSSION, A., JUCHMES, J. & MARCELLE, R. (1989). Contribution of peripheral blood pooling to central hemodynamic disturbances during endotoxin insult in intact dogs. *Crit. Care Med.*, **17**, 1314–1319.

ECHT, M., DUWELING, J., GAUER, O.H. & LANGE, L. (1974). Effective compliance of the total vascular bed and the intrathoracic compartment derived from changes in central venous pressure induced by volume changes in man. *Circ. Res.*, **34**, 61–68.

ELSNER, D., STEWART, D.J., SOMMER, O., HOLTZ, J. & BASSENGE, E. (1986). Postsynaptic α1- and α2-adrenergic receptors in adrenergic control of capacitance vessel tone in vivo. *Hypertension*, **8**, 1003–1014.

GANZ, W. & SWAN, H.J.C. (1972). Measurement of blood flow by thermodilution. *Am. J. Cardiol.*, **29**, 241–246.

GILBERT, R.P. (1960). Mechanisms of the hemodynamic effects of endotoxin. *Physiol. Rev.*, **40**, 245–279.

JACOBS, E.R. SOULSBY, M.E., BONE, R.C., WILSON, F.J. Jr. & HILLER, F.C. (1982). Ibuprofen in canine endotoxin shock. *J. Clin. Invest.*, **70**, 536–541.

JULOU-SCHAEFFER, G., GRAY, G.A., FLEMING, I., SCHOTT, C., PARRATT, J.R. & STOCLET, J.C. (1990). Loss of vascular responsiveness induced by endotoxin involves L-arginine pathway. *Am. J. Physiol.*, **259**, H1038–H1043.

KENZORA, J.L., PEREZ, J.E., BERGMAN, S.R. & LANGE, L.G. (1984). Effects of acetyl glyceryl ether phosphorylcholine (platelet activating factor) on ventricular preload, afterload, and contractility in dogs. *J. Clin. Invest.*, **74**, 1193–1203.

KLABUNDE, R.E. & RITGER, R.C. (1991). N^G-monomethyl-L-arginine (NMA) restores arterial blood pressure but reduces cardiac output in a canine model of endotoxic shock. *Biochem. Biophys. Res. Commun.*, **178**, 1135–1140.

MIURA, K., YUKIMURA, T., YAMASHITA, Y., SHIMMEN, T., OKUMURA, M., YAMANAKA, S., IMANISHI, M. & YAMAMOTO, K. (1991). Renal and femoral vascular responses to endothelin-1 in dogs: role of prostaglandins. *J. Pharmacol. Exp. Ther.*, **256**, 11–17.

MOORE, J.M., EARNEST, M.A., DISIMONE, A.G., ABUMRAD, N.N. & FLETCHER, J.R. (1991). A PAF receptor antagonist, bn 52021, attenuates thromboxane release and improves survival in lethal canine endotoxemia. *Circ. Shock*, **35**, 53–59.

MORRISON, D.C. & RYAN, J.L. (1987). Endotoxins and disease mechanisms. *Annu. Rev. Med.*, **38**, 417–432.

MOZES, T., ZIJLSTRA, F.J., HEILIGERS, J.P., TAK, C.J., BEN EFRAIM, S., BONTA, I.L. & SAXENA, P.R. (1991). Sequential release of tumour necrosis factor, platelet activating factor and eicosanoids during endotoxin shock in anaesthetized pigs: protective effects of indomethacin. *Br. J. Pharmacol.*, **104**, 691–699.

MÜNZEL, T., STEWART, D.J., HOLTZ, J. & BASSENGE, E. (1988). Preferential venoconstriction by cyclooxygenase inhibition in vivo without attenuation of nitroglycerin venodilatation. *Circulation*, **78**, 407–415.

PAPADAKIS, E.J. & ABEL, F.L. (1988). Left ventricular performance in canine endotoxin shock. *Circ. Shock*, **24**, 123–131.

RAYMOND, R.M. (1990). When does the heart fail during shock? *Circ. Shock*, **30**, 27–41.

SANCHEZ CRESPO, M. & FERNANDEZ GALLARDO, S. (1991). Pharmacological modulation of PAF: a therapeutic approach to endotoxin shock. *J. Lipid Mediat.*, **4**, 127–143.

SIEGEL, J.H., GREENSPAN, M. & DEL GUERCIO, L.R.M. (1967). Abnormal vascular tone, defective oxygen transport and myocardial failure in human septic shock. *Ann. Surg.*, **165**, 504–517.

TERASHITA, Z., IMURA, Y., NISHIKAWA, K. & SUMIDA, S. (1985). Is platelet activating factor (paf) a mediator of endotoxin shock? *Eur. J. Pharmacol.*, **109**, 257–261.

TERASHITA, Z., KAWAMURA, M., TAKATANI, M., TSUSHIMA, S., IMURA, Y. & NISHIKAWA, K. (1992). Beneficial effects of TCV-309, a novel potent and selective platelet activating factor antagonist in endotoxin and anaphylactic shock in rodents. *J. Pharmacol. Exp. Ther.*, **260**, 748–755.

THIEMERMANN, C. & VANE, J. (1990). Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *Eur. J. Pharmacol.*, **182**, 591–595.

YAMANAKA, S., MIURA, K., YUKIMURA, T., OKUMURA, M. & YAMAMOTO, K. (1992). Putative mechanism of hypotensive action of platelet-activating factor in dogs. *Circ. Res.*, **70**, 893–901.

ZAR, J.H. (1984). *Biostatistical Analysis*, 2nd ed. Englewood Cliffs, N.J.: Prentice-Hall.

(Received February 15, 1993
Revised July 31, 1993
Accepted August 2, 1993)

Br. J. Pharmacol. (1993), **110**, 1508–1514 © Macmillan Press Ltd, 1993

Protection by scoparone against the alterations of plasma lipoproteins, vascular morphology and vascular reactivity in hyperlipidaemic diabetic rabbit

Huei-Chen Huang, Yu-I Weng, Chai-Rong Lee, Tong-Rong Jan, †Yuh-Lien Chen & *Yuan-Teh Lee

Department of Pharmacology, and *Department of Internal Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan and †Department of Anatomy, National Yang-Ming Medical College, Taipei, Taiwan

1 The *in vivo* pharmacological effects of scoparone (6,7-dimethoxycoumarin) in a hyperlipidaemic diabetic rabbit model were investigated.

2 Three groups of rabbits were studied: (1) normal, (2) hyperlipidaemic and diabetic-untreated and (3) hyperlipidaemic and diabetic-scoparone treated. The hyperlipidaemic diabetic rabbits were fed with 1% cholesterol and treated with alloxan, a diabetogenic agent. The plasma levels of total cholesterol, total triglyceride, very low-density lipoprotein (VLDL) cholesterol, low density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol were markedly increased as soon as the rabbit became diabetic at the second week. Scoparone-treatment (5 mg kg^{-1} day^{-1}, s.c.) significantly reduced the plasma lipid and lipoprotein cholesterol levels of the hyperlipidaemic diabetic rabbit to 73.3% of total cholesterol, 48.3% of total triglyceride, 66.0% of VLDL cholesterol, 55.7% of LDL cholesterol and 79.5% of HDL cholesterol.

3 Six weeks after cholesterol-feeding, the aortic arch and thoracic aorta were dissected for morphological and functional studies. In vascular rings from the untreated hyperlipidaemic diabetic rabbit, there was intimal thickening with accumulation of fatty streaks, foam cells and migration of smooth muscle cells to the intima. In the rabbits treated with scoparone, there were fewer pathological morphology changes found in vascular segments than in the untreated hyperlipidaemic diabetic rabbits.

4 In the vascular reactivity experiments, the phenylephrine-induced contraction and nitroprusside-induced dilatation did not differ significantly among the three rabbit groups, except that the contraction was enhanced in the thoracic aorta of hyperlipidaemic diabetic rabbits either untreated or treated with scoparone, as compared to the normal group, and the sensitivity to nitroprusside was increased in the thoracic aorta of the scoparone-treated group as compared to the untreated group.

5 The endothelium-dependent dilatation induced by acetylcholine was significantly attenuated in both the aortic arch and thoracic aorta from the hyperlipidaemic diabetic rabbits as compared to the normal rabbits. This attenuation was partially prevented, when scoparone (5 mg kg^{-1}) was administered daily.

6 These results suggest that scoparone protects against some alterations of plasma lipoproteins, vascular morphology and vascular reactivity in the hyperlipidaemic diabetic rabbit. These protective effects of scoparone may be partly related to its free radical scavenging property.

Keywords: Scoparone; coumarin derivative; hyperlipidaemic diabetic rabbit; hypolipidaemic effect; antiatherogenic effect; vascular reactivity

Introduction

Scoparone (6,7-dimethoxycoumarin), an active principle from the hypolipidaemic Chinese herb *Artemisia scoparia*, has been found to possess free-radical scavenging properties (Huang *et al.*, 1992b) and to exhibit vasodilator and antiproliferative effects (Huang *et al.*, 1991a; 1992a,b; 1993). In this study, the *in vivo* pharmacological effects of scoparone in the hyperlipidaemic diabetic rabbit were investigated. Hyperlipidemia is often associated with diabetes and the abnormal lipoprotein metabolism may be one of the reasons for this (Rosenko *et al.*, 1980; Bierman, 1991). The development of drugs with a cholesterol-lowering effect on hyperlipidaemic diabetic subjects is important for preventing the vascular complications of atherosclerosis and diabetes. During the course of studying the pharmacological actions of hypolipidaemic Chinese herbs, we evaluated the hypolipidaemic effects of some crude extracts and active principles from Chinese herbs in a hyperlipidaemic diabetic rabbit model. In this model, the cholesterol-fed rabbit was treated with diabetogenic agent, alloxan, to elevate more markedly the plasma lipid and lipoprotein lipid levels at the earlier weeks as compared to the animal fed with cholesterol only (Huang *et al.*, 1991b). The effects of scoparone on plasma lipid and lipoprotein lipid levels were investigated in this rabbit model. Moreover, as hypercholesterolaemia is known to induce important morphological and functional changes in the arterial wall, we also examined the vascular morphology and reactivity of the thoracic aorta and aortic arch from untreated and scoparone-treated hyperlipidaemic diabetic rabbits.

[1] Author for correspondence.

Methods

Animals

The effects of scoparone were evaluated by using three rabbit groups, normal, hyperlipidaemic diabetic (cholesterol-fed alloxan-diabetic) and scoparone-treated (scoparone-treated cholesterol-fed alloxan-diabetic) rabbit groups. Each group contained six rabbits. The three groups of rabbits were age-matched and weight-matched. Hyperlipidaemic cholesterol-fed alloxan-diabetic rabbits were prepared in our laboratory (Huang *et al.*, 1991b). Rabbits (2.0–2.5 kg) were fed with a

specific diet containing 1% cholesterol and 20% corn oil. In the second week, diabetes was induced by intravenous administration of a 10% solution of alloxan monohydrate freshly prepared in saline (60 mg kg^{-1}). In the scoparone-treated rabbit group, cholesterol-fed alloxan-diabetic rabbits were treated by daily injection of scoparone (5 mg kg^{-1}, s.c.) starting at the time of the administration of alloxan. Six weeks after cholesterol feeding, the aortic arch and thoracic aorta were dissected for morphological and functional studies.

Plasma lipid levels, blood pressure and blood glucose

The body weight, blood pressure, blood glucose concentration and plasma lipid, lipoprotein lipid concentrations of rabbits were measured every week. The arterial blood pressure was measured by the tail plethysmographic method (PE 300, Narco Biosystem, U.S.A.) (Huang, 1984). Before the start of experiments, rabbits (restrained, at room temperature, with 7/16 inch i.d. cuff) were trained to enable reproducible control values to be obtained. The blood glucose concentration was measured with a glucose analyser (YSI model 23A, Scientific Division, U.S.A.). Lipoprotein fractions were isolated by repeated ultracentrifugations according to the method of Buege & Aust (1978). Plasma was adjusted to the desired density by the addition of NaBr. Lipoprotein fractions including very low-density lipoprotein (VLDL; $d < 1.019$ g ml^{-1}), low-density lipoprotein (LDL; $d = 1.019-1.063$ g ml^{-1}) and high-density lipoprotein (HDL; $d = 1.063-1.21$ g ml^{-1}) were obtained sequentially by differential ultracentrifugation (110,000 *g* at 10°C). The plasma total cholesterol, total triglyceride and lipoprotein cholesterol levels were measured by Merck assay kits (Darmstadt, Germany).

Vascular morphology

Vascular segments were fixed in 10% formalin. The fixed tissue samples were dehydrated and embedded in paraffin, cut into 5 μm sections and stained with Movat's pentachrome stain. Histological sections were projected, and the intima/media index (intimal/media area times 100) was determined by computerized planimetry.

Vascular reactivity

The aortic arch and thoracic aorta were excised. The vessel was dissected free from connective tissue and ring segments (3 to 5 mm) were prepared. The vascular ring was mounted between hooks and placed in a 5 ml organ bath containing Krebs solution at 37°C and bubbled with 5% CO_2 in O_2. Isometric tension was measured with a transducer (Harvard Bioscience, South Natick, MA, U.S.A.) attached to tissue bath computer (Buxco electronics, Sharon, CT, U.S.A.). Rings were stretched to the optimal length for isometric contraction (Huang & Lee, 1985). The preparations were then allowed to equilibrate for 120 min.

Vascular responses to vasoconstrictor (phenylephrine) and vasodilators (acetylcholine and nitroprusside) were studied. Concentration-response curves of the vasoconstrictor were obtained by cumulative additions of phenylephrine. In order to demonstrate the effect of the vasodilators, a dose of phenylephrine ($3 \times 10^{-6}-10^{-5}$ M) producing a sustained contraction was used to precontract the vascular ring. After the addition of phenylephrine (when a stable plateau was reached) cumulative doses of vasodilator were added. The contractions elicited by phenylephrine was expressed as the gram tension increase. The dilatations elicted by acetylcholine and nitroprusside were expressed as percentages of the maximal tension increase produced by phenylephrine. The concentration evoking 50% of the maximal response (EC_{50} value) for each experiment was estimated graphically from the dose-response curve.

Materials

Scoparone was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). (−)-Phenylephrine HCl, acetylcholine chloride, sodium nitroprusside, cholesterol, and sodium bromide were purchased from Sigma Co. (St. Louis, MO, U.S.A.). New Zealand rabbits were supplied from the animals centre of National Taiwan University, Taipei, Taiwan.

Statistics

The data were expressed as means ± s.e.mean. For statistical analysis, Student's *t* test for unpaired observation was used. *P* values less than 0.05 were considered to be significant.

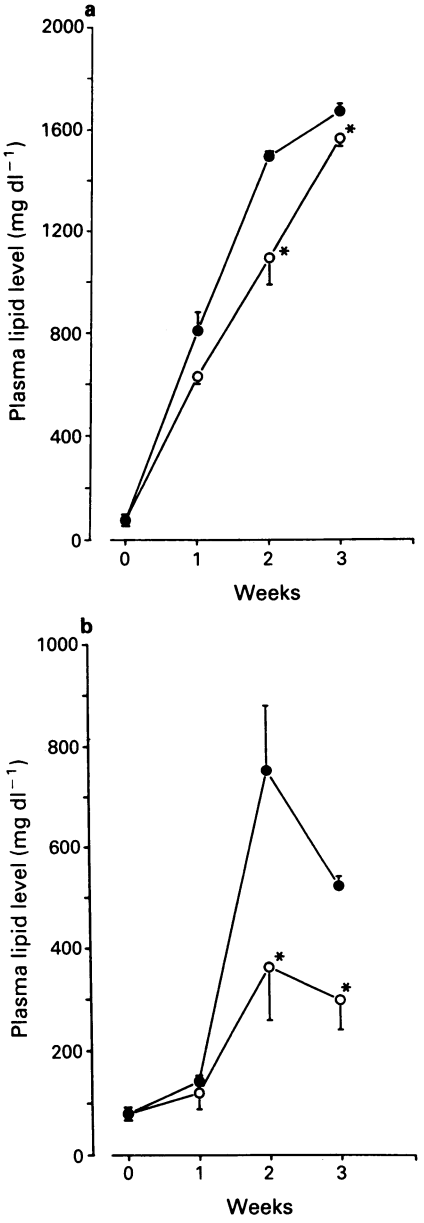


Figure 1 Effects of scoparone on plasma (a) total cholesterol and (b) total triglyceride concentrations in hyperlipidaemic diabetic rabbits. The plasma levels of total cholesterol and total triglyceride in hyperlipidaemic diabetic (●) and scoparone-treated (○) groups were measured. Hyperlipidaemic diabetic rabbits were fed with a specific diet containing 1% cholesterol and 20% corn oil and diabetes was then induced by 10% alloxan solution (60 mg kg^{-1}) at the second week. The scoparone-treated hyperlipidaemic diabetic group was treated by daily injection of scoparone (5 mg kg^{-1}, s.c.) after the administration of alloxan. Points represent the means ± s.e.mean ($n = 6$). *$P < 0.05$, compared to the hyperlipidaemic diabetic group.

Table I Effect of scoparone on plasma lipid and lipoprotein lipid concentrations in the hyperlipidaemic diabetic rabbit

	n	*Total cholesterol* (mg dl^{-1})	*Total triglyceride* (mg dl^{-1})	*Lipoprotein cholesterol* (mg dl^{-1})		
				VLDL	*LDL*	*HDL*
Normal group	6	76.0 ± 2.2	80.0 ± 7.5	29.7 ± 2.0	21.8 ± 2.5	33.0 ± 5.2
Hyperlipidaemic diabetic group	6	1492.7 ± 6.0[a]	752.8 ± 128.3[a]	1182.3 ± 24.6[a]	219.1 ± 30.6[a]	58.0 ± 1.3[a]
Scoparone-treated group	6	1093.8 ± 105.1[b] (73.3%)	363.3 ± 103.5[b] (48.3%)	778.8 ± 20.8[b] (66.0%)	122.1 ± 27.5[b] (55.7%)	46.1 ± 0.7[b] (79.5%)

The values are means ± s.e.mean of plasma lipid and lipoprotein lipid concentrations at the end of the second week. The values in parentheses express percentage of values in the hyperlipidaemic diabetic group. The hyperlipidaemic diabetic group was fed with a specific diet containing 1% cholesterol and 20% corn oil and diabetes was then induced by bolus intravenous injection of 10% alloxan solution (60 mg kg^{-1}) at the second week. The scoparone-treated group was fed with the specific diet, injected with alloxan and treated by daily injection of scoparone (5 mg kg^{-1} day^{-1}, s.c.) starting at the time of the administration of alloxan.
[a]$P<0.05$, compared to normal group; [b]$P<0.05$, compared to hyperlipidaemic diabetic group.

Table 2 Responsiveness of vascular rings from normal, untreated and scoparone-treated hyperlipidaemic diabetic rabbits to vasoactive agents

			Phenylephrine		*Acetylcholine*		*Nitroprusside*	
	Vessel	n	EC_{50} (10^{-7} M)	T_{max} (g)	EC_{50} (10^{-8} M)	*Max. response* (%)	EC_{50} (10^{-7} M)	*Max response* (%)
Normal group	Aortic arch	6	5.8 ± 0.5	9.2 ± 0.6	5.5 ± 1.7	50.0 ± 2.8	14.8 ± 5.3	85.8 ± 3.7
	Thoracic aorta	12	3.7 ± 0.6	5.8 ± 0.2	6.9 ± 0.7	77.5 ± 1.8	8.3 ± 2.1	96.5 ± 0.9
Hyperlipidaemic diabetic group	Aortic arch	5	6.5 ± 1.4	7.5 ± 1.4	55.0 ± 20.0[a]	35.6 ± 1.2[a]	11.8 ± 4.4	85.0 ± 3.3
	Thoracic aorta	12	2.5 ± 0.8	6.8 ± 0.3[a]	11.3 ± 0.3[a]	59.7 ± 2.6[a]	5.9 ± 0.5	93.2 ± 1.8
Scoparone-treated group	Aortic arch	5	6.3 ± 1.7	8.0 ± 0.5	5.7 ± 0.6[b]	37.5 ± 3.3[a]	5.0 ± 1.1	88.8 ± 0.3
	Thoracic aorta	12	2.8 ± 0.5	6.8 ± 0.3[a]	7.7 ± 0.7[b]	72.1 ± 4.0[b]	3.1 ± 0.7[b]	95.0 ± 1.0

The values are means ± s.e.mean. The hyperlipidaemic diabetic group was fed with a specific diet containing 1% cholesterol and 20% corn oil and diabetes was then induced by intravenous injection of 10% alloxan solution (60 mg kg^{-1}) at the second week. The scoparone-treated group was fed with the specific diet, injected with alloxan and treated by daily injection of scoparone (5 mg kg^{-1} day^{-1}, s.c.) after the administration of alloxan. Six weeks after cholesterol-feeding vessels were dissected out from three rabbit groups for vascular reactivity experiments.
[a]$P<0.05$, compared to normal group. [b]$P<0.05$, compared to hyperlipidaemic diabetic group.

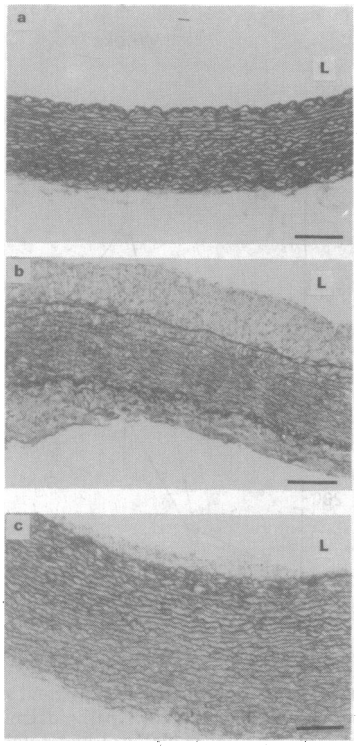


Figure 2 Light microscopical features of cross sections of the aortic arches obtained from (a) normal, (b) hyperlipidaemic diabetic and (c) scoparone-treated hyperlipidaemic diabetic rabbit groups. Movat's pentachrome stain, 150 ×. Bar = 70 μm. Marked intimal thickening, significant fibrous plaque and foam cells were found in vessels from the hyperlipidaemic diabetic group. In vessels from the scoparone-treated group, less intimal thickening, no significant fibrous plaque and fewer foam cells were observed. L: lumen.

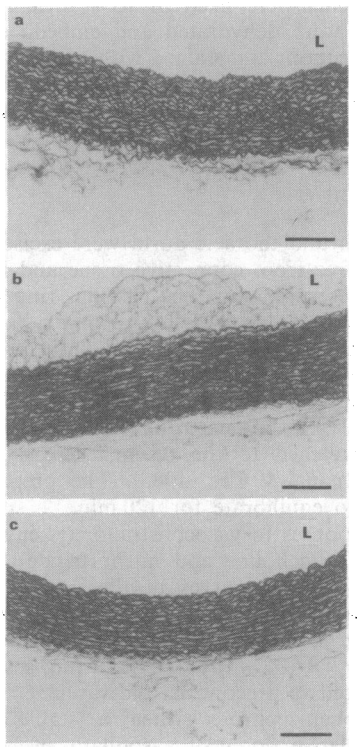


Figure 3 Light microscopical features of cross sections of the thoracic aortae obtained from (a) normal, (b) hyperlipidaemic diabetic and (c) scoparone-treated hyperlipidaemic diabetic rabbit groups. Movat's pentachrome stain, 150 ×. Bar = 70 μm. Marked intimal thickening, significant fibrous plaque and foam cells were found in vessels from the untreated hyperlipidaemic diabetic group. In vessels from the scoparone-treated group, less intimal thickening, less fibrous plaque and fewer foam cells were observed. L: lumen.

Results

Baseline data

During treatment in rabbits, scoparone (5 mg kg^{-1} day^{-1}) was well tolerated and no serious side effect was observed. Although the body weight increased with time during the experimental period (2.3 ± 0.1 to 2.5 ± 0.1 kg), there was no significant difference in body weight between the scoparone-treated and the untreated hyperlipidaemic diabetic rabbits (2.3 ± 0.1 to 2.5 ± 0.1 vs. 2.3 ± 0.1 to 2.6 ± 0.1 kg). There was no significant difference in the blood pressure between scoparone-treated and untreated rabbits (98.9 ± 5.0 mmHg for basal control values; 107.6 ± 4.6 vs. 118.6 ± 7.2 mmHg at the 6th week). The mean values of arterial blood pressure of the scoparone-treated group were lower than those of the untreated hyperlipidaemic diabetic group after the 3rd week (105.2 ± 5.7 vs. 118.9 ± 5.9 mmHg at the 4th week). However, the differences in blood pressure between treated and untreated groups were inconsistent and did not achieve significance during the 6-week period. Upon administration of alloxan in cholesterol-fed rabbit at the second week, the blood glucose concentration was elevated to a level above 250 mg dl^{-1} within 48 h. The blood glucose concentration of untreated hyperlipidaemic diabetic rabbit was elevated from 141.2 ± 3.5 to 262.0 ± 46.6 mg dl^{-1}. Scoparone-treatment reduced the blood glucose to 223.5 ± 28.0 mg dl^{-1}. However, the differences in blood glucose concentrations between treated and untreated groups did not achieve significance.

In the normal group, the plasma total cholesterol, total triglyceride and lipoprotein cholesterol concentrations did not change significantly during the 6-week period. In the untreated cholesterol-fed alloxan-diabetic group, the total plasma cholesterol and triglyceride levels were markedly increased as soon as the rabbit became diabetic at the second week. The increases in the plasma cholesterol and triglyceride in cholesterol-fed alloxan-diabetic rabbits were more marked than those in rabbits fed with cholesterol only (Huang *et al.*, 1991b). The plasma total cholesterol concentrations were greatly increased at the second week (1492.7 ± 6.0 mg dl^{-1}) (Figure 1a) and continued to rise throughout the study (2123.8 ± 266.8 mg dl^{-1} at the 6th week). The plasma total triglyceride had a peak value at the second week (752.8 ± 128.3 mg dl^{-1}) and decreased at the third week (Figure 1b), then remained stable (523.0 ± 19.1 mg dl^{-1} at the 6th week). Table 1 shows the lipid profiles of three rabbit groups at the end of the second week. Scoparone-treatment (5 mg kg^{-1} day^{-1}) significantly reduced the plasma lipid and lipoprotein cholesterol concentrations of hyperlipidaemic diabetic rabbits at the second week to 73.3% of total cholesterol, 48.3% of total triglyceride, 66.0% of VLDL cholesterol, 55.7% of LDL cholesterol and 79.5% of HDL cholesterol. At the 6th week, scoparone-treatment still significantly reduced the plasma total cholesterol to 80%, but did not affect the plasma total triglyceride.

Vascular morphology

Six weeks after cholesterol feeding, the aortic arches and thoracic aortae were dissected out from the three rabbit groups. Microscopic evaluations of segments of aortic arch and thoracic aorta are shown in Figures 2 and 3. No lesion was noted in the blood vessels from normal group (I/M index = 0, $n = 6$, for both aortic arch and thoracic aorta) (Figures 2a and 3a). In contrast, there was intimal thickening with accumulation of fatty streaks, foam cells and migration of smooth muscle cells to the intima in vascular segments from the untreated hyperlipidaemic diabetic group (I/M index = 50 ± 3, $n = 5$, and 41 ± 7, $n = 6$, for aortic arch and thoracic aorta, respectively) (Figures 2b and 3b). Of the arteries obtained from the hyperlipidaemic diabetic rabbits, the aortic arch was more affected by the lesion than the thoracic aorta. In the rabbits treated with scoparone, there was a significantly less fatty lesion found in both vessels (I/M index = 7 ± 1, $n = 5$, and 3 ± 0.2, $n = 6$, for aortic arch and thoracic aorta, respectively) as compared to the untreated hyperlipidaemic diabetic rabbits (Figures 2c and 3c). Less protective effect of scoparone-treatment was observed in the aortic arch than in the thoracic aorta.

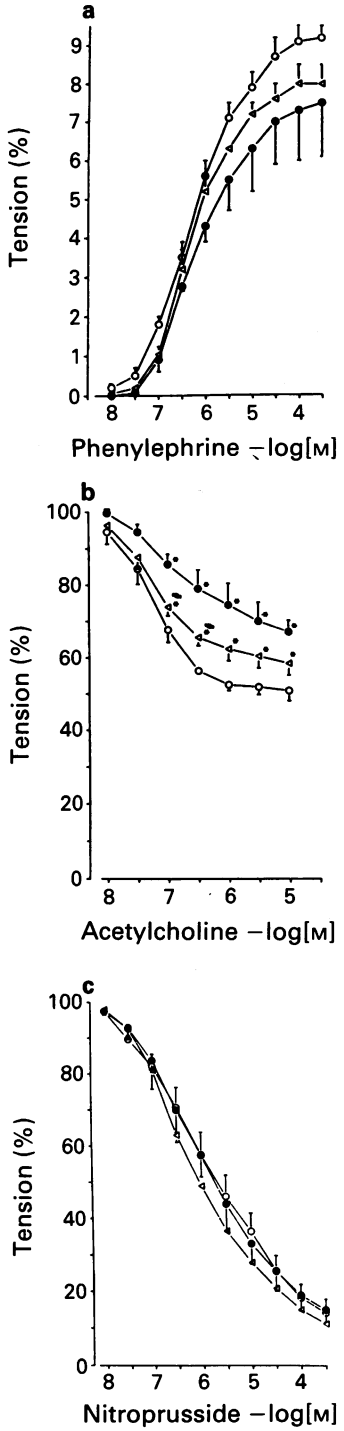


Figure 4 Concentration-response curves to (a) phenylephrine, (b) acetylcholine and (c) nitroprusside in aortic arch rings obtained from normal, hyperlipidaemic diabetic and scoparone-treated hyperlipidaemic diabetic rabbit groups. Contractile or dilator responses were recorded in vascular rings from normal (○), untreated hyperlipidaemic diabetic (●) and scoparone-treated hyperlipidaemic diabetic (△) groups. Contractile response was expressed in grams (Tension, g). Dilator response was expressed as percentage of the maximum tension increase produced by phenylephrine (Tension, %). Points represent the means ± s.e.mean ($n \geq 5$). *$P < 0.05$, compared to the normal group.

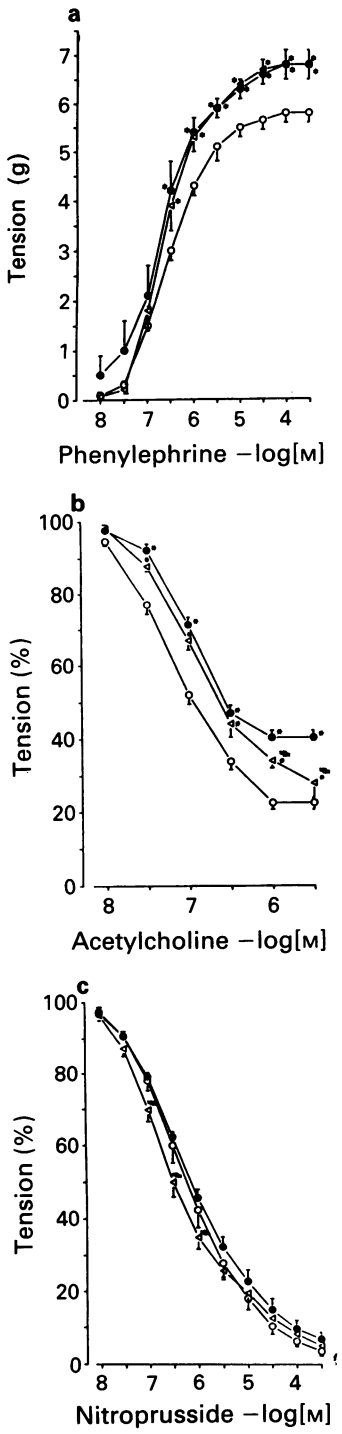


Figure 5 Concentration-response curves to (a) phenylephrine, (b) acetylcholine and (c) nitroprusside in thoracic aortic rings obtained from normal, hyperlipidaemic diabetic and scoparone-treated hyperlipidaemic diabetic rabbit groups. Contractile or dilator responses were recorded in vascular rings from normal (○), untreated hyperlipidaemic diabetic (●) and scoparone-treated hyperlipidaemic diabetic (△) groups. Contractile response was expressed in grams (Tension, g). Dilator response was expressed as a percentage of the maximum tension increase produced by phenylephrine (Tension, %). Points represent the means ± s.e.mean ($n = 12$). *$P < 0.05$, compared to normal group. †$P < 0.05$, compared to untreated hyperlipidaemic diabetic group.

Vascular reactivity

Increasing concentrations of phenylephrine (10^{-8}–10^{-4} M) evoked concentration-dependent contractions in vascular rings of the aortic arch and thoracic aorta from three rabbit groups (Figure 4a and 5a). The EC_{50} and maximal tension (T_{max}) values of responses to phenylephrine are listed in Table 2. The EC_{50} and T_{max} values in aortic arch were not significantly changed between the hyperlipidaemic diabetic and the scoparone-treated rabbit groups. In the thoracic aorta, the contraction responses to phenylephrine were significantly enhanced in hyperlipidaemic diabetic rabbits either untreated or treated with scoparone (Figure 5a and Table 2).

When acetylcholine was added to vascular rings precontracted with phenylephrine, concentration-dependent dilatations were obtained in the aortic arch and thoracic aorta at the concentration ranges of 10^{-8}–10^{-5} M and 10^{-8}–3×10^{-6} M, respectively (Figures 4b and 5b). In the aortic arch and thoracic aorta, the dilator responses to acetylcholine were significantly attenuated at almost all concentrations in the untreated hyperlipidaemic diabetic group as compared to the normal group. The attenuations were partially prevented when scoparone was administered daily (Figures 4b and 5b). As shown in Table 2, the sensitivity and maximal dilatation in the aortic arch and thoracic aorta were significantly reduced in the hyperlipidaemic diabetic group and scoparone-treatment significantly restored the sensitivity in both vessels, but restored only the maximal dilatation in the thoracic aorta. A dose-dependency for the scoparone-induced protective effect was observed at the dose range of 1–10 mg kg^{-1} day^{-1}. At 5 and 10 mg kg^{-1} day^{-1}, scoparone exhibited the maximum protective activity against impaired endothelium-dependent relaxation.

Nitroprusside induced a concentration-dependent dilatation in vascular rings of the aortic arch and thoracic aorta at the concentration range of 10^{-8}–3×10^{-4} M in three rabbit groups (Figures 4c and 5c). The EC_{50} and maximal response values of nitroprusside-induced dilatations did not differ significantly among the three rabbit groups (Table 2), except that the EC_{50} was decreased in the thoracic aorta of scoparone-treated group as compared to the untreated group.

Discussion

We have evaluated the *in vivo* effects of scoparone in a hyperlipidaemic diabetic rabbit model. The results demonstrate that scoparone not only reduced the elevation of plasma lipid and lipoprotein lipid levels, but also protected against some pathological alterations of vascular morphology and reactivity in the aortic arch and thoracic aorta. The hyperlipidaemic cholesterol-fed alloxan-diabetic rabbit, provides a useful model to examine the effects of compounds on lipid profile, atherogenic lesion and vascular reactivity in the hyperlipidaemic diabetic animal. Upon alloxan administration in the cholesterol-fed rabbit, the concentrations of plasma total lipid and lipoprotein cholesterol were markedly increased as soon as the rabbit became diabetic at the second week. Therefore, the hypolipidaemic effect of drugs can be easily evaluated at the earlier weeks in this animal model as compared to the animal fed with cholesterol only. By using this model we have evaluated the hypolipidaemic effects of some crude extracts and active principles from Chinese herbs. In this study, scoparone-treatment significantly reduced the elevated levels of total cholesterol, total triglyceride, very low-density lipoprotein (VLDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol in hyperlipidaemic diabetic rabbits. The reduction in HDL cholesterol was less than the other fractions. Six weeks after cholesterol feeding, the significant morphological and functional alterations were observed in vascular tissues. These pathological vascular changes may be induced by hyperlipidaemia or diabetes. Administration of scoparone to this rabbit model has been found to be associated with reduction of plasma lipids, the regression of atheroma and protection of vascular reactivity. The protective effects of scoparone on the vascular morphological and functional changes in the hyperlipidaemic diabetic rabbit were

more prominent in the thoracic aorta than in the aortic arch. Probucol, an effective antioxidant, has been reported to lower LDL and HDL levels, enhance the fractional catabolic rate of LDL, lower plasma LDL levels, enhance various components of the reverse cholesterol transport system through modifications to HDL and apo E synthesis, and prevent the oxidative modification of LDL (Schwartz, 1988). Scoparone possessing a free radical scavenging property (Huang *et al.*, 1992b) may also affect the lipoprotein levels and atherosclerotic changes as does probucol.

In our vascular activity experiments, the enhancement of phenylephrine-induced contraction was observed in the thoracic aorta from hyperlipidaemic diabetic rabbits and scoparone treatment had no effect on responses to phenylephrine. It has been reported that the contractions caused by the selective α_1-adrenoceptor agonist, phenylephrine, are not altered in arteries obtained from hypercholesterolaemic rabbits (Henry & Yokoyama, 1980; Yokoyama *et al.*, 1983), indicating that the diabetic state of the animal may be the important factor for the enhancement of phenylephrine-induced contraction in the present study. Similarly, an increased vascular reactivity to α-adrenoceptor agonists has been reported in aorta and carotid artery from alloxan-diabetic rabbits (Cseuz *et al.*, 1973; Agrawal *et al.*, 1985). It has been demonstrated that there are α_2-adrenoceptors but not α_1, on endothelial cells that mediate the release of endothelium-derived relaxing factor (EDRF) (Angus *et al.*, 1986). The enhancement of phenylephrine-induced contraction may be less related to endothelial dysfunction. It has been indicated that the difference between induced contractions of healthy and diabetic vessels became evident only after denudation of endothelium and injury of endothelial cells may also contribute to the hyper-reactivity of diabetic arteries (Gebremedhin *et al.*, 1987). Several changes, such as impairment of adrenergic nerve fibres, change in calcium homeostasis and increased activity of calcium channels have also been proposed as responsible for the altered contractile responsiveness of diabetic vascular smooth muscle to α-adrenergic stimulation (Gebremedhin *et al.*, 1987). Further studies are needed to elucidate the mechanisms of the enhancement of phenylephrine-induced contraction in our animal model. On the other hand, the endothelium-dependent dilatation to acetylcholine in rabbit aorta has been reported to be impaired by cholesterol feeding (Jayakody *et al.*, 1985). In the present study, the endothelium-dependent dilatations induced by acetylcholine were significantly attenuated in both aortic arch and thoracic aorta from the hyperlipidaemic diabetic rabbit. The impairment of the acetylcholine-induced dilatation was partially restored by daily treatment with scoparone. Components related to atherogenesis, such as endothelial cells and inflammatory cells, can produce superoxide anion, and this anion modified LDL at the extracellular space (Morel *et al.*, 1984; Parthasarathy *et al.*, 1986) and inactivates the endothelium-derived relaxing factor (EDRF) (Tagawa *et al.*, 1991). Oxidized LDL may lead to an acceleration of atherosclerotic lesion. The oxidative modification of EDRF has been reported to be a possible mechanism for the impairment of endothelium-dependent dilatation to acetylcholine in the cholesterol-fed rabbit (Mügge *et al.*, 1991). Since scoparone has been found by us to exhibit free radical scavenging activity in the vascular ring (Huang *et al.*, 1992b), it may protect against the atherosclerotic lesion and the impairment of vascular dilatation through reducing the oxidative modification of LDL and EDRF in the hyperlipidaemic diabetic rabbit. There was a decrease in the EC_{50} of nitroprusside-induced dilatation in the thoracic aorta of the scoparone-treated group as compared to the untreated group, suggesting that the endothelium-independent dilatation in the aorta may also be affected. This effect may be partly mediated through the direct activation of guanylate cyclase by scoparone (Huang *et al.*, 1992b).

In summary, scoparone-treatment reduced the elevation of plasma lipid and lipoprotein cholesterol concentrations and protected against some *in vivo* vascular morphological and functional alterations in hyperlipidaemic cholesterol-fed alloxan-diabetic rabbit. These protective effects of scoparone may be partly related to its free radical scavenging property.

This investigation was supported by grants from the National Science Council and Department of Health, Taiwan.

References

AGRAWAL, D.K., BHIMJI, S. & MCNEILL, J.H. (1985). Increased vascular reactivity to agonists and prostaglandins in alloxan-treated diabetic rabbits. *Proc. Can. Fed. Biol. Soc.*, **28**, PA91.

ANGUS, J.A., COCKS, T.M. & SATOH, K. (1986). The α adrenoceptors on endothelial cells. *Fed. Proc.*, **45**, 2355–2359.

BIERMAN, E.L. (1991). Atherosclerosis and other forms of arteriosclerosis. In *Harrison's Principles of Internal Medicine*. 12th ed., eds. Wilson, J.D., Braunwald, E., Isselbacher, K.J., Petersdorf, R.G., Martin, J.B., Fauci, A.S. & Root, R.K. pp. 992–1001. New York: McGraw-Hill Inc.

BUEGE, J.A. & AUST, S.D. (1978). Microsomal lipid peroxidation. *Methods Enzymol.*, **52**, 302–310.

CSEUZ, R., WENGER, T.L., KUNOS, G. & SZENTIVANYI, M. (1973). Changes in adrenergic reaction pattern in experimental diabetes mellitus. *Endocrinology*, **93**, 752–755.

GEBREMEDHIN, D., KOLTAI, M.Z., POGATSA, G., MAGYAR, K. & HADHAZY, P. (1987). Differential contracile responsiveness of femoral arteries from healthy and diabetic dogs: role of endothelium. *Arch. Int. Pharmacodyn.*, **288**, 100–108.

HENRY, P.D. & YOKOYAMA, M. (1980). Supersensitivity of atherosclerotic rabbit aorta to ergonovine. Mediation by a serotonergic mechanism. *J. Clin. Invest.*, **66**, 306–313.

HUANG, H.C. (1984). Effects of phospholipases A_2 from *Vipera russelli* snake venom on blood pressure, plasma prostacyclin level and renin activity in rats. *Toxicon.*, **22**, 253–264.

HUANG, H.C., CHU, S.H. & LEE CHAO, P.D. (1991a). Vasorelaxants from Chinese herbs, emodin and scoparone, possess immunosuppressive properties. *Eur. J. Pharmacol.*, **198**, 211–213.

HUANG, H.C., HUANG, Y.L., CHANG, J.H., CHEN, C.C. & LEE, Y.T. (1992a). Possible mechanism of immunosuppressive effect of scoparone (6, 7-dimethoxy coumarin). *Eur. J. Pharmacol.*, **217**, 143–148.

HUANG, H.C., LAI, M.W., WANG, H.R., CHUNG, Y.L., HSIEH, L.M. & CHEN, C.C. (1993). Antiproliferative effect of esculetin on vascular smooth muscle cells: possible roles of signal transduction pathways. *Eur. J. Pharmacol.*, **237**, 39–44.

HUANG, H.C. & LEE, C.Y. (1985). Relaxant effect of phospholipase A_2 from *Vipera russelli* snake venom on rat aorta. *Eur. J. Pharmacol.*, **118**, 139–146.

HUANG, H.C., LEE, C.R., WENG, Y.I., LEE, M.C. & LEE, Y.T. (1992b). Vasodilator effect of scoparone (6,7-dimethoxycoumarin) from a Chinese herb. *Eur. J. Pharmacol.*, **218**, 123–128.

HUANG, H.C., WENG, Y.I., LEE, C.R., JAN, T.R. & LEE CHAO, P.D. (1991b). Mechanisms of antiatherogenic action of *Polygonum multiflorum*. *The Sixth Joint Annual Conference of Biomedical Science*, Taipei, Taiwan, p. 207.

JAYAKODY, R.L., SENARATNE, M.P., THOMSON, A.B. & KAPPAGODA, C.T. (1985). Cholesterol feeding impairs endothelium-dependent relaxation of rabbit aorta. *Can. J. Physiol. Pharmacol.*, **63**, 1206–1209.

MOREL, D.W., DICORLETO, P.E. & CHISOLM, G.M. (1984). Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis*, **4**, 357–364.

MÜGGE, A., ELWELL, J.H., PETERSON, T.E., HOFMEYER, T.G., HEISTAD, D.D. & HARRISON, D.G. (1991). Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ. Res.*, **69**, 1293–1300.

PARTHASARATHY, S., PRINTZ, D.J., BOYD, D., JOY, L. & STEINBERG, D. (1986). Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis*, **6**, 505–510.

ROSENKO, J.M., BRESLOW, J.L., MIETTINEN, O.S. & GABBAY, K.H. (1980). Hyperglycemia and plasma lipid levels. A prospective study of young insulin-dependent diabetic patients. *N. Engl. J. Med.*, **302**, 650–654.

SCHWARTZ, C.J. (1988). The probucol experience: a review of the past and a look at the future. *Am. J. Cardiol.*, **62**, 1B–5B.

TAGAWA, H., TOMOIKE, H. & NAKAMURA, M. (1991). Putative mechanisms of the impairment of endothelium-dependent relaxation of the aorta with atheromatous plaque in heritable hyperlipidemic rabbits. *Circ. Res.*, **68**, 330–337.

YOKOYAMA, M., AKITA, H., MIZUTANI, T., FUKUZAKI, H. & WATANABE, Y. (1983). Hyperreactivity of coronary arterial smooth muscles in response to ergonovine from rabbits with hereditary hyperlipidemia. *Circ. Res.*, **53**, 63–71.

(Received May 10, 1993
Revised July 26, 1993
Accepted August 2, 1993)

Br. J. Pharmacol. (1993), **110**, 1515–1521 © Macmillan Press Ltd, 1993

Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin

[1]M.M. Teixeira, T.J. Williams & P.G. Hellewell

Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 Oedema formation in skin is dependent on a synergism between mediators that increase vascular permeability and mediators that enhance local blood flow. Leukocyte accumulation is also enhanced by mediators that increase local blood flow. In this study, we have investigated whether nitric oxide (NO), an important endogenous vasodilator, could modulate oedema formation and leukocyte accumulation in guinea-pig skin.

2 Local administration of the NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), dose-dependently inhibited the oedema formation induced in response to intradermal injection of bradykinin or histamine. L-NAME, but not N^G-nitro-D-arginine methyl ester (D-NAME), also inhibited oedema formation in response to i.d. injection of platelet-activating factor (PAF), zymosan-activated plasma (ZAP) and in a passive cutaneous anaphylactic (PCA) reaction.

3 N-iminoethyl-L-ornithine (L-NIO) was less effective and about 100 times less potent than L-NAME in inhibiting bradykinin-induced oedema formation. The cyclo-oxygenase inhibitor, ibuprofen, had little effect on oedema responses induced by bradykinin, PAF and in a PCA reaction. On the other hand, histamine-induced oedema formation was significantly suppressed by ibuprofen.

4 The inhibition by L-NAME of bradykinin-induced oedema formation was reversed by co-injection of sodium nitroprusside (SNP) or prostaglandin E_1 (PGE_1).

5 L-NAME inhibited ^{111}In-eosinophil and ^{111}In-neutrophil accumulation induced by i.d. injection of ZAP. ^{111}In-eosinophil accumulation induced by PAF and in the PCA reaction was also inhibited by L-NAME but not by D-NAME.

6 Co-injection of SNP or PGE_1, reversed the inhibition by L-NAME of ZAP-induced oedema formation and ^{111}In-neutrophil accumulation. SNP, but not PGE_1, also reversed the effects of L-NAME on ZAP-induced ^{111}In-eosinophil accumulation.

7 L-NAME caused a significant decrease in basal cutaneous blood flow when injected alone or with bradykinin. Again, SNP or PGE_1 reversed the effects of L-NAME suggesting that the inhibitory action of L-NAME on oedema formation and cell accumulation was due to an inhibition of vasodilator tone in the microcirculation.

8 Thus, it appears that in guinea-pig skin the inhibition of the production of endogenous NO inhibits both leukocyte accumulation and oedema formation induced by different mediators of inflammation. Since administration of L-NAME also causes a local decrease in basal blood flow, we suggest that this is the mechanism by which it exerts anti-inflammatory effects in this model.

Keywords: Nitric oxide; prostaglandins; eosinophils; neutrophils; inflammation; oedema; vasodilatation; N^G-nitro-L-arginine methyl ester

Introduction

Oedema formation, one of the features of the cutaneous inflammatory response, is dependent on a synergism between mediators that increase vascular permeability and mediators that increase blood flow (Williams & Morley, 1973; Williams & Peck, 1977). Thus, drugs such as indomethacin may inhibit inflammatory responses by virtue of their ability to inhibit production of vasodilator prostaglandins which are thought to act on arterioles to increase local blood flow at the site of injury or insult. Indeed, in rabbit skin, indomethacin inhibits oedema formation in various inflammatory reactions (Williams & Peck, 1977; Williams *et al.*, 1986; Needham *et al.*, 1988; Hellewell *et al.*, 1992). This synergism between mediators also implies that mediators which decrease local blood flow may attenuate oedema formation. In support of this, it has been shown that vasoconstrictor agents such as endothelin (Brain *et al.*, 1988) may decrease oedema formation induced by various mediators of inflammation (Brain *et al.*, 1989). The accumulation of leukocytes into sites of inflammation in the skin of various animals is also modulated by an increase in local blood flow (Issekutz & Movat, 1979; Issekutz, 1981; Buckley *et al.*, 1991). Thus, the regulation of blood flow at the site of an inflammatory insult appears to be an important mechanism in determining the outcome of the response.

Since first described by Furchgott and Zawadzki (1980), endothelium-derived relaxing factor has received great attention as an important endogenous dilator. The major vasodilator activity accounting for the effects of endothelium-derived relaxing factor has been attributed to nitric oxide (NO) (Palmer *et al.*, 1987). NO is a short-lived molecule which has been shown to have a role in the control of microvascular tone under basal conditions and under stress conditions (Ignarro, 1989; Moncada *et al.*, 1991). After appropriate stimulation, many different cells, including leukocytes, can express an inducible form of NO synthase which generates large amounts of NO (Moncada *et al.*, 1991). Both the endothelial (constitutive) and leukocyte (inducible) forms of NO synthase can be inhibited by L-arginine analogues and this has made it possible to investigate the role of NO in various pathophysiological conditions (for review see Moncada *et al.*, 1991).

In guinea-pig skin, local oedema formation and neutrophil accumulation are enhanced by intradermally-injected prostaglandins although the synergism is not as marked as that observed in rabbit skin (Williams & Morley, 1973; Williams

[1] Author for correspondence.

& Peck, 1977; Teixeira et al., 1993). This may be a reflection of higher basal flow in guinea-pig skin than in the rabbit. In this study we have investigated the role of endogenous prostaglandins and NO as modulators of acute inflammatory responses in guinea-pig skin.

Methods

Preparation of zymosan-activated plasma

Guinea-pig heparinized (10 u ml^{-1}) plasma was incubated with zymosan (5 mg ml^{-1}) at 37°C. After 30 min, zymosan was removed by centrifugation (2 × 10 min at 3000 *g*). The activated plasma obtained was then desalted using a PD-10 Sephadex G-25M column and stored in aliquots at − 20°C. Zymosan-activated plasma (ZAP) was used as a 10% solution in saline unless stated otherwise.

Preparation of passive cutaneous anaphylaxis sera and reactions

Details of the preparation of IgG_1-rich anti-sera are described elsewhere (Weg et al., 1991). Briefly, male guinea-pigs (Harlan Porcellus, Oxon, 350–400 g) were immunized with BGG in Freund's complete adjuvant (0.2 mg BGG 0.2 ml^{-1} of adjuvant s.c.). On day 21, these animals received a boost of antigen in Freund's incomplete adjuvant and the serum was collected and prepared on day 30. Skin sites were sensitized for passive cutaneous anaphylatic (PCA) reactions by giving an i.d. injection of a 1/50 dilution of anti-BGG anti-serum. After allowing antibody to fix to tissue cells for 16–20 h, PCA reactions were induced by an i.d. injection of antigen (BGG, 1 μg of antigen per site).

Induction, purification and radiolabelling of guinea-pig-eosinophils

The method is described in detail by Faccioli et al. (1991). Briefly, ex-breeder female guinea-pigs (Harlan Porcellus, Oxon; 700–800 g) were treated with horse serum (1 ml) i.p. every other day for two weeks. A boost was given on the day prior to collection of the cells. The animals were killed by exposure to CO_2 and their peritoneal cavities washed out with heparinized saline (10 u ml^{-1}). The cells obtained were layered onto a discontinuous Percoll-HBSS (calcium- and magnesium-free) gradient prepared on the day of the procedure and the eosinophils collected from the 1.085/1.090 and 1.090/1.095 interfaces. Cells were used only if purity was greater than 95%. The main contaminating cells were mononuclear cells and the presence of neutrophils (>1%) was a major exclusion criteria for the use of the preparation. Viability was assessed in some experiments by trypan blue exclusion and was found to be greater than 98%. The purified eosinophils (1.5 to 3.0 × 10^7 cells) were radiolabelled by incubation with ^{111}Indium (100 μCi in 10 μl) chelated to 2-mercaptopyridine-N-oxide (40 μg in 0.1 ml of 50 mM PBS, pH 7.4) for 15 min at room temperature. The cells were then washed twice in HBSS (calcium and magnesium-free) containing 10% guinea-pig platelet-poor plasma and resuspended at a final concentration of 10^7 cells ml^{-1} prior to i.v. injection.

Induction, purification and radiolabelling of guinea-pig neutrophils

Neutrophils were elicited in the peritoneal cavity of naive ex-breeder guinea-pigs by the i.p. injection of 15 ml of a 5% (w/v) solution of casein. After 12–16 h, the animals were killed and the peritoneal cavity washed with heparinized saline (10 u ml^{-1}). The rest of the procedure was followed as described for the eosinophils. The cells were also collected from the 1.085/1.090 and 1.090/1.095 interfaces. The purity of the preparation was always greater than 99% and the rare contaminants were eosinophils and occasional mononuclear cells. Viability, tested by trypan blue exclusion, was greater than 98%. Labelling of the cells was carried out as described above for the eosinophils.

Measurement of local oedema formation and ^{111}In-leukocyte accumulation in guinea-pig skin

Radiolabelled leukocyte infiltration and oedema formation were measured simultaneously in the skin. Guinea-pigs (Harlan Porcellus, Oxon, 350–400 g) were anaesthetized with Hypnorm (0.2 ml, i.m.) and received an i.v. injection of ^{111}In-labelled eosinophils or neutrophils (5 × 10^6 cells per animal) together with ^{125}I-human serum albumin (5 μCi). After 5 min, inflammatory mediators or antigen were injected i.d. into the dorsal skin of shaved animals with or without the NO synthase (NOS) or cyclo-oxygenase inhibitors. In some experiments, PGE_1 (3 × 10^{-10} mol per site) or SNP (10^{-7} mol per site) were also added. All drugs were mixed before the i.d. injection. Each animal received a duplicate of each treatment following a randomized injection plan and the inflammatory response (^{111}In-labelled cell accumulation and oedema formation) was assessed after 2 h. A maximum of 15 different treatments was given per animal and all experiments assessing the effects of an inhibitor were done in the same animals. Experiments were carried out over 2 h since previous time course studies indicated that the majority of plasma leakage or leukocyte accumulation was complete at this time (Faccioli et al., 1991; Weg et al., 1992; Collins et al., 1993). At 2 h, a blood sample was obtained by cardiac puncture and plasma prepared; the animals were killed by an overdose of sodium pentobarbitone and the dorsal skin was removed. Skin sites were punched out with a 17 mm punch and, together with plasma samples, counted in an automatic 5-head gamma-counter (Canberra Packard Ltd, Pangbourne, Berks.) with cross-channel correction for the two isotopes.

The number of leukocytes accumulating at the site of inflammation was expressed as ^{111}In-labelled cells per skin site based on the specific activity of the leukocytes (i.e. counts per cell) in each experiment. Oedema formation was expressed as μl of plasma, obtained by dividing ^{125}I counts of the skin sample by the ^{125}I counts in 1 μl of plasma.

Measurement of skin blood flow

Guinea-pigs (350–400 g) were shaved and depilated the day before the experiment to avoid any non-specific effect of the depilating agent (Immac, Reckitt & Colman Products, Hull). The animals were allowed to acclimatize to the laboratory temperature for at least 1 h and then anaesthetized (Hypnorm. 0.15 ml, i.m.). Temporal alterations of red blood cell flux, taken as an index of skin blood flow, were determined with a Perimed II laser-Doppler flow meter (Perimed, Stockholm, Sweden) and recorded on a MacLab device (ADI Instruments, Reading, Berks.) coupled to an Apple Macintosh Classic (Apple Computer Inc., CA, U.S.A.). Triplicate basal readings of the site to be injected were obtained and the drugs (mixed prior to injection when necessary) were injected i.d. Ten min later, triplicate readings of the same sites were obtained again and averaged. Each animal received up to 6 injections. Results were expressed as % change of basal flow (i.e. flow in the site prior to the i.d. injection).

Reagents

The following compounds were purchased from Sigma Chemical Company (Poole, Dorset): bradykinin, casein, bovine gamma globulin (BGG), L-arginine, D-arginine, N^G-nitro-L-arginine-methyl ester (L-NAME), sodium nitroprusside (SNP) and zymosan. N^G-nitro-D-arginine-methyl ester (D-NAME) and PAF (C16) were purchased from Bachem (Saffron Walden, Essex). N(5)-(1-iminoethyl)-L-ornithine (L-

NIO, batch CZ92) was purchased from Cookson Chemicals Ltd, Southampton). Hanks balanced salt solution (HBSS), HEPES and horse serum were purchased from Gibco Limited (Paisley, Renfrewshire). Percoll was from Pharmacia (Milton Keynes, Bucks.) and prostaglandin E_1 (PGE_1) from Janssen Pharmaceuticals (Belgium). Ibuprofen was a gift from Boots Chemical Company plc (Nottingham, Notts.). $^{111}InCl_3$ (>10 mCi per μg in 0.04 M HCl) and ^{125}I-human serum albumin (2.5 μCi per mg in sterile isotonic saline) were purchased from Amersham International (Amersham, Herts.).

Statistics

Data are presented as means ± s.e.mean for the number of experiments indicated. Data were analyzed by two-way analysis of variance on normally distributed data. For blood flow experiments, Student's paired *t* test was used comparing the values before and after the local treatment. Values of $P<0.05$ were considered statistically significant.

Results

Effect of ibuprofen on oedema formation induced by bradykinin, histamine, PAF and in a PCA reaction

The potentiation of bradykinin-induced oedema formation by low doses of arachidonic acid (AA, 1.5×10^{-9} mol per site) has been shown previously to be dependent on the production of vasodilator prostaglandins (Williams & Peck, 1977). Ibuprofen, at a dose (10^{-7} mol per site) which inhibited this potentiation by >95% had little effect on oedema formation induced by bradykinin alone, PAF or in a PCA reaction (Table 1). In contrast, ibuprofen significantly suppressed histamine-induced oedema formation by 30% (Table 1).

Effect of L-NAME on oedema formation induced by bradykinin, histamine, PAF and in a PCA reaction

The concomitant injection of increasing doses of L-NAME with bradykinin (10^{-10} mol per site) caused a dose-dependent inhibition of bradykinin-induced oedema formation (Figure 1). At a dose of 10^{-6} mol per site, L-NAME inhibited bradykinin-induced oedema formation by 89% (Figure 1). Interestingly, oedema formation induce by histamine, at a dose (2.5×10^{-8} mol per site) selected to induce a similar response to bradykinin (10^{-10} mol per site), was not inhibited

Table 1 Effect of ibuprofen on oedema formation in guinea-pig skin

	Control (μl of plasma)	*Ibuprofen* (μl of plasma)
BK	32.2 ± 2.6	31.0 ± 2.8
BK + AA	44.8 ± 2.4	31.6 ± 1.7*
PAF	50.4 ± 2.8	48.6 ± 5.9
PCA	29.3 ± 7.0	25.6 ± 3.6
Hist	54.4 ± 5.2	38.4 ± 7.0*
Saline	7.4 ± 1.8	5.6 ± 0.7
AA	9.4 ± 2.1	ND

The mediators or antigen were mixed with ibuprofen (10^{-7} mol per site) prior to their i.d. injection and oedema formation assessed 2 h later. The following stimuli were used: bradykinin (BK, 10^{-10} mol per site), arachidonic acid (AA, 1.5×10^{-9} mol per site), platelet-activating factor (PAF, 10^{-9} mol per site), histamine (Hist, 2.5×10^{-8} mol per site) and a passive cutaneous anaphylaxis (PCA) reaction (1 μg of BGG per site). Results are mean ± s.e. mean of at least 4 animals in each group. *$P<0.05$ when compared to control values. ND, not determined.

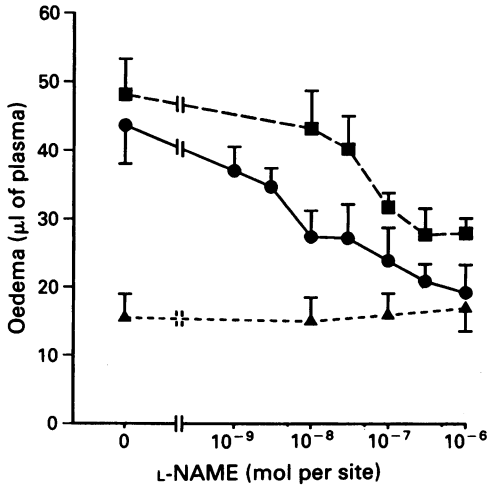


Figure 1 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on oedema formation induced by i.d. injection of bradykinin (10^{-10} mol per site, ●) or histamine (2.5×10^{-8} mol per site, ■) in guinea-pig skin. The effect of L-NAME alone (injected in saline) is shown by (▲). Oedema formation was assessed 2 h after the i.d. injections. Values are mean ± s.e.mean from 4–6 guinea-pigs.

to the same extent with a maximal inhibition of 62% (Figure 1). The dose of L-NAME used in all subsequent experiments was 10^{-6} mol per site. The inhibition was specific for L-NAME since D-NAME, at a dose of 10^{-6} mol per site, had no effect on bradykinin-induced oedema formation (bradykinin 10^{-10} mol per site, 33.8 ± 4.5 μl; bradykinin + D-NAME 10^{-6} mol per site, 29.6 ± 2.0 μl; bradykinin + L-NAME 10^{-6} mol per site, 12.6 ± 1.4 μl; saline, 8.6 ± 0.6 μl, $n = 4$). L-NAME also effectively suppressed the oedema formation induced by PAF (10^{-9} mol per site) and a PCA reaction by up to 68% and 81%, respectively (Table 2). However, these responses were not altered by D-NAME (Table 2). There was no additional inhibition of histamine- and bradykinin-induced oedema formation if ibuprofen was added to L-NAME (data not shown).

L-NIO has been shown previously to be a potent inhibitor of NO synthase in leukocytes and rat skin (McCall *et al.*, 1991; Mulligan *et al.*, 1992). Therefore we decided to compare the activity of L-NIO with that of L-NAME on bradykinin-induced oedema formation in the guinea-pig skin. Figure 2 shows the effects of L-NIO and L-NAME on oedema formation induced by bradykinin in guinea-pig skin. In these experiments, bradykinin-induced oedema responses were suppressed by up to 80% and 30% by L-NAME and L-NIO, respectively. Oedema formation induced by histamine was also less effectively inhibited by L-NIO than L-NAME (data not shown). The L-NIO preparation used was highly effective as assessed by its ability to block the relaxation induced by acetylcholine (10^{-6} M) of rat aortic rings pre-contracted with phenylephrine (5×10^{-7} M). In this preparation, L-NAME (10^{-6} M) and L-NIO (10^{-6} M) inhibited acetylcholine-induced relaxation by 89% and 80%, respectively ($n = 2$).

Effect of PGE_1 and SNP on the inhibition by L-NAME of bradykinin-induced oedema formation

The addition of PGE_1 or SNP to the mixture of bradykinin and L-NAME prior to their injection reversed the inhibitory effects of L-NAME on bradykinin-induced oedema formation (Figure 3). L-Arginine, in doses up to 50 times greater than L-NAME, only partially reversed the inhibitory effects of L-NAME (data not shown). The D-enantiomer of arginine (5×10^{-6} mol per site) displayed inflammatory effects when injected alone, so it was not possible to draw any conclusions from its use (data not shown).

Table 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) and D-NAME on ^{111}In-eosinophil accumulation and oedema formation in guinea-pig skin

	Oedema (μl of plasma)			*^{111}In-eosinophils*		
	Control	*L-NAME*	*D-NAME*	*Control*	*L-NAME*	*D-NAME*
ZAP 10%	31.4 ± 6.8	12.6 ± 2.2*	29.1 ± 5.1	6172 ± 781	738 ± 143*	5898 ± 914
30%	41.5 ± 2.8	23.0 ± 2.6*	ND	9618 ± 1325	4552 ± 918*	ND
PCA	29.1 ± 5.1	14.2 ± 2.5*	28.2 ± 7.5	6391 ± 844	3605 ± 876*	7141 ± 1920
PAF	48.3 ± 6.3	22.9 ± 2.6*	43.2 ± 12.9	9186 ± 1698	1267 ± 242*	9087 ± 1136
BK	33.8 ± 4.5	12.6 ± 1.4*	29.6 ± 2.0	252 ± 51	198 ± 33	555 ± 114
Sal	10.4 ± 1.5	10.6 ± 1.4	11.0 ± 2.5	454 ± 41	368 ± 42	362 ± 73

The mediators or antigen were mixed with L-NAME (10^{-6} mol per site) or D-NAME (10^{-6} mol per site) prior to i.d. injection and oedema formation and ^{111}In-eosinophil accumulation assessed after 2 h. The following stimuli were used: zymosan-activated plasma (ZAP, 10% and 30% in saline), a passive cutaneous anaphylaxis (PCA) reaction (1 μg of BGG per site), platelet-activating factor (PAF, 10^{-9} mol per site), bradykinin (BK, 10^{-10} mol per site) and in saline injected sites (Sal). Results are expressed as the mean ± s.e.mean for 4–6 animals. *$P<0.05$ when compared to control values. ND, not determined.

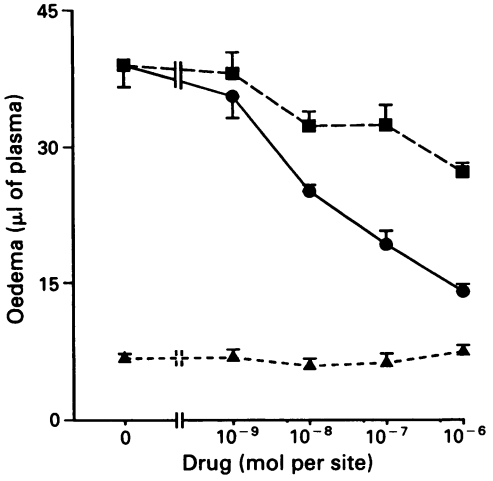


Figure 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME, ●) and N(5)-(1-iminoethyl)-L-ornithine (L-NIO, ■) on oedema formation induced by i.d. injection of bradykinin (10^{-10} mol per site) in guinea-pig skin. The effect of L-NIO alone (injected in saline) is shown by (▲). Oedema formation was assessed 2 h after the i.d. injections. Values are mean ± s.e.mean from 4 guinea-pigs.

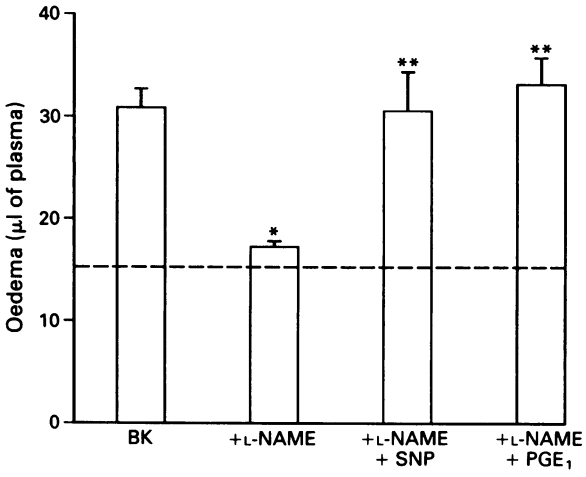


Figure 3 Reversal of the inhibitory effect of N^G-nitro-L-arginine methyl ester (L-NAME) on bradykinin-induced oedema formation in guinea-pig skin by sodium nitroprusside (SNP) or prostaglandin E_1 (PGE_1). Bradykinin (BK, 10^{-10} mol per site) was injected alone, with L-NAME (10^{-6} mol per site) and with L-NAME plus either SNP (10^{-7} mol per site) or PGE_1 (3×10^{-10} mol per site) and oedema formation was assessed after 2 h. The dashed line across the graph represents the background value obtained after i.d. injection of saline. Values are means ± s.e.mean from 7 guinea-pigs. *$P<0.05$ when compared to bradykinin; **$P<0.05$ when compared to bradykinin + L-NAME.

Effect of L-NAME on ZAP-induced oedema formation and leukocyte accumulation

The local injection of ZAP in guinea-pig skin has been shown previously to induce an inflammatory response in which the accumulation of leukocytes is a prominent feature (Faccioli *et al.*, 1991). When ZAP was injected with L-NAME, a significant inhibition of both oedema formation and cell accumulation was observed (Table 2, Figure 4), but D-NAME had no effect on ZAP-induced responses (Table 2). The inhibitory effects of L-NAME on ZAP-induced oedema formation (Figure 4a) and neutrophil accumulation (Figure 4b) were reversed by co-injection of PGE_1 (3×10^{-10} mol per site) or SNP (10^{-7} mol per site). However, the inhibition of ZAP-induced eosinophil accumulation by L-NAME was reversed by SNP, but not by PGE_1 (Figure 4c).

Eosinophil accumulation induced by PAF (10^{-9} mol per site) and in a PCA reaction were also suppressed by co-injection of L-NAME (Table 2). In addition, L-NAME suppressed PAF-induced neutrophil accumulation by 72%. Neither eosinophil accumulation nor oedema formation induced by these mediators was affected by D-NAME (Table 2).

Effect of L-NAME on blood flow in guinea-pig skin

In order to determine whether the inhibitory effects of L-NAME on oedema formation and cell accumulation could be due to a local decrease in blood flow, we measured cutaneous blood flow using a laser-Doppler flow system. Induction of anaesthesia with Hypnorm did not alter blood flow in uninjected sites (7.6 ± 5.1% increase in flow, $n = 3$, $P>0.05$) so all subsequent experiments were carried out in anaesthetized animals. As shown in Figure 5, L-NAME caused a significant decrease in basal flow, measured 10 min after its i.d. injection, compared with saline which did not change blood flow from the basal level. The co-injection of L-NAME with bradykinin also led to a significant decrease of local basal flow which was reversed by both PGE_1 and SNP (Figure 5). Basal flow in conscious animals was also inhibited by L-NAME (10^{-6} mol per site; 48.7 ± 7.1% decrease in flow, $n = 3$, $P<0.05$).

Discussion

Basal release of NO from endothelial cells has been previously shown and it is thought to contribute to the control of microvascular tone (Ignarro, 1989). By inhibiting NO production, an increase in vascular tone is observed and this may lead to systemic hypertension (Rees *et al.*, 1989). NO inhibition can also affect the inflammatory response induced by different mediators or reactions under different conditions (Garside *et al.*, 1992; Ialenti *et al.*, 1992; Lippe *et al.*, 1993; Miller *et al.*, 1993).

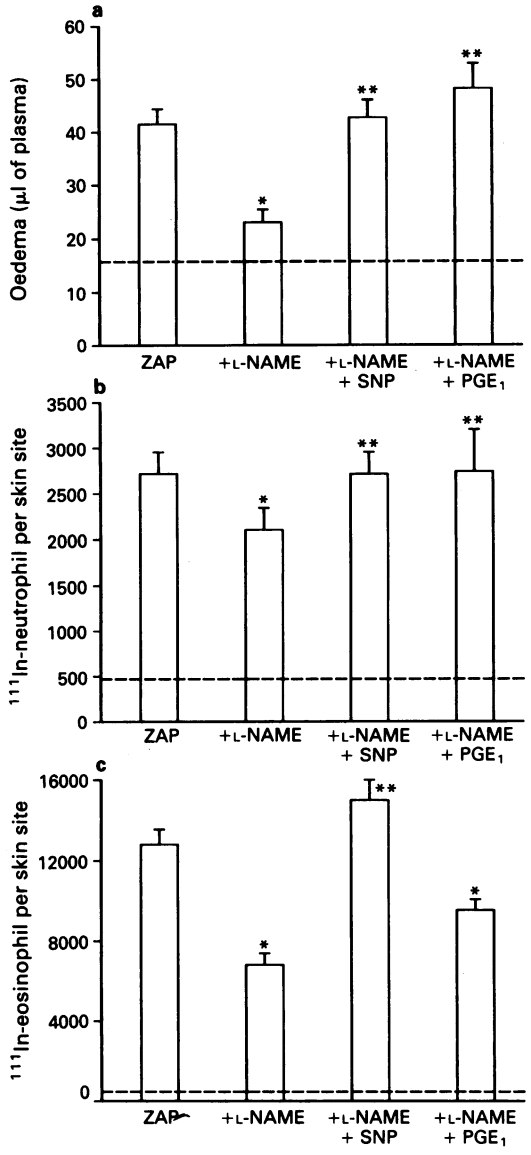


Figure 4 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on zymosan-activated plasma (ZAP)-induced (a) oedema formation, (b) neutrophil accumulation and (c) eosinophil accumulation and reversal by sodium nitroprusside (SNP) and prostaglandin E_1 (PGE_1). ZAP (10% in saline) was injected alone, with L-NAME (10^{-6} mol per site) and with L-NAME plus either SNP (10^{-7} mol per site) or PGE_1 (3×10^{-10} mol per site) and oedema formation or radiolabelled cell accumulation assessed after 2 h. The dashed line across each graph represents the background value obtained after i.d. injection of saline. Values are means ± s.e.mean from 4–8 guinea-pigs. $*P<0.05$ when compared to ZAP alone; $**P<0.05$ when compared to ZAP + L-NAME.

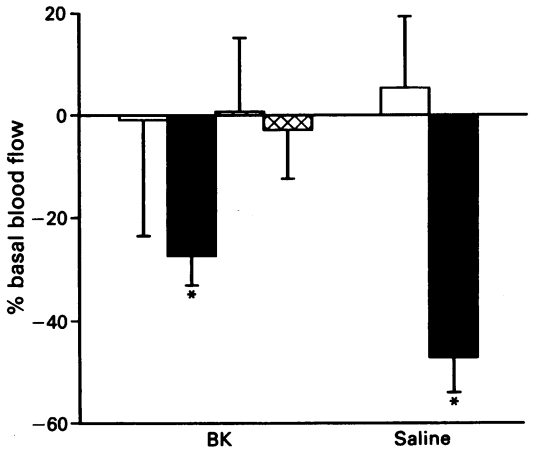


Figure 5 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on blood flow in guinea-pig skin and reversal by sodium nitroprusside (SNP) or prostaglandin E_1 (PGE_1). Triplicate measurements were obtained in each site before and 10 min after the i.d. injections. Bradykinin (BK 10^{-10} mol per site) or saline were injected alone (open columns), with L-NAME (10^{-6} mol per site, solid columns) and with L-NAME plus SNP (10^{-7} mol per site, hatched column) or PGE_1 (3×10^{-10} mol per site, cross-hatched column). Values are means ± s.e.mean of 4–5 guinea-pigs. $*P<0.05$ when compared to basal flow before the i.d. injection.

When the NO inhibitor, L-NAME, was co-injected with bradykinin, we observed dose-dependent inhibition of bradykinin-induced oedema formation in guinea-pig skin. This inhibitory effect of L-NAME, but not D-NAME, on oedema formation was also observed when histamine, PAF, a PCA reaction or ZAP were used as inflammatory stimuli. Nevertheless, the degree of inhibition of PAF- and histamine-induced oedema formation was less marked than that towards bradykinin. This observation has been previously noted, at least for histamine (Paul *et al.*, 1992), and it is thought to be due to differences in mechanisms by which these mediators produce oedema formation in guinea-pig skin. For example, it is possible that L-arginine liberated on breakdown of bradykinin in skin may be converted to NO, contributing to further vasodilatation. However, injection of L-arginine (10^{-5} mol per site) with bradykinin (10^{-10} mol per site) or ZAP (10%) had no effect on plasma leakage, arguing against such a possibility. It is also possible that histamine (and also PAF) may induce local release of prostaglandins which may contribute to the local maintenance of blood flow. In fact, histamine-induced oedema was significantly inhibited (by 30%) by ibuprofen at a dose that blocked cyclo-oxygenase in skin by >95% (as assessed by inhibition of responses to bradykinin potentiated by arachidonic acid). However, oedema responses induced by bradykinin, PAF and in a PCA reaction were unaltered by cyclo-oxygenase inhibition. This is in contrast to inflammatory responses in rabbit skin where local oedema formation induced by polycations, a PCA reaction and a reversed passive Arthus reaction are reduced by approximately 50% by administration of indomethacin (Williams *et al.*, 1986; Needham *et al.*, 1988; Hellewell *et al.*, 1992).

L-NIO has been described as a potent inhibitor of both the inducible and the constitutive form of NO synthase (Moncada *et al.*, 1991). In an immune complex-induced vasculitis model in rat skin, L-NIO was much more potent in inhibiting oedema formation than other inhibitors of NO synthase (Mulligan *et al.*; 1992). L-NIO has also been reported to be more potent than other inhibitors in suppressing NO production by activated leukocytes (McCall *et al.*, 1991) which might explain its potent effects on plasma leakage induced by deposition of immune complexes in rat skin, a response known to be neutrophil-dependent (Mulligan *et al.*, 1992). In our model, L-NIO was about 100 times less potent than L-NAME in inhibiting oedema formation induced by bradykinin. However, the same batch of the compound effectively inhibited relaxation of rat aorta induced by acetylcholine. The relative refractoriness of guinea-pig skin to the inhibitory action of L-NIO has not been described previously.

The inhibitory effect of L-NAME on bradykinin-induced oedema was reversed by SNP, a nitrovasodilator which releases NO as its active moiety (Ignarro, 1989). PGE_1 also effectively reversed L-NAME inhibition of bradykinin-induced oedema formation. Prostaglandins act on EP prostanoid receptors which are linked to G-proteins and may lead to an increase in adenosine 3':5'-cyclic monophosphate (cyclic) AMP levels (via EP_2 receptors) or activate phospholipase C (via EP_1 receptors) (Coleman & Humphrey,

1993). We are not aware of any study showing that prostaglandins may alter cyclic GMP levels within any type of cell. The most likely explanation for reversal of the effects of L-NAME on bradykinin-induced oedema formation by PGE_1 is local vasodilation. In rabbit skin, PGE_1 has been shown to have direct vasodilator activities as demonstrated using a xenon clearance technique (Williams, 1979). However, in guinea-pig skin using the laser Doppler method, we were able to detect only minor and inconsistent increases in local blood flow above basal levels when PGE_1 was injected alone ($19.6 \pm 14.8\%$ increase, $n = 4$). This could mean that the microcirculation was almost maximally dilated (under the control of endogenous NO) or that the laser Doppler technique is not as sensitive as xenon clearance. A more likely explanation is that the laser-Doppler method measures flow in superficial tissues of the skin, whereas the xenon clearance method measures flow in the deeper dermis in the region of direct exposure to PGE_1.

L-Arginine was weak in reversing the effects of L-NAME on bradykinin-induced oedema formation. This also seems to be the case in rabbit and human skin (S. Larkin, personal communication). It is possible that L-arginine does not achieve a sufficiently high concentration at skin sites to reverse the effect of L-NAME. However, the fact that D-NAME was ineffective in inhibiting oedema formation by any of the mediators tested provided good evidence that the inhibition of inflammation by L-NAME was due to inhibition of NO synthase rather than a non-specific action (Moncada et al., 1991).

ZAP is a potent leukocyte chemoattractant in guinea-pig skin (Faccioli et al., 1991). When ZAP was co-injected with L-NAME, not only was an inhibition of ZAP-induced oedema formation noticed but also a significant inhibition of ZAP-induced eosinophil and neutrophil accumulation (Figure 4). PAF- and the PCA reaction-induced eosinophil accumulation were also inhibited by L-NAME, but not by D-NAME (Table 2), again suggesting the stereospecific nature of the inhibition. Interestingly, the inhibition of ZAP-induced neutrophil accumulation was also reversed by SNP and PGE_1. Since neutrophil accumulation has been previously shown to depend on the local flow at the inflammatory site (Issekutz & Movat, 1979; Buckley et al., 1991), it is also possible that both SNP and PGE_1 are reversing the inhibition of ZAP-induced neutrophil accumulation by reversing the decrease in local basal flow induced by L-NAME. The inhibition of ZAP-induced eosinophil accumulation by L-NAME was reversed by SNP, whereas PGE_1 was much less effective. Since both PGE_1 and SNP appeared to reverse the L-NAME-induced decrease in local flow to the same extent (Figure 5), it is unlikely that a difference in flow could explain the different effects of these two vasodilators on eosinophil accumulation. We have recently described the observation that i.d. administration of PGE_1 dose-dependently inhibits eosinophil accumulation induced by various stimuli in guinea-pig skin (Teixeira et al., 1993). This effect, which occurred at the same time that PGE_1 enhanced oedema formation and neutrophil accumulation, is thought to be due to the capacity of PGE_1 to increase cyclic AMP levels in the target tissue (which could be the eosinophil itself or the venular endothelial cell) since the inhibitory effect was mimicked by isoprenaline (Teixeira et al., 1993). Thus, in the case of leukocyte accumulation, PGE_1 can have two opposing effects.

There has been considerable interest in the study of the involvement of NO in inflammatory responses since the discovery that, with appropriate stimuli, inflammatory cells may produce large amounts of NO and that NO can be toxic to tissues, tumour cells and parasites (see Moncada et al., 1991). The participation of NO in endotoxic shock has also been recently highlighted (Moncada et al., 1991) as has its participation in different acute inflammatory models (Ialenti et al., 1992; Mulligan et al., 1992; Lippe et al., 1993). The precise cellular target for the anti-inflammatory effects of NO inhibitors *in vivo* are nevertheless unknown. Recent work suggests that the inhibition of the production of NO in a model of adjuvant arthritis in rats may be due to inhibitory effect on T lymphocyte proliferation (Ialenti et al., 1993).

In our model, the capacity of an NO inhibitor (L-NAME) to reduce inflammation appears to be linked to its ability to reduce skin blood flow. Others have found no suppression of leukocyte accumulation on a model of acute inflammation in the skin of rats even though oedema formation and red blood cell extravasation were potently inhibited (Mulligan et al., 1991). In that series of experiments, the authors used rats rather than guinea-pigs and it is possible that the dependence of skin blood flow on NO is greater in the latter.

Kubes et al. (1991) and Kubes & Granger (1992) found that inhibitors of NO led to an increased oedema formation and leukocyte accumulation in a cat mesenteric preparation. They suggested that, by inhibiting NO production, there would be a loss of a protective (anti-adherence) effect of NO on neutrophils which would lead to their increased adhesiveness and injury to endothelial cells (Kubes et al., 1991) and thence to increased vascular permeability. An antibody against CD11/CD18 partially blocked the increase in plasma exudation induced by inhibition of NO (Kubes & Granger, 1992). Vascular permeability was also enhanced in the rat coronary circulation after pre-treatment with L-NAME (Filep et al., 1993). The neutrophil-dependence of hypoxic injury (Romson et al., 1983) and the capacity of NO inhibitors to increase expression of CD11/CD18 may explain the increase in vascular permeability observed in these models. It is also possible that NO does not play a major role in modulating basal vascular flow in the cat mesentery or rat heart, though in the latter there is some evidence for NO involvement in control-ling perfusion pressure *in vitro* (Baydoun & Woodward, 1991).

We have shown that an NO inhibitor, L-NAME, significantly inhibits oedema formation and leukocyte accumulation in guinea-pig skin. These effects were reversed by SNP and by PGE_1, except in the case of eosinophil accumulation where PGE_1 was not as effective as SNP. Since both SNP and PGE_1 were also capable of inhibiting the L-NAME-induced reduction of basal flow, we suggest that the inhibitory effects of L-NAME on guinea-pig skin are related to its ability to reduce basal blood flow. This may also explain part of the inhibitory effects of NO inhibitors in other inflammatory models. The observation that cyclo-oxygenase inhibitors may inhibit inflammation through the inhibition of vasodilator prostaglandins (Williams & Peck, 1977) suggests that inhibitors of NO may also have a role as anti-inflammatory drugs.

We thank the National Asthma Campaign and Sandoz, Switzerland for support. We thank Miss A. Wilson for helpful technical support with rat aorta experiments.

References

BAYDOUN, A.R. & WOODWARD, B. (1991). Effects of bradykinin in the rat isolated perfused heart: role of kinin receptors and endothelium-derived relaxing factor. *Br. J. Pharmacol.*, **103**, 1829–1833.

BRAIN, D.S., CROSSMAN, D.C., BUCKLEY, T.L. & WILLIAMS, T.J. (1989). Endothelin-1: demonstration of potent effects on the microcirculation of humans and other species. *J. Cardiovasc. Pharmacol.*, **13** (Suppl 5), S147–S149.

BRAIN, S.D., TIPPINS, J.R. & WILLIAMS, T.J. (1988). Endothelin induces potent microvascular constriction. *Br. J. Pharmacol.*, **95**, 1005–1007.

BUCKLEY, T.L., BRAIN, S.D., COLLINS, P.D. & WILLIAMS, T.J. (1991). Inflammatory edema induced by interactions between interleukin-1 and the neuropeptide calcitonin gene-related peptide. *J. Immunol.*, **146**, 3424–3430.

COLEMAN, R.A. & HUMPHREY, P.P.A. (1993). Prostanoid receptors: their function and classification. In *Therapeutic Applications of Prostaglandins*. ed. Vane J. & O'Grady J. London: Edward Arnold, (in press).

COLLINS, P.D., WEG, V.B., FACCIOLI, L.H., WATSON, M.L., MOQBEL, R. & WILLIAMS, T.J. (1993). Eosinophil accumulation induced by human interleukin-8 in the guinea pig in vivo. *Immunology*, **79**, 312–318.

FACCIOLI, L.H., NOURSCHARGH, S., MOQBEL, R., WILLIAMS, F.M., SEHMI, R., KAY, A.B. & WILLIAMS, T.J. (1991). The accumulation of ^{111}In-eosinophils induced by inflammatory mediators in vivo. *Immunology*, **73**, 222–227.

FILEP, J.G., FOLDES-FILEP, E. & SIROIS, P. (1993). Nitric oxide modulates vascular permeability in the rat coronary circulation. *Br. J. Pharmacol.*, **108**, 323–326.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARSIDE, P., HUTTON, A.K., SEVERN, A., LIEW, F.Y. & MOWATT, A.M. (1992). Nitric oxide mediates intestinal pathology in graft-versus-host disease. *Eur. J. Immunol.*, **22**, 2141–2145.

HELLEWELL, P.G., JOSE, P.J. & WILLIAMS, T.J. (1992). Inflammatory mechanisms in the passive cutaneous anaphylactic reaction in the rabbit: evidence that novel mediators are involved. *Br. J. Pharmacol.*, **107**, 1163–1172.

IALENTI, A., IANARO, A., MONCADA, S. & DI ROSA, M. (1992). Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.*, **211**, 177–182.

IALENTI, A., MONCADA, S. & DI ROSA, M. (1993). Modulation of adjuvant arthritis by the L-arginine: NO pathway. *Br. J. Pharmacol.*, **108**, 10P.

IGNARRO, L.J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released form artery and vein. *Circ. Res.*, **65**, 1–21.

ISSEKUTZ, A.C. (1981). Effect of vasoactive agents on polymorphonuclear leukocyte emigration in vivo. *Lab. Invest.*, **45**, 234.

ISSEKUTZ, A.C. & MOVAT, H.Z. (1979). The effect of vasodilator prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. *Am. J. Pathol.*, **107**, 300–309.

KUBES, P. & GRANGER, D.N. (1992). Nitric oxide modulates microvascular permeability. *Am. J. Physiol.*, **262**, H611–H615.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4651–4655.

LIPPE, I.T., STABENTHEINER, A. & HOLZER, P. (1993). Participation of nitric oxide in the mustard oil-induced neurogenic inflammation of the rat paw skin. *Eur. J. Pharmacol.*, **232**, 113–120.

MCCALL, T.B., FEELISCH, M., PALMER, R.M.J. & MONCADA, S. (1991). Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.*, **102**, 234–238.

MILLER, M.J.S., SADOWASKA-KROWICKA, H., CHOTINARUEMOL, S., KAKKIS, J.L. & CLARK, D.A. (1993). Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J. Pharmacol. Exp. Ther.*, **264**, 11–16.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MULLIGAN, M.S., HEVEL, J.M., MARLETTA, M.A. & WARD, P.A. (1991). Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6338–6342.

MULLIGAN, M.S., MONCADA, S. & WARD, P.A. (1992). Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis. *Br. J. Pharmacol.*, **107**, 1159–1162.

NEEDHAM, L., HELLEWELL, P.G., WILLIAMS, T.J. & GORDON, J.L. (1988). Endothelial functional responses and increased vascular permeability induced by polycations. *Lab. Invest.*, **59**, 538–548.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PAUL, W., LAWRENCE, L., DOUGLAS, G.J., SCHACHTER, M. & PAGE, C.P. (1992). Modulators of the L-arginine-nitric oxide pathway: differential effects on cutaneous permeability to bradykinin and histamine in the guinea pig. *Br. J. Pharmacol.*, **107**, 287P.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3379.

ROMSON, J.L., HOOK, B.G., KUNKEL, S.L., ABRAMS, G.D., SCHORK, M.A. & LUCCHESI, B.R. (1983). Reduction of the extent of ischemic myocardial injury by neutrophil in the dog. *Circulation*, **67**, 1016–1023.

TEIXEIRA, M.M., WILLIAMS, T.J. & HELLEWELL, P.G. (1993). E-type prostaglandins enhance local oedema formation and neutrophil accumulation but suppress eosinophil accumulation in guinea pig skin. *Br. J. Pharmacol.*, **110**, 416–422.

WEG, V.B., WATSON, M.L., CORDEIRO, R.S.B. & WILLIAMS, T.J. (1991). Histamine, leukotriene D_4 and platelet activating factor in guinea pig passive cutaneous anaphylaxis. *Eur. J. Pharmacol.*, **204**, 157–163.

WEG, V.B., WATSON, M.L., FACCIOLI, L.H. & WILLIAMS, T.J. (1992). [^{111}In]-eosinophil accumulation during passive cutaneous anaphylaxis in the guinea pig. *Br. J. Pharmacol.*, **105**, 127P.

WILLIAMS, T.J. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *Br. J. Pharmacol.*, **65**, 517–524.

WILLIAMS, T.J., HELLEWELL, P.G. & JOSE, P.J. (1986). Inflammatory mechanisms in the Arthus reaction. *Agents Actions*, **19**, 66–72.

WILLIAMS, T.J. & MORLEY, J. (1973). Prostaglandin as potentiators of increased vascular permeability in inflammation. *Nature*, **246**, 215–217.

WILLIAMS, T.J. & PECK, M.J. (1977). Role of prostaglandin-mediated vasodilatation in inflammation. *Nature*, **270**, 530–532.

(Received June 14, 1993
Revised July 15, 1993
Accepted August 2, 1993)

Br. J. Pharmacol. (1993), **110**, 1522–1526 © Macmillan Press Ltd, 1993

Evidence for functional dissociation of dependence and tolerance in guinea-pig isolated ileal segments following 20 hour exposure to morphine *in vitro*

C. David, N. Davis, R. Mason & [1]V.G. Wilson

Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 In the present study we have examined the relationship between tolerance and dependence in isolated ileal segments from the guinea-pig under three different conditions: fresh preparations not previously exposed to morphine (fresh/morphine naive); preparations stored overnight at 4°C in modified Krebs-Henseleit saline (overnight-stored/morphine-naive); preparations stored overnight at 4°C in Krebs-Henseleit saline containing 10 μM morphine and extensively washed with modified Krebs-Henseleit saline to remove residual morphine (overnight-stored/morphine-exposed).

2 Morphine produced a concentration-dependent inhibition of the response of ileal segment to 0.1 Hz, 1 ms and 10 V transmural field stimulation in fresh/morphine-naive, overnight-stored/morphine-naive and overnight-stored/morphine-exposed preparations. The maximum effect observed was similar in all three preparations – approximately 80% inhibition. Although, morphine was significantly more potent in the fresh/morphine-naive preparations (pD_2 6.72 ± 0.05, $n = 8$) than either the overnight-stored/morphine-naive (pD_2 6.42 ± 0.11, $n = 8$) or the overnight-stored/morphine-exposed (pD_2 6.44 ± 0.14, $n = 8$), there was no significant difference between the overnight models in their sensitivity to morphine. The latter observation indicates that overnight exposure to ileal segments to 10 μM morphine at 4°C failed to induce tolerance to morphine.

3 The μ opiate receptor antagonist, naloxone (10 μM), produced contractions in both fresh/morphine-naive and overnight-stored/morphine-naive ileal segments following acute exposure to 10 μM morphine. Naloxone (10 μM) also produced contractions in 2/9 fresh/morphine-naive, 1/9 overnight-stored/morphine-naive and 7/9 overnight-stored/morphine-exposed preparations in the absence of morphine. The greater incidence of naloxone-induced contractions in overnight-stored/morphine-exposed preparations, suggests that dependence in this model is the product of adaptive changes that outlive the presence of morphine.

4 The selective α_2-adrenoceptor agonists, clonidine (0.3 μM) and 5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate (UK-14304, 1 μM), inhibited naloxone-induced contractions in overnight-stored/morphine-exposed preparations of ileal segments ($n = 4$ preparations for each agonist), suggesting that the response is due to transmitter release from the myenteric plexus.

5 The findings in the present study indicate that tolerance and dependence to morphine in ileal segments of the guinea-pig can be functionally dissociated by overnight exposure to morphine at 4°C. The development of tolerance to morphine, unlike dependence, appears to be a temperature-dependent process. This also raises the possibility that naloxone possesses intrinsic negative agonism at morphine-sensitive receptors, which is manifested as a functional response only after adaptive changes in the myenteric plexus following exposure to morphine.

Keywords: Morphine; naloxone; tolerance; dependence; guinea-pig ileum; withdrawal contraction

Introduction

Opiate dependence is generally viewed as the 'physiological abnormalities when morphine is removed from its receptors either by discontinuing its administration or by using a specific receptor antagonist' (Johnson & Fleming, 1989), and there are numerous examples consistent with this proposal. For example, a contraction of the guinea-pig isolated ileum is observed following short-term exposure to morphine (minutes) and subsequent washout (Chahl, 1986; 1990). Similarly, either short-term or chronic exposure of the guinea-pig isolated ileum to morphine, followed by a challenge with naloxone, is also associated with a contraction (Miguel & Rodriguez, 1981; Johnson *et al.*, 1987; Chahl, 1990).

There are, however, examples of naloxone-induced contractions of guinea-pig isolated ileum which clearly do not involve removal of morphine from its receptor. For example, Alfaro *et al.* (1990) reported that isolated ileal segments from morphine-treated guinea-pigs responded to naloxone with a contraction even after repeated washing of the preparation with opiate-free solution. Similarly, Collier *et al.* (1981a) noted that naloxone produced a contraction in guinea-pig isolated ileal segments exposed to normorphine for 22 h at 22°C which had also been exposed to an opiate-free solution for 90 min prior to the addition of the antagonist. Significantly, in both of these reports the ileal segments also exhibited evidence of tolerance, as judged by the reduction in either the potency of, and/or the maximum inhibition produced by, opiates against the electrically-evoked twitch response. Based upon these findings, and earlier reports of a close association between the development of opiate dependence and tolerance (see Collier, 1980), Johnson & Fleming (1989) proposed that the ability of naloxone to produce a contraction in the guinea-pig isolated ileum is a consequence of non-specific 'supersensitivity' within the myenteric plexus, which is associated with the development of tolerance to agents that inhibit acetylcholine release from cholinergic neurones (see also, Taylor *et al.*, 1988).

In preliminary experiments we observed that overnight

[1] Author for correspondence.

incubation of the guinea-pig isolated ileum with morphine at 4°C, rather than 22°C, and subsequent washing to remove residual morphine, was not associated with any evidence of tolerance to the effects of morphine against electrically-evoked contractions. Hence, the possibility exists that the development of tolerance to opiates is a temperature-sensitive process. In the present study we have examined this model in greater detail to determine whether the development of dependence to morphine is also a temperature-sensitive phenomenon. Our results indicate the tolerance to and dependence on morphine in the guinea-pig isolated ileum are differentially affected by a reduction in temperature and, therefore, are not mutually-dependent phenomena.

Methods

Male or female guinea-pigs, 350–450 g, were killed by cervical dislocation and the intestines exteriorized. The ileo-caecal junction was located and 5 cm of the terminal ileum discarded. Approximately 70 cm of the ileum was removed and divided into four segments and the lumen flushed with modified Krebs-Henseleit saline. Two of the segments were placed in Krebs-Henseleit saline which were gassed with 95% O_2/5% CO_2 and maintained at room temperature ('fresh/morphine-naive' preparations) until required for experimentation (less than 4 h). The remaining two segments were placed in 250 ml of either Krebs-Henseleit saline ('stored/morphine-naive') or Krebs-Henseleit saline with 10 μM morphine ('stored/morphine-exposed') and stored for 20–24 h at 4°C. Both solutions were pre-gassed with 95% O_2/5% CO_2 before receiving the ileal segments.

Five to six cm length segments of the ileum were secured onto a glass holder supporting a wire electrode which protruded into the lumen of the preparation. The tissue and electrode were placed in a 50 ml isolated organ bath containing modified Krebs-Henseleit saline which was maintained at 37°C and gassed with 95% O_2/5% CO_2. The upper end of the tissue was attached to a Washington isotonic transducer which was counterbalanced with a 1 g weight. Contractions of the tissue were elicited by transmural stimulation (1 ms duration, 0.1 Hz, 10 V) with a SRI stimulator. Responses were recorded on a George Washington pen recorder.

After 15 min equilibration, all ileal segments were stimulated for a minimum of 45 min to establish reproducible responses to electrical field stimulation. At the end of this period the stimulus intensity was adjusted to achieve a 'just submaximal' response.

Fresh/morphine-naive preparations

Agonist concentration-response curves were constructed by cumulative addition of either morphine (1 nM–30 μM), clonidine (1 nM–3 μM), or UK-14304 (1 nM–3 μM) with approximately 0.5 $\log_{10}$ unit increments. A minimum of 3 min was allowed between individual concentrations. Only one agonist was used per tissue. In another series of experiments, the effect of 10 μM naloxone was examined in either the absence or after a 3 min exposure to 10 μM morphine, with the stimulator turned off 1 min prior to the addition of naloxone.

Stored/morphine naive and stored/morphine-exposed preparations

On the day of experimentation, the lumen of ileal segments stored overnight were gently flushed through with 10 ml of Krebs-Henseleit saline. Segments were then placed in 250 ml of fresh Krebs-Henseleit saline stored at room temperature and gassed with 95% O_2/5% CO_2. This washout procedure was repeated a further 3 times over an 80 min period prior to setting up the ileal segment in isolated organ baths.

Agonist concentration-response curves were constructed by cumulative addition of morphine (1 nM–30 μM) with approximately 0.5 $\log_{10}$ unit increments, allowing a minimum of 3 min between individual concentrations. In a separate series of experiments with stored/morphine/naive preparations, the effect of 10 μM naloxone was examined in either the absence or after a 3 min exposure to 10 μM morphine. The effect of 10 μM naloxone was also examined in stored/morphine-exposed ileal segments in the presence and absence of either 0.3 μM clonidine or 1 μM UK-14304.

Analysis of data

All responses have been calculated as a percentage of the response to electrical field stimulation and expressed as the mean ± standard error of the mean (s.e.mean). The logarithm of the concentration of morphine producing 50% of the maximum response (pD_2) was calculated using the logistic equation (DeLean *et al.*, 1978).

Differences between mean values were considered statistically significant when $P<0.05$ using either a paired or unpaired Student's t test.

Drugs and solutions

The composition of the modified Krebs-Henseleit saline was as follows (mM): NaCl 118, KCl 4.8, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25 and glucose 11.1. The following drugs were used: morphine sulphate (Evans), naloxone (Endo Laboratories), clonidine HCl (Sigma); UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer). All drugs were dissolved in distilled water and added in volumes less than 0.1% of the organ bath volume.

Results

The effect of morphine against electrically-evoked contractions of the guinea-pig isolated ileum

Following 20–24 h storage at 4°C, and repeated washing in fresh Krebs-Henseleit saline at room temperature for a further 3 h, morphine-naive and morphine-exposed preparations of the guinea-pig ileum responded to electrical field stimulation (10 V, 1 ms, 0.1 Hz) with stable contractions.

Morphine produced a concentration-dependent inhibition of the electrically-evoked twitch response in fresh/morphine-naive, stored/morphine-naive and stored/morphine-exposed segments of the guinea-pig isolated ileum (Figure 1). As shown in Table 1, morphine was 2 fold more potent in the

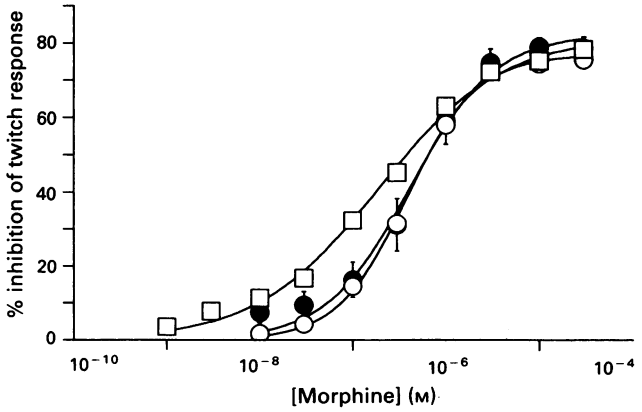


Figure 1 The effect of morphine against electrically-evoked contractions of fresh/morphine-naive (□), overnight-stored/morphine-naive (●) and overnight-stored/morphine-exposed (○) segments of the guinea-pig isolated ileum. The inhibition produced by morphine has been expressed as a percentage of the electrically-evoked twitch response and the values shown are the mean (± s.e.mean) of eight observations.

fresh/morphine-naive preparations, than in the two overnight storage models. However, there was no significant difference in either the potency of, or the maximum effect produced by, morphine in the stored/morphine-naive and stored/morphine-exposed preparations.

The effect 10 μM naloxone on the guinea-pig isolated ileum in the presence of 10 μM morphine

Following acute exposure to 10 μM morphine, the electrically-evoked contractions of fresh/morphine-naive and stored/morphine-naive ileal segments were inhibited by 83.5 ± 5.6% ($n = 7$) and 82.4 ± 5.9% ($n = 6$), respectively. Subsequent addition of 10 μM naloxone produced contractions in both preparations (131.3 ± 10% and 209 ± 32% of the twitch response in fresh/morphine-naive ($n = 7$) and stored/morphine-naive ($n = 6$) ileal segments, respectively).

The effect of 10 μM naloxone on the guinea-pig isolated ileum in the absence of morphine

In a separate series of experiments, fresh/morphine-naive, stored/morphine-naive and stored/morphine-exposed ileal segments (not used to determine the potency of acute exposure to morphine) were challenged with 10 μM naloxone after stable electrically-evoked responses were established. Contractions to 10 μM naloxone were observed in 2/9 fresh/morphine-naive preparations, 1/9 stored/morphine-naive preparations and 7/9 stored/morphine-exposed preparations. Responses in the stored/morphine-exposed preparations varied between 75% and 363% of the electrically-evoked twitch response. Because of the highly variable nature of the responses, the results have been represented graphically in Figure 2 in the form of a scattergram.

Table 1 pD_2 and E_{max} values for morphine against electrically-evoked contraction of isolated ileal segments of the guinea-pig ($n = 8$)

	pD_2	E_{max}
Fresh/morphine-naive	6.72 ± 0.05*	0.80 ± 0.04
Overnight-stored/ morphine-naive	6.42 ± 0.11	0.82 ± 0.03
Overnight-stored/ morphine-exposed	6.44 ± 0.14	0.77 ± 0.04

*Significantly different from overnight-stored/morphine-naive and overnight-stored/morphine-exposed preparations ($P < 0.05$, paired Student's t test).

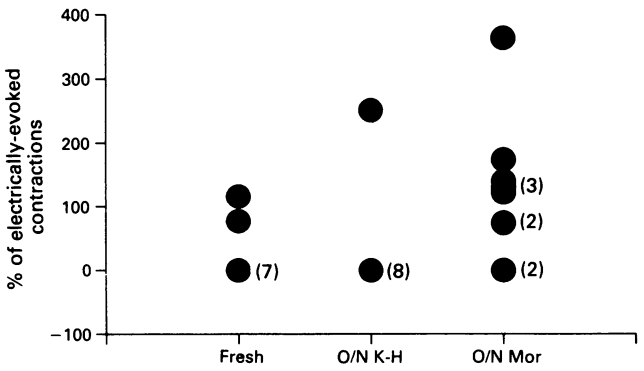


Figure 2 The effect of 10 μM naloxone in fresh/morphine-naive (Fresh), overnight-stored/morphine-naive (O/N K-H) and overnight-stored/morphine-exposed (O/N Mor) segments of the guinea-pig isolated ileum. Responses have been expressed as a percentage of the electrically-evoked contractions and are represented by (●). The values in parentheses indicate where a number of individual responses overlap.

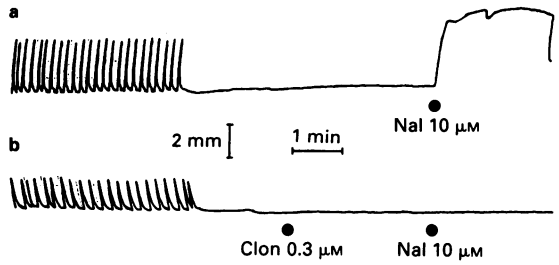


Figure 3 Representative trace recordings of the effect of 10 μM naloxone in overnight-stored/morphine-exposed preparations of the guinea-pig isolated ileum in (a) the absence of clonidine and (b) the presence of 0.3 μM clonidine.

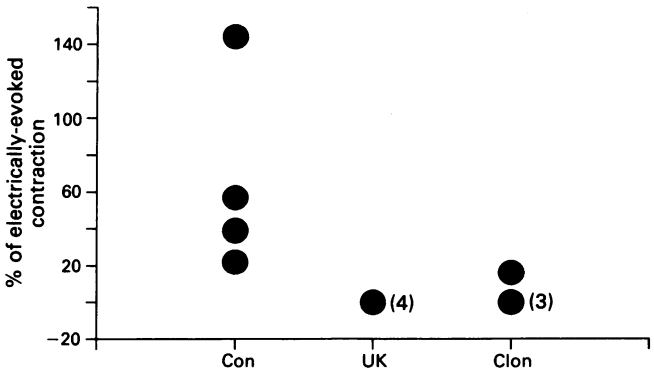


Figure 4 The effect of 10 μM naloxone in overnight-stored/morphine-exposed segments of the guinea-pig isolated ileum in the presence of saline (Con), 1 μM UK-14304 (UK) and 0.3 μM clonidine (Clon). Responses have been expressed as a percentage of the electrically-evoked contractions and are represented by the closed circles. The values in parentheses indicate where a number of individual responses overlap.

The effect of α_2-adrenoceptor-activation on naloxone-induced contractions of stored/morphine-exposed preparations

The α_2-adrenoceptor agonists clonidine (0.3 nM − 1 μM) and UK-14304 (1 nM − 1 μM) produced concentration-dependent inhibition of the twitch responses in stored/morphine-naive preparations of the guinea-pig ileum. The maximum inhibition produced in both agonists was greater than 90%.

Clonidine (0.3 μM, 100 fold greater than the EC_{50}) completely inhibited naloxone-induced contraction of stored/morphine-exposed preparations in 3 out of 4 preparations (Figure 3). As shown in Figure 4, 1 μM UK-14304 (100 fold greater than the EC_{50}) was similarly effective against contractions evoked by 10 μM naloxone.

Discussion

Functional dissociation of tolerance and dependence

In the present study we have shown that overnight incubation of the guinea-pig isolated ileum in Krebs-Henseleit saline at 4°C (stored/morphine-naive) does not impair the ability of the preparation to respond either to electrical field stimulation or to 10 μM naloxone after acute exposure to 10 μM morphine; this agrees with an earlier report by Collier and coworkers (1981a). However, compared to fresh/morphine-naive preparations, there was a significant reduction in the potency of morphine as an inhibitor of the electrically-evoked contractions even though the maximum inhibition

produced was unaffected. The basis of this difference in sensitivity to morphine is not known.

A comparison of stored/morphine-naive and stored/morphine-exposed preparations failed to reveal any difference in either the potency or maximum inhibition produced by morphine against the electrically-evoked contractions. Hence, there was no evidence for the development of tolerance following overnight exposure of the guinea-pig isolated ileum to 10 μM morphine at 4°C. These findings also indicate that the washout procedure adopted (4 washes over 3 h at room temperature) reduces the receptor biophase concentration of morphine in stored/morphine-exposed preparations to below 10 nM – the threshold concentration for inhibition of the electrically-evoked response. Our results contrast sharply with the earlier report that 18–22 h incubation of the guinea-pig isolated ileum with 10 nM normorphine at 22°C was associated with a 10 fold rightward displacement of the normorphine concentration-response curve (Collier *et al.*, 1981a). It seems likely that the adaptive processes responsible for receptor-specific tolerance, e.g. a reduction in receptor number and/or uncoupling of the receptor (Johnson & Fleming, 1989), are temperature-sensitive.

In spite of the lack of evidence for the development of tolerance to morphine in stored/morphine-exposed guinea-pig ileal preparations, seven out of nine segments responded to 10 μM naloxone with a contraction. This represents a significant increase over the incidence of naloxone-induced contractions in fresh/morphine-naive preparations (2/9) and stored/morphine-naive preparations (1/9; see Figure 2). Since the naloxone-induced contractions in stored/morphine-exposed preparations were practically abolished by the α_2-adrenoceptor agonists, clonidine and UK-14304, it seems likely that these contractions, like those observed following acute exposure to morphine and naloxone (Chahl, 1990), involves the release of acetylcholine from cholinergic neurones. Furthermore, these observations are also consistent with the known efficacy of α_2-adrenoceptor agonists against withdrawal responses in opiate-dependent states in experimental models (Alfaro *et al.*, 1990; Coupar 1991) and in man (Gold *et al.*, 1978). Significantly, the effectiveness of clonidine against both the electrically-evoked contractile responses and the naloxone-induced contractile response (>90% inhibition) also supports the view that overnight exposure to morphine at 4°C failed to induce tolerance. Taylor *et al.* (1988) and Alfaro *et al.* (1991) both reported that tolerance following long-term exposure to opiates was not receptor specific, and α_2-adrenoceptors effects against the electrically-evoked contractions of the guinea-pig isolated ileum or the naloxone-induced contractions were similarly blunted.

Taken together, the findings in the present study clearly indicate that opiate dependence and tolerance are not mutually-dependent processes, a view consistent with earlier suggestions by Schulz and coworkers (1985). Since naloxone was able to elicit a contraction in the stored/morphine-exposed guinea-pig isolated ileum in the absence of both morphine and the development of tolerance, the view that this response is a consequence of adaptive processes associated with tolerance (Johnson & Fleming, 1989) is no longer tenable and an alternative explanation is required. One possibility is that 'dependence' in the guinea-pig ileum as judged by the appearance of a 'naloxone-induced' contraction, can be precipitated by concentrations of morphine that are sub-threshold for the inhibition of the electrically-evoked contractions (>10 nM). Another possibility is that the antagonist, naloxone, possesses inverse agonist properties which are manifest only after receptor-specific adaptive changes in the myenteric plexus have been induced by morphine.

Does naloxone possess inverse agonist properties at morphine-sensitive receptors?

A number of recent observations indicate that naloxone may possess negative agonism at opiate receptors. Costa & Herz (1989) reported that antagonists for δ opiate receptors on the neuroblastoma/glioma NG-108-15 cell line can be divided into two groups based upon their effect on guanosine-triphosphatase (GTPase) activity. The antagonist (−)-5,9-diethyl-2-(3-furyl-methyl)-2'-hydroxybenzomorphan (MR 2266) had no effect on GTPase activity but produced a competitive inhibition of the GTPase stimulated by δ opiate receptor agonist [D-Ala^2, D-Leu^5[enkephalin (DADLE). MR-2266 can be considered a pure δ receptor antagonist. In contrast, the antagonists ICI 174864 ([N,*N'*-diallyl-Tyr^1,Aib^{2,3}] Leu^5 enkephalin), ICI 154129 ([N,*N'*-diallyl-Tyr^1, φ-(CH_2S) Phe^4,Leu^5] enkephalin) and naloxone produced a concentration-dependent inhibition of GTPase activity and, in the case of ICI 174854, the 'response' was sensitive to MR 2266. Thus, these antagonists can be considered as possessing negative agonist activity at δ opiate receptors. The essential difference between 'pure' antagonists and those possessing 'negative agonist' properties was underlined in studies by Millan *et al.* (1988) and Morris & Millan (1991), who showed that unlike naloxone long-term exposure to MR 2266 failed to up-regulate central μ, δ or κ opiate binding sites or to produce supersensitivity to opiate agonists.

If naloxone also possesses negative agonism at μ opiate receptors, this could account for the contractile activity in response to naloxone observed in both stored/morphine-exposed and in morphine-naive preparations of the guinea-pig isolated ileum (see also Rodriguez *et al.*, 1980; Miguel & Rodriguez, 1981). An assumption underlying this suggestion is that μ opiate receptors undergo a 'temperature-insensitive' adaptive change following overnight exposure to morphine (that outlives the presence of the agonist) in order to account for the greater incidence of naloxone-induced contractions. Since there is no evidence for cross-dependence between μ opiate receptors and α_2-adrenoceptors following overnight exposure to either morphine or clonidine (Collier *et al.*, 1991b), this adaptive change could be either at the receptor binding site or at some point in the receptor-coupling mechanism proximal to the point of interaction with neuronal α_2-adrenoceptors. Support for this working model would be provided by the failure of both MR 2266 and an α_2-adrenoceptor antagonist, e.g. phentolamine, to elicit a withdrawal response in stored/morphine-exposed preparations of the guinea-pig isolated ileum.

In conclusion, we have demonstrated that overnight exposure of the guinea-pig isolated ileum to morphine at 4°C results in the functional dissociation of dependence and tolerance, suggesting that the processes underlying the latter adaptive response are temperature-sensitive. Our findings also raise the possibility that naloxone-induced contractions in 'stored/morphine-exposed' segments of the guinea-pig ileum arise from either residual, 'subthreshold' concentrations of morphine or is a consequence of a combination of intrinsic inverse agonism of naloxone at μ opiate receptors on cholinergic neurones and receptor-specific adaptive changes.

References

ALFARO, M.J., COLODA, M.I., LOPEZ, F. & MARTIN, M.I. (1990). Effects of clonidine, nimodipine and diltiazem on the *in vitro* opioid withdrawal response in the guinea-pig ileum. *Br. J. Pharmacol.*, **101**, 958–961.

CHAHL, L.A. (1986). Withdrawal responses of the guinea-pig isolated ileum following brief exposure to opiates and opioid peptides. *Naunyn Schmied Arch. Pharmacol.*, **333**, 387–392.

CHAHL, L.A. (1990). Effects of putative neurotransmitters and related drugs on withdrawal contractures of the guinea-pig isolated ileum following brief contact with [Met^5]-enkephalin. *Br. J. Pharmacol.*, **101**, 908–912.

COLLIER, H.O.J. (1980). Cellular site of opiate dependence. *Nature*, **283**, 625–629.

COLLIER, H.O.J., CUTHBERT, N.J. & FRANCIS, D.L. (1981a). Model of opiate dependence in the guinea-pig ileum. *Br. J. Pharmacol.*, **73**, 921–933.

COLLIER, H.O.J., CUTHBERT, N.J. & FRANCIS, D.L. (1981b). Clonidine dependence in the guinea-pig ileum. *Br. J. Pharmacol.*, **73**, 741–749.

COSTA, T. & HERZ, A. (1989). Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 7321–7325.

COUPAR, I.M. (1992). Effect of alpha$_2$-adrenoceptor agonist on the expression of morphine-withdrawal in rats. *Naunyn Schmied Arch. Pharmacol.*, **345**, 553–557.

DELEAN, A., MUNSON, P.J. & RODBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: applications to bioassay, radioligand assay and physiological dose response curves. *Am. J. Physiol.*, **235**, E97–E102.

GOLD, M.S., REDMOND, D.E. & KLEBER, H.D. (1978). Clonidine in opiate withdrawal. *Lancet*, **i**, 929–930.

JOHNSON, S.M. & FLEMING, W.W. (1989). Mechanisms of cellular adaptive sensitivity changes: application to opioid tolerance and dependence. *Pharmacol. Rev.*, **41**, 435–488.

JOHNSON, S.M., WILLIAMS, J.T., COSTA, M. & FURNESS, J.B. (1987). Naloxone-induced depolarization and synaptic activation of myenteric neurones in morphine-dependent guinea-pig ileum. *Neuroscience*, **21**, 595–602.

MIGUEL, L. & RODRIGUEZ, R. (1981). Pharmacological characterization of opiate physical dependence in the isolated ileum of the guinea-pig. *Br. J. Pharmacol.*, **73**, 859–866.

MILLAN, M.J., MORRIS, B.J. & HERZ, A. (1986). Antagonist-induced opioid receptor up-regulation I: Characterization of supersensitivity to selective mu and kappa agonists. *J. Pharmacol. Exp. Ther.*, **247**, 721–728.

MORRIS, B.J. & MILLAN, M.J. (1991). Inability of opioid antagonist lacking negative intrinsic activity to induce opioid receptor up-regulation *in vivo*. *Br. J. Pharmacol.*, **102**, 883–887.

RODRIGUEZ, R., LUJAN, M. & VARGAS-ORTEGA, E. (1980). Circannual variation in sensitivity of the guinea-pig isolated ileum to naloxone. *J. Pharm. Pharmacol.*, **32**, 363–365.

SCHULTZ, R., SEIDL, E. & HERZ, A. (1985). Opioid dependence in the guinea-pig myenteric plexus is controlled by non-tolerant and tolerant opioid receptors. *Eur. J. Pharmacol.*, **110**, 335–341.

TAYLOR, D.A., LEEDHAM, J.A., DOAK, N. & FLEMING, W.W. (1988). Morphine tolerance and non-specific subsensitivity of the longitudinal muscle, myenteric plexus preparations of guinea-pig to inhibitory agonist. *Naunyn Schmied. Arch. Pharmacol.*, **338**, 553–559.

(Received March 29, 1993
Revised July 30, 1993
Accepted August, 2 1993)

Br. J. Pharmacol. (1993), **110**, 1527–1531 © Macmillan Press Ltd, 1993

Different pathways of calcium sensitization activated by receptor agonists and phorbol esters in vascular smooth muscle

[1]Masatoshi Hori, Koichi Sato, Shigeki Miyamoto, Hiroshi Ozaki & Hideaki Karaki

Department of Veterinary Pharmacology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

1 It has been shown that receptor agonists and activators of protein kinase C, phorbol esters, increase Ca^{2+} sensitivity of contractile elements in vascular smooth muscle. To discover if protein kinase C is involved in the agonist-mediated Ca^{2+} sensitization, we examined the effects of receptor agonists in the rat isolated aorta in which protein kinase C activity had been diminished by pretreatment with phorbol 12-myristate 13-acetate for 24 h.

2 In the aorta with protein kinase C activity, a high concentration (1 μM) of 12-deoxyphorbol 13-isobutyrate induced contraction and a low concentration (100 nM) potentiated high K^+-induced contraction. In addition, prostaglandin $F_{2\alpha}$ induced greater contractions than high K^+ at a given cytosolic Ca^{2+} level. The maximally effective concentrations of noradrenaline and endothelin-1 also induced greater contraction than high K^+. In the aorta without protein kinase C activity, the contraction induced by 12-deoxyphorbol 13-isobutyrate and its potentiation of the high K^+-induced contraction were abolished. However, prostaglandin $F_{2\alpha}$, noradrenaline and endothelin-1 still induced a greater contraction than high K^+.

3 In the aorta without protein kinase C activity, noradrenaline, endothelin-1 and prostaglandin $F_{2\alpha}$, but not 12-deoxyphorbol 13-isobutyrate, induced contractions in the presence of the Ca^{2+} channel blocker, verapamil, or in the absence of external Ca^{2+}, by increasing Ca^{2+} sensitivity.

4 In the permeabilized preparations, inhibition of protein kinase C activity abolished the effect of potentiation of the Ca^{2+}-induced contraction by 12-deoxyphorbol 13-isobutyrate although the potentiation of the contraction by prostaglandin $F_{2\alpha}$ did not change.

5 These results suggest that there are two pathways for Ca^{2+} sensitization in rat aorta; a protein kinase C-dependent pathway which is activated by phorbol esters, and a protein kinase C-independent pathway which is activated by receptor agonists.

Keywords: Protein kinase C; cystolic Ca^{2+} level; receptor agonists; Ca^{2+} sensitization; vascular smooth muscle (rat aorta)

Introduction

Although smooth muscle contraction is induced by increasing the cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) and phosphorylating the myosin light chain (MLC), receptor agonists cause greater contraction than high K^+-depolarization at a given $[Ca^{2+}]_i$ (For review see Karaki, 1989; Somlyo & Himpens, 1989). In muscle permeabilized with bacterial α-toxin, receptor agonists potentiate Ca^{2+}-induced contraction in the presence of GTP (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989). Since Ca^{2+} sensitization induced by receptor agonists is accompanied by an increase in MLC phosphorylation in intact and permeabilized smooth muscle preparations (Rembold & Murphy, 1988; Suematsu *et al.*, 1991; Kitazawa *et al.*, 1991; Hori *et al.*, 1992), it has been suggested that an altered balance between the activities of MLC kinase and MLC phosphatase results in the Ca^{2+} sensitization of MLC phosphorylation (Stull *et al.*, 1990; Gong *et al.*, 1992). In addition, the existence of a regulatory mechanism that is not dependent on MLC phosphorylation is also proposed and this mechanism may also be involved in the Ca^{2+} sensitization in smooth muscle (Hori *et al.*, 1992).

Activators of protein kinase C, phorbol esters, also induce contraction and increase Ca^{2+} sensitivity in intact and permeabilized smooth muscle preparations (Nishimura & van Breemen, 1989; Anabuki *et al.*, 1990; Singer 1990; Sato *et al.*, 1992). Since various receptor agonists activate inositol phosphate metabolism to generate the endogenous activator of protein kinase C, diacylglycerol (Berridge, 1984; Nishizuka 1984), it is possible that activation of protein kinase C is involved in Ca^{2+} sensitization mediated by the receptor agonists. In the present experiments, this possibility was examined in rat isolated aorta in which protein kinase C activity had been abolished. For this purpose, we treated the muscle with phorbol ester because such treatment has been shown to decrease protein kinase C activity by decreasing the amount of protein kinase C molecules in the cells (Young *et al.*, 1987). An account of some of these results has been published in abstract form (Sato & Karaki, 1992).

Methods

Muscle preparations

Male Wistar rats (250–300 g) were killed by a sharp blow on the neck and exsanguination. The thoracic aorta was isolated and cut into helical strips. The endothelium was removed by gently rubbing the intimal surface with a finger moistened with normal physiological salt solution (PSS) which contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, EDTA 0.01 and glucose 5.5. High K^+ solution was made by substituting 72.7 mM NaCl with equimolar KCl. These solutions were saturated with 95% O_2 and 5% CO_2 mixture at 37°C to maintain the pH at 7.4.

Muscle strips were stimulated by the maximally effective concentrations of noradrenaline (1 μM; Sato *et al.*, 1988), endothelin-1 (30 nM; Sakata *et al.*, 1989), 12-deoxyphorbol 13-isobutyrate (DPB; 1 μM; Sato *et al.*, 1992) or KCl (72.7 mM; Karaki *et al.*, 1986). A low concentration of DPB (100 nM) was also used to modulate high K^+-induced contractions.

[1] Author for correspondence.

Pretreatment with phorbol ester

To decrease protein kinase C activity, thoracic aortic strips were incubated in Dulbecco's modified Eagle medium with 10% foetal calf serum containing penicillin-streptomycin (GIBCO, New York, U.S.A.) for 24 h at 37°C in a CO_2 incubator in the presence or absence of 3 μM phorbol 12-myristate 13-acetate (TPA). At the end of the incubation, tissues were washed with normal PSS for more than 60 min to remove TPA from the surface of the tissue. In a preliminary experiment, we also used 3 μM DPB and found that the effect of DPB was less than that of TPA. For this reason, we used TPA to decrease protein kinase C activity in the present experiments.

Measurement of protein kinase C activity

Cytosolic and membrane fractions were prepared from rat aorta as described previously (Haller *et al.*, 1990). After a 20 min incubation with test agents, muscle strips were quickly frozen in liquid nitrogen and crushed. The muscle powder was dissolved in buffer containing (mM): Tris-HCl (pH 7.4) 20, sucrose 300, EDTA 5, EGTA 10, phenylmethylsulphonyl fluoride 0.3, benzamidine 10 and 0.3% β-mercaptoethanol. The suspension was centrifuged at 100,000 *g* at 4°C for 60 min. The supernatant was used as cytosolic fraction. The precipitate was re-suspended in the same buffer containing 0.1% Triton X-100 and kept on ice for 30 min. The suspension treated with Triton X-100 was centrifuged at 30,000 *g* for 20 min at 4°C. The supernatant was used as the membrane fraction.

Protein kinase C activity was measured by a commercial assay system (RPN 77A, Amersham Japan, Tokyo, Japan). The cytosolic and membrane fractions were dissolved in a reaction medium containing: Tris-HCl (pH 7.5) 50 mM, calcium acetate 1 mM, magnesium acetate 15 mM, L-α-phosphatidyl-L-serine 0.67 mol %, TPA 2 μg ml^{-1}, synthetic peptide 75 μM, sodium azide 0.05% w/v and dithiothreitol 2.5 mM. The reaction was started by adding 50 μM ATP containing 0.2 μCi [γ-^{32}P]-ATP. After treatment at 25°C for 15 min, the reaction was stopped and the radioactivity in the synthetic peptide was counted with a liquid scintillation spectrometer. In some experiments, calcium acetate, TPA and L-α-phosphatidyl-L-serine were removed from the reaction medium to inhibit the protein kinase C activity and to determine the background level of the measuring system.

Simultaneous measurement of force and $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured simultaneously with muscle force by use of the fluorescent Ca^{2+} indicator, fura-2, as reported previously (Sato *et al.*, 1988). The muscle strips (2 mm wide and 7 mm long) were loaded with 5 μM acetoxymethyl ester of fura-2 for 4–5 h at room temperature (23–25°C). The muscle strip was illuminated alternately (48 Hz) with 340 nm and 380 nm light and 500 nm emission was detected with a fluorimeter (CAF-100, JASCO, Tokyo, Japan). The ratio of 500 nm fluorescence excited at 340 nm and 380 nm (R340/380) was used as an indicator of $[Ca^{2+}]_i$. Abolsute Ca^{2+} concentration was not calculated because the dissociation constant of fura-2 in cytoplasm may be different from that obtained *in vitro* (Karaki, 1989). Contractile force was measured by a force displacement transducer under a resting force of 10 mN.

Permeabilized muscle

Permeabilized muscle preparations were made by treating the TPA-pretreated muscle strips (1 mm wide and 4 mm long) with *Staphylococcus aureus* α-toxin (80 μg ml^{-1}). Relaxing solution contained; potassium propionate 130 mM, $MgCl_2$ 4 mM, Na_2ATP 2 mM, Tris maleate (pH 6.8) 20 mM, EGTA 2 mM, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) 1 μM, creatine phosphate 5 mM, creatine phosphokinase 10 u ml^{-1} and leupeptine 1 μg ml^{-1}. Free Ca^{2+} concentration was changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^6 M^{-1}. Force of contraction was measured by an isometric transducer under a resting force of 5 mN. Intracullelar Ca^{2+} stores were depleted by adding 10 μM ionomycin for 20 min (Nishimura *et al.*, 1988).

Materials

Prostaglandin $F_{2\alpha}$ verapamil and FCCP (Sigma Chemical, St. Louis, MO, U.S.A.), TPA and DPB (Funakoshi, Tokyo, Japan), noradrenaline bitartrate, dithiothreitol and β-mercaptoethanol (Wako Pure Chemicals, Tokyo, Japan), cremophor EL (Nacalai Tesque, Kyoto, Japan), forskolin (Nihon Kayaku, Tokyo, Japan), GTP (Boehringer Mannheim Corp. New York, NY, U.S.A.), endothelin-1 (donated by Ciba-Geigy Japan, Takarazuka, Japan), fura-2/AM (donated by Dojindo Laboratories, Kumamoto, Japan), and α-toxin (donated by Dr Iwao Kato, Chiba University) were used.

Results

Preparations without protein kinase C activity

Figure 1 shows the effect of DPB on the protein kinase C activity in rat aorta. Protein kinase C activity was expressed as pmol ATP incorporated in synthetic peptide per mg of smooth muscle protein per min (pmol mg^{-1} min^{-1}). In the muscle without TPA-pretreatment (control), protein kinase C activity in the cytosolic fraction was higher than that in the membrane fraction. After incubation with 1 μM DPB for 20 min, protein kinase C activity in the cytosolic fraction decreased and that in the membrane fraction increased, suggesting that DPB activated protein kinase C. In the muscle pretreated with TPA for 24 h, protein kinase C activities in both fractions were reduced to a level similar to that

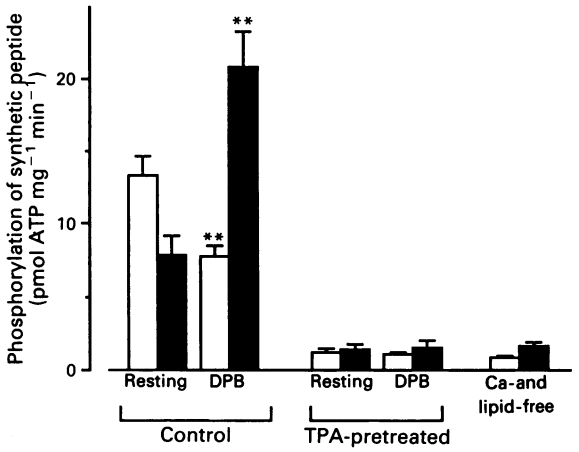


Figure 1 Effect of 12-myristate 13-acetate (TPA)-pretreatment on protein kinase C activity in rat aorta. Muscle strips without (control) or with TPA-pretreatment were incubated with 1 μM 12-deoxyphorbol 13-isobutyrate (DPB) for 20 min. Protein kinase C activity was shown by pmol ATP incorporated into synthetic peptide per mg of smooth muscle protein per min (pmol mg^{-1} min^{-1}). In some experiments, protein kinase C activity was measured in the absence of Ca^{2+}, TPA and lipid (Ca and lipid-free) to determine the background level of the measurement. Open and closed columns show cytosolic and membrane fractions, respectively. Each column represents mean of 4–8 experiments and s.e.mean is shown by vertical bar. **Significantly different from the value in resting muscle with $P<0.01$ using Student's *t* test.

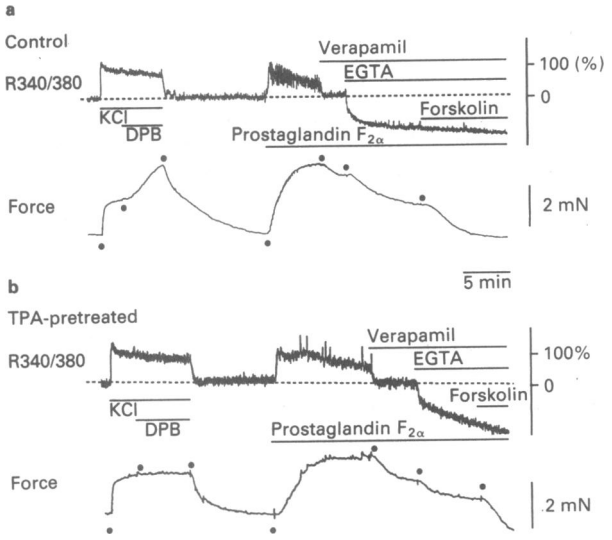


Figure 2 Changes in $[Ca^{2+}]_i$ (upper trace; indicated by R340/380) and contractile force (lower trace) in the muscle pretreated with vehicle (control; a) or phorbol 12-myristate 13-acetate (TPA) (b) KCl 72.7 mM; 12-deoxyphorbol 13-isobutyrate (DPB) 100 nM; prostaglandin $F_{2\alpha}$ 30 μM; verapamil 10 μM; EGTA 4 mM; forskolin 1 μM. 100% represents the levels of $[Ca^{2+}]_i$ stimualted by 72.7 mM KCl. Traced from typical results out of 4 experiments.

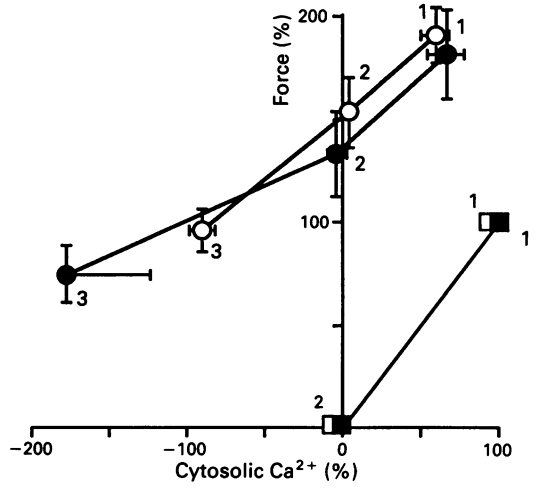


Figure 3 $[Ca^{2+}]_i$ force relationship in the muscle pretreated with vehicle (control; open symbol) or phorbol 12-myristate 13-acetate (TPA) (closed symbol): (□, ■) 72.7 mM KCl; (○, ●) 30 μM prostaglandin $F_{2\alpha}$. (1) In normal solution; (2) in the presence of 10 μM verapamil; (3) in the presence of 10 μM verapamil and 4 mM EGTA. 100% represents the levels of contractile force and $[Ca^{2+}]_i$ stimulated by 72.7 mM KCl. Each point represents mean ± s.e.mean of 4–6 experiments.

obtained in the absence of Ca^{2+}, TPA and lipid (background level). In these preparations, DPB was ineffective. These results suggest that treatment with TPA for 24 h abolished protein kinase C activity in the aorta.

Effects of prostaglandin $F_{2\alpha}$ and other agonists

Figure 2 shows the effects of DPB and prostaglandin $F_{2\alpha}$ on $[Ca^{2+}]_i$ and contractions in the muscles pretreated with vehicle (control) or TPA for 24 h. KCl (72.7 mM) induced sustained increases in contractile force and $[Ca^{2+}]_i$ in both types of preparations. DPB (100 nM) changed neither resting force nor

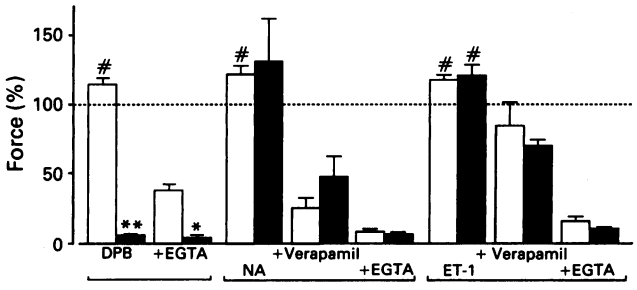


Figure 4 Effects of maximally effective concentrations of 12-deoxy phorbol 13-isobutyrate (DPB, 1 μM), noradrenaline (NA: 1 μM) and endothelin-1 (ET-1: 30 nM) on the contractile force in the muscle pretreated with vehicle (control; open column) or phorbol 12-myristate 13-acetate (TPA) (closed column). Experiments were done as shown in Figure 2 except that $[Ca^{2+}]_i$ was not measured. 100% (dotted line) represents the level of contractile force stimulated by maximally effective concentration of KCl (72.7 mM). Each column represents mean with s.e.mean of 4–6 experiments. * and **: Significantly different from control muscles with $P<0.05$ and 0.01, respectively, using Student's t test. #: Significantly greater than high K^+-induced contraction in control muscles with $P<0.05$ using Student's t test. Verapamil: 10 μM. EGTA: 4 mM.

$[Ca^{2+}]_i$ in control and in TPA-pretreated preparations ($n=4$ each, data not shown). In control muscles, the same concentrations of DPB potentiated the high K^+-induced contraction without changing $[Ca^{2+}]_i$ (Figure 2a). In the TPA-pretreated muscles, in contrast, 100 nM DPB was ineffective (Figure 2b).

Prostaglandin $F_{2\alpha}$ (30 μM) also increased $[Ca^{2+}]_i$ and force in the preparations pretreated with vehicle (control) or TPA (Figure 2). Prostaglandin $F_{2\alpha}$ induced greater force than high K^+ at a given $[Ca^{2+}]_i$ in both types of preparations (Figure 3). Verapamil (10 μM) inhibited the high K^+-stimulated increase in $[Ca^{2+}]_i$ and force to their respective resting levels (Figure 3). The same concentration of verapamil inhibited the prostaglandin $F_{2\alpha}$-stimulated $[Ca^{2+}]_i$ to a level near a resting level. However, the prostaglandin $F_{2\alpha}$-induced contraction was only partially inhibited (Figures 2 and 3). Sequential addition of EGTA (4 mM) further decreased $[Ca^{2+}]_i$ and force although a part of the developed force was not inhibited in both types of preparations. Contraction resistant to EGTA was inhibited by forskolin without changing $[Ca^{2+}]_i$ (Figures 2 and 3). These results are similar to those reported previously in rat aorta (Hori *et al.*, 1992; Sato *et al.*, 1992). The $[Ca^{2+}]_i$ force relationship (Figure 3) indicated that prostaglandin $F_{2\alpha}$ shifted this relationship to a lower Ca^{2+} level compared to that obtained in the presence of high K^+, irrespective of the pretreatment with TPA.

Figure 4 shows the effects of DPB, noradrenaline and endothelin-1 on contractile force in the aorta pretreated with vehicle (control) or TPA. The experiment was done as shown in Figure 2 except that $[Ca^{2+}]_i$ was not measured. In control preparations, DPB (1 μM) induced a sustained contraction greater than that induced by high K^+. DPB-induced contraction was partially inhibited by the addition of 4 mM EGTA. In control preparations, maximally effective concentrations of noradrenaline (1 μM; see Methods) and endothelin-1 (30 nM; see Methods) also induced contractions greater than those induced by 72.7 mM K^+. Addition of 10 μM verapamil partially inhibited the contractions induced by these receptor agonists. Sequential addition of EGTA further decreased contractile force although a part of the contraction was not inhibited.

In TPA-pretreated preparations, in contrast, 1 μM DPB did not induce contraction in the presence or absence of external Ca^{2+}. However, the contractions to noradrenaline and endothelin-1 in the absence or presence of EGTA and/or verapamil were not modified by the pretreatment with TPA (Figure 4).

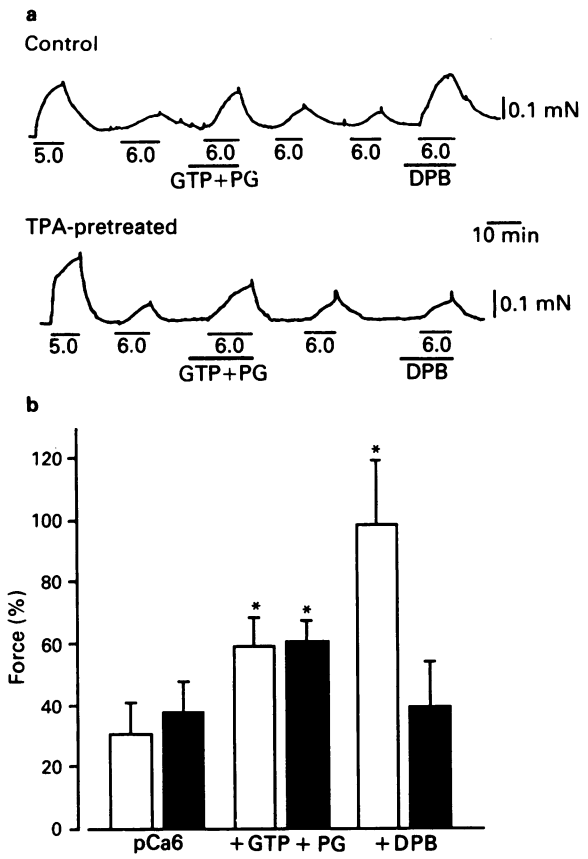


Figure 5 Ca^{2+}-induced contraction in permeabilized aorta pretreated with vehicle (control) or phorbol 12-myristate 13-acetate (TPA). (a) Trace of typical result, one of four experiments. Numbers indicate the concentrations of Ca^{2+} in $-\log M$ (pCa); GTP 100 μM; prostaglandin $F_{2\alpha}$ 10 μM; 12-deoxyphorbol 13-isobutyrate (DPB) 1 μM. (b) Summary of the results in (a). Open column: control. Closed column: TPA-pretreated. Each column represents the mean with s.e.mean of 4 experiments. 100% represents the level of contraction induced by 10 μM Ca^{2+}. *Significantly different from the contraction induced by 1 μM Ca^{2+} (pCa6) with $P<0.05$ using Student's t test.

Permeabilized preparation

As shown in Figure 5, addition of Ca^{2+} induced contraction in the permeabilized aorta pretreated with vehicle (control). This contraction was potentiated by 10 μM prostaglandin $F_{2\alpha}$ in the presence of 100 μM GTP. DPB (1 μM) had a similar effect to prostaglandin $F_{2\alpha}$ in the vehicle-treated preparations. In the permeabilized aorta pretreated with TPA, addition of Ca^{2+} also induced contraction. In TPA-pretreated preparations, the potentiation of the Ca^{2+}-induced contraction by DPB was abolished. However, 10 μM prostaglandin $F_{2\alpha}$ still potentiated the Ca^{2+}-induced contractions in the TPA-pretreated preparations. Thus, DPB showed similar effects to prostaglandin $F_{2\alpha}$ only in control preparations.

Discussion

To obtain preparations without protein kinase C activity, we treated the rat isolated aorta with tissue culture medium containing TPA for 24 h. In the preparations pretreated with TPA, there was no protein kinase C activity and the translocation of protein kinase C activity from cytosol to membrane by DPB was abolished. It has been reported that suppression of protein kinase C activity by phorbol esters is due to a decrease in protein kinase C molecules resulting from an increased rate of degradation, with no change in the amount of mRNA or rates of synthesis of protein kinase C (Young *et al.*, 1987).

In a previous paper (Sato *et al.*, 1992), we have shown that DPB induced greater contraction and greater myosin light chain (MLC) phosphorylation than in preparations stimulated by high K^+ for a given increase in $[Ca^{2+}]_i$ and that DPB induced contraction in the absence of external Ca^{2+} which is accompanied by an increase in neither $[Ca^{2+}]_i$ nor MLC phosphorylation. These results suggest that DPB has dual effects, increasing $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of contractile elements. It is also suggested that Ca^{2+} sensitization is due not only to the increase in Ca^{2+} sensitivity of MLC phosphorylation but also to the activation of a mechanism that is not dependent on the increase in MLC phosphorylation. In the present experiments, we found that DPB potentiated the high K^+-induced contraction without changing $[Ca^{2+}]_i$ in intact muscle and potentiated the Ca^{2+}-induced contraction in permeabilized muscle. All of the effects of DPB were abolished by pretreatment with TPA. These results suggest that the contraction and increase in Ca^{2+} sensitivity induced by DPB are due to activation of protein kinase C.

It has been shown that receptor agonists such as noradrenaline, prostaglandin $F_{2\alpha}$ and endothelin-1 caused greater contractions than those induced by high K^+ at a given $[Ca^{2+}]_i$ in rat aorta (Hori *et al.*, 1992). In permeabilized arterial muscles, it has also been reported that receptor agonists such as phenylephrine, noradrenaline and endothelin-1 enhance Ca^{2+}-induced contraction in the presence of GTP (Kitazawa *et al.*, 1989; 1991; Nishimura *et al.*, 1989; 1992; Anabuki *et al.*, 1990; Hori *et al.*, 1992). These receptor agonists induced contraction in the presence of the Ca^{2+} channel blocker, verapamil, or in the absence of external Ca^{2+} (with EGTA), and these contractions were not accompanied by an increase in MLC phosphorylation (Hori *et al.*, 1992). From these results, it has been suggested that receptor agonists have similar effects to phorbol esters: (1) increasing $[Ca^{2+}]_i$ and MLC phosphorylation, (2) increasing Ca^{2+} sensitivity of MLC phosphorylation, and (3) increasing Ca^{2+} sensitivity by activating a mechanism that is not dependent on MLC phosphorylation (Hori *et al.*, 1992).

Although receptor agonists and phorbol esters have similar effects in increasing Ca^{2+} sensitivity, we found that pretreatment with TPA selectively inhibited the effects of phorbol esters. After TPA-treatment, prostaglandin $F_{2\alpha}$ still induced greater contraction than high K^+ at a given $[Ca^{2+}]_i$. The maximally effective concentrations of noradrenaline and endothelin-1 also induced greater contractions than high K^+. In addition, these agonists induced contractions in the absence of external Ca^{2+}. In the permeabilized muscle without protein kinase C activity, prostaglandin $F_{2\alpha}$ potentiated Ca^{2+}-induced contraction. These results suggest that Ca^{2+} sensitization induced by receptor agonists is not attributable to the activation of protein kinase C.

A difference between receptor agonists and phorbol esters has been reported using protein kinase C inhibitors. For example, calphostin C selectively inhibited the contraction induced by phorbol ester without changing the contraction induced by endothelin-1 (Shimamoto *et al.*, 1992) and calphosin C did not inhibit Ca^{2+} sensitization induced by acetylcholine in permeabilized guinea-pig stomach (Oishi *et al.*, 1992). In contrast, it has also been shown that various protein kinase C inhibitors inhibited the contraction and Ca^{2+} sensitization induced by receptor agonists (Heaslip & Sickels, 1989; Nishimura *et al.*, 1992; Murray *et al.*, 1992; Katsuyama & Morgan, 1993). This discrepancy may be explained as follows. The first possibility is that these could be a problem with the selectivity of the protein kinase C inhibitors; some protein kinase C inhibitors inhibit kinases other than protein kinase C (Garland *et al.*, 1987; Jefferson & Schulman, 1988), resulting in non-selective inhibition of smooth muscle contractions. The second possibility is that different agonists may

activate different signal transduction pathways. Murray *et al.* (1992) reported that contractions induced by phorbol 12, 13-dibutyrate and 5-hydroxytryptamine but not those induced by prostaglandin $F_{2\alpha}$ were inhibited by protein kinase C inhibitors, sphingosine and H-7, in rat basilar artery. The third possibility is that these are tissue differences. Although the increases in Ca^{2+} sensitivity in rat aorta due to prostaglandin $F_{2\alpha}$ and endothelin-1 did not seem to be mediated by protein kinase C (present results), protein kinase C inhibitors selectively inhibited Ca^{2+} sensitization induced by prostaglandin $F_{2\alpha}$ and endothelin-1 in permeabilized rabbit mesenteric artery (Nishimura *et al.*, 1992), rat basilar artery (Murray *et al.*, 1992) and ferret aorta (Katsuyama & Morgan, 1993).

From these results, we suggest that there are two pathways of Ca^{2+} sensitization in smooth muscle, a protein kinase C-dependent pathway and a protein kinase C-independent pathway. Although we did not determine whether receptor agonists activate protein kinase C in rat aorta, present results suggest that this it not the case. Thus, it seems likely that phorbol esters selectively activate the protein kinase C-dependent pathways whereas receptor agonists selectively activate a protein kinase C-independent pathway in rat aorta. In other smooth muscles, however, some receptor agonists may generate enough diacylglycerol to activate protein kinase C to a level similar to that activated by phorbol esters and in turn also activate the protein kinase C-dependent pathway. Further studies are necessary to clarify the mechanisms of protein kinase C-dependent and protein kinase C-independent pathways of Ca^{2+} sensitization in smooth muscle.

We are grateful to Dr T. Okada (International Research Laboratories, CIBA-GEIGY Japan), Dr I. Kato (Chiba University), and Dojindo Laboratories (Kumamoto, Japan) for the generous gifts of endothelin-1, α-toxin and fura-2/AM. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

ANABUKI, J., HORI, M., OZAKI, H., KATO, I. & KARAKI, H. (1990). Mechanism of pinacidil-induced vasodilatation. *Eur. J. Pharmacol.*, **190**, 373–379.

BERRIDGE, M.J. (1984). Inositol triphosphate and diacylglycerol as second messengers. *Biochemical. J.*, **220**, 345–360.

GARLAND, L.G., BONSER, R.W. & THOMPSON, N.T. (1987). Protein kinase C inhibitors are not selective. *Trends Pharmacol. Sci.*, **8**, 334.

GONG, M.C., FUGLSANG, A., ALESSI, D., KOBAYASHI, S., COHEN, P., SOMLYO, A.V. & SOMLYO, A.P. (1992). Arachidonic acid inhibits myosin light chain phosphatase and sensitizes smooth muscle to calcium. *J. Biol. Chem.*, **267**, 21492–21498.

HALLER, H., SMALLWOOD, J.I. & RASMUSSEN, H. (1990). Protein kinase C translocation in intact vascular smooth muscle strips. *Biochem. J.*, **270**, 375–381.

HEASLIP, R.J. & SICKELS, B.D. (1989). Evidence that prostaglandins can contract the rat aorta via a novel protein kinase C-dependent mechanism. *J. Pharmacol. Exp. Ther.*, **250**, 44–51.

HORI, M., SATO, K., SAKATA, K., OZAKI, H., TAKANO-OHMURO, H., TSUCHIYA, T., SUGI, H., KATO, I. & KARAKI, H. (1992). Receptor agonists induce myosin phosphorylation-dependent and phosphorylation-independent contractions in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **261**, 506–512.

JEFFERSON, A.B. & SCHULMAN, H. (1988). Sphingosine inhibits calmodulin-dependent enzymes. *J. Biol. Chem.*, **263**, 15241–15244.

KARAKI, H. (1989). Ca^{2+} localization and sensitivity in vascular smooth muscle. *Trends Pharmacol. Sci.*, **10**, 320–325.

KARAKI, H., NAGASE, H., OZAKI, H., URAKAWA, N. & WEISS, G.B. (1986). Effects of the calcium channel facilitator, CGP28,382, on different modes of contraction in smooth muscle of rabbit and rat aortae and guinea-pig taenia caeci. *Br. J. Pharmacol.*, **89**, 423–429.

KATSUYAMA, H. & MORGAN, K.G. (1993). Mechanisms of Ca^{2+}-independent contraction in single permeabilized ferret aorta cells. *Circ. Res.*, **72**, 651–657.

KITAZAWA, T., GAYLINN, B.D., DENNY, G.H. & SOMLYO, A.P. (1991). G-protein mediated Ca^{2+} sensitization of smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.*, **266**, 1708–1715.

KITAZAWA, T., KOBAYASHI, S., HORIUCHI, K., SOMLYO, A.V. & SOMLYO, A.P. (1989). Receptor-coupled, permeabilized smooth muscle. *J. Biol. Chem.*, **264**, 5339–5342.

MURRAY, M.A., FARACI, F.M. & HEISTAD, D.D. (1992). Role of protein kinase C in constrictor responses of the rat basilar artery *in vivo*. *J. Physiol.*, **445**, 169–179.

NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTPγS increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.*, **157**, 677–683.

NISHIMURA, J. MORELAND, S., AHN, H.Y., KAWASE, T., MORELAND, R.S. & VAN BREEMEN, C. (1992). Endothelin increases myofilament Ca^{2+} sensitivity in α-toxin-permeabilized rabbit mesenteric artery. *Circ. Res.*, **71**, 951–959.

NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements by second messenger. *Biochem. Biophys. Res. Commun.*, **163**, 929–935.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature*, **308**, 693–698.

OISHI, K., MITA, M., ONO, T., HASIMOTO, T. & UCHIDA, M.K. (1992). Protein kinase C-independent sensitization of contractile proteins to Ca^{2+} in α-toxin-permeabilized smooth muscle cells from the guinea-pigs stomach. *Br. J. Pharmacol.*, **107**, 908–909.

REMBOLD, C.M. & MURPHY, R.A. (1988). $[Ca^{2+}]_i$-dependent myosin phosphorylation in phorbol ester stimulated smooth muscle contraction. *Am. J. Physiol.*, **255**, C719–723.

SAKATA, K., OZAKI, H., KWON, S.-C. & KARAKI, H. (1989). Effects of endothelin on the mechanical activity and cytosolic calcium levels of vascular types of smooth muscle. *Br. J. Pharmacol.*, **98**, 483–492.

SATO, K., HORI, M., OZAKI, H., TAKANO-OHMURO, H., TSUCHIYA, T., SUGI, H. & KARAKI, H. (1992). Myosin phosphorylation-independent contraction induced by phorbol ester in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **261**, 497–505.

SATO, K. & KARAKI, H. (1992). Does protein kinase C play an important role in the vascular contraction induced by receptor agonists? *Jpn. J. Pharmacol.*, **58**, 189P.

SATO, K., OZAKI, H. & KARAKI, H. (1988). Changes in cytosolic calcium level in vascular smooth muscle strips measured simultaneously with contraction using fluorescent calcium indicator Fura-2. *J. Pharmacol. Exp. Ther.*, **246**, 294–300.

SHIMAMOTO, H., SHIMAMOTO, Y., KWAN, C.-Y. & DANIEL, E.E. (1992). Participation of protein kinase C in endothelin-1-induced contraction in rat aorta: studies with new tool, calphostin C. *Br. J. Pharmacol.*, **107**, 282–287.

SINGER, H.A. (1990). Phorbol ester-induced stress and myosin light chain phosphorylation in swine medial smooth muscle. *J. Pharmacol. Exp. Ther.*, **252**, 1068–1074.

SOMYLO, A.P. & HIMPENS, B. (1989). Cell calcium and its regulation in smooth muscle. *FASEB J.*, **3**, 2266–2276.

STULL, J.T., HSU, L.-C., TANSEY, M.G. & KAMM, K.E. (1990). Myosin light chain kinase phosphorylation in tracheal smooth muscle. *J. Biol. Chem.*, **265**, 16683–16690.

SUEMATSU, E., RESNICK, M. & MORGAN, K.G. (1991). Change of Ca^{2+}-requirement for myosin phosphorylation by prostaglandin $F_{2\alpha}$. *Am. J. Physiol.*, **261**, C253–C258.

YOUNG, S., PARKER, P.J., ULLRICH, A. & STABEL, S. (1987). Down-regulation of protein kinase C is due to an increased rate of degradation. *Biochem. J.*, **244**, 775–779.

(Received June 1, 1993
Revised August 10, 1993
Accepted August 12, 1993)

Br. J. Pharmacol. (1993), **110**, 1532–1536 © Macmillan Press Ltd, 1993

Regional haemodynamic responses to intravenous and intraarterial endothelin-1 and big endothelin-1 in conscious rats

[1]S.M. Gardiner, P.A. Kemp & T. Bennett

Department of Physiology and Pharmacology, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 In the same chronically-instrumented conscious Long Evans rats, we assessed regional haemodynamic responses to i.v. and i.a. injections of endothelin-1 (ET-1) and big endothelin-1 at doses of 0.05 ($n = 4$) and 0.5 nmol kg^{-1} ($n = 7$).

2 For ET-1, the cardiovascular effects (initial hypotension and tachycardia with transient hindquarters vasodilatation, followed by hypertension, bradycardia and renal, mesenteric and hindquarters vasoconstrictions) of i.v. and i.a. injections were not different.

3 Similarly, for big ET-1, the cardiovascular effects (hypertension, bradycardia and renal, mesenteric, and hindquarters vasoconstrictions) of i.v. and i.a. injections were not different.

4 At a dose of 0.5 nmol kg^{-1}, i.v. or i.a., the pressor effects of ET-1 and big ET-1 were not different (area under curve [$AUC_{0-30\ min}$), ET-1 i.v. = 786 ± 115 mmHg min; ET-1 i.a. = 880 ± 165 mmHg min; big ET-1 i.v. = 878 ± 93 mmHg min; big ET-1 i.a. = 833 ± 103 mmHg min); but ET-1 caused greater renal, and lesser hindquarters, vasoconstrictions than big ET-1 (renal ET-1 i.v., AOC = 1550 ± 211% min; ET-1 i.a., AOC = 1746 ± 139% min; big ET-1 i.v., AOC = 1097 ± 128% min; big ET-1 i.a., AOC = 1041 ± 119% min; hindquarters, ET-1 i.v., AOC = 758 ± 176% min; ET-1 i.a. AOC = 787 ± 184% min; big ET-1 i.v., AOC = 1270 ± 88% min; big ET-1 i.a., AOC = 1173 ± 77% min), while the mesenteric vasoconstrictor effects of both peptides were not significantly different (ET-1 i.v. AOC = 1419 ± 132% min; ET-1 i.a., AOC = 1526 ± 172% min; big ET-1 i.v., AOC = 1099 ± 166% min; big ET-1 i.a., AOC = 1155 ± 120% min).

5 Under the conditions of our experiments, pulmonary clearance of ET-1, or pulmonary activation of big ET-1, was not apparent from the haemodynamic responses to i.v. and i.a. administration of the peptides. The results are consistent with previous findings indicating that local, rather than systemic, conversion of exogenous big ET-1 to ET-1 is responsible for its regional haemodynamic actions.

Keywords: Endothelin-1; big endothelin-1; pulmonary clearance; haemodynamics

Introduction

There is disagreement about the importance of pulmonary clearance for the elimination of endothelin-1 (ET-1) from the circulation. For example, in rats, guinea-pigs, rabbits and man, there is evidence for substantial pulmonary clearance of exogenous ET-1 (de Nucci *et al.*, 1988; Änggård *et al.*, 1989; Shiba *et al.*, 1989; Sirviö *et al.*, 1990; Rimar & Gillis, 1992; Gasic *et al.*, 1992; Wagner *et al.*, 1992). However, in other studies in cats, pigs, rabbits, dogs and man it was concluded that the lung was not important in the clearance of ET-1 (Lippton *et al.*, 1989; 1991; Pernow *et al.*, 1989; 1990; Emmeluth & Bie, 1992; Ray *et al.*, 1992). While, to some extent, these disparities may be attributable to species differences, it is also feasible that differences in experimental procedures have contributed to the opposing findings. Thus, as pointed out by Sirviö *et al.* (1990), infusion of ET-1 could saturate pulmonary clearance mechanisms and thereby produce results indicating that pulmonary clearance of ET-1 was unimportant, whereas administration of ET-1 by bolus injection could lead to the opposite conclusion.

It is also possible that pharmacokinetic data do not relate directly to the functional effects of ET-1 *in vivo*, considering the multiplicity of factors responsible for the regional haemodynamic actions of ET-1 (de Nucci *et al.*, 1988; Gardiner *et al.*, 1989; 1990; Ohlstein *et al.*, 1990). However, in the original study of de Nucci *et al.* (1988), it was found that ET-1 (0.2–2.2 nmol kg^{-1}), given to anaesthetized or pithed rats, had greater effects on systemic arterial blood pressure when the bolus injection was into the left ventricle rather than the right atrium, consistent with the effects of ET-1 being limited by its removal in the pulmonary circulation. Since anaesthesia or pithing have profound effects on haemodynamic status, we considered it important to determine if i.a. and i.v. bolus injections of ET-1 had different effects in conscious rats. Moreover, in order to assess more fully the effects of ET-1 in these experiments, we monitored changes in renal, mesenteric and hindquarters blood flows, in addition to systemic arterial blood pressure and heart rate.

There is good evidence that the haemodynamic effects of big ET-1 *in vivo* are due to its conversion to ET-1 through the action of a phosphoramidon-sensitive enzyme (Fukuroda *et al.*, 1990; Matsumara *et al.*, 1990; Gardiner *et al.*, 1991; McMahon *et al.*, 1991). Previous studies have indicated that local, rather than pulmonary, conversion of big ET-1 to ET-1 might be responsible for the *in vivo* regional haemodynamic effects of big ET-1 (Gardiner *et al.*, 1991; 1992c). However, no data are available regarding the comparative regional haemodynamic effects of i.a. and i.v. bolus injections of big ET-1. Therefore, the second objective of the present study was to provide such data. Some of this work has been presented to the British Pharmacological Society (Gardiner *et al.*, 1992b).

Methods

All experiments were carried out on male, Long Evans rats (400–450 g) bred in the Medical School Biomedical Services Unit. Animals were anaesthetized (sodium methohexitone, Brietal, Lilly; 40–60 mg kg^{-1}, i.p., supplemented as required) and had pulsed Doppler probes (Haywood *et al.*, 1981) implanted to monitor renal, mesenteric and hindquarters

[1] Author for correspondence.

blood flows (Gardiner *et al.*, 1991; 1992a,c). At least 7 days later, animals were anaesthetized (sodium methohexitone, 40 mg kg^{-1}, i.p.) and had catheters implanted in the left carotid artery and right jugular vein (for peptide administration), and in the distal abdominal aorta, via the ventral caudal artery (for recording arterial blood pressure and heart rate). The tip of the catheter inserted into the left carotid artery was advanced 3 cm caudally to lie close to the aortic arch, such that injections were delivered into the systemic arterial circulation. Animals were given free access to food and water in their individual home cages, with the monitoring systems arranged as described in detail previously (Gardiner *et al.*, 1991; 1992a,c) to allow measurements to be made without restraining or disturbing the animals. Experiments were not begun until at least 1 day after catheters had been implanted. Animals ($n = 7$) were randomized to receive (at intervals of about 2 h) i.a. and i.v. bolus injections of ET-1 and big ET-1 (0.5 nmol kg^{-1}). This dose was chosen since it gave reproducible responses, and was within the dose-range used by de Nucci *et al.* (1988). Moreover, in preliminary studies we found this dose of ET-1 and big ET-1 had well-matched pressor effects. From the results of these experiments (see Results) we found responses to i.a. and i.v. injections were not different, raising the possibility that the 0.5 nmol kg^{-1} dose of ET-1 or big ET-1 might have been saturating any putative pulmonary clearance or activating mechanisms, respectively. Therefore, in a subsidiary experiment we followed the protocol as above, but giving ET-1 and big ET-1 at a 10 fold lower dose, i.e., 0.05 nmol kg^{-1}, i.a. and i.v. in 4 Long Evans rats.

Data analysis

In each animal, variables were measured immediately before and at 0.25, 0.5, 0.75, 1, 5, 10, 15, 20, 25 and 30 min after ET-1 at a dose of 0.5 nmol kg^{-1}; in those animals given ET-1 at 0.05 nmol kg^{-1} measurements were not made beyond 15 min post-injection because variables were back to baseline by then. For big ET-1, measurements were made as for ET-1, except the first post-injection was at 1 min since there were no rapid onset responses to big ET-1.

At each time point, measurements were made of mean arterial blood pressure and heart rate and mean renal, mesenteric and hindquarters Doppler shift signals. Percentage changes in the latter were taken as indices of flow changes; % changes in regional vascular conductances were calculated from the mean Doppler shift and the mean blood pressure values (Gardiner *et al.*, 1991; 1992a,c).

Responses to ET-1 or big ET-1 were compared to baseline values using Friedman's test. Comparison of responses to i.v. and i.a. administration of ET-1 or of big ET-1 was by Wilcoxon's test applied to integrated responses (areas under or over curves, AUC or AOC respectively). Comparison of responses to i.v. ET-1 and big ET-1 and i.a. ET-1 and big ET-1 was performed in a similar way. A P value < 0.05 was taken as significant.

Peptides

Human ET-1 and big ET-1 (1–38) were obtained from Bachem (UK); the peptides were dissolved in distilled water and diluted with sterile isotonic saline (154 mmol l^{-1} NaCl) containing 1% bovine serum albumin (Sigma U.K.). Bolus injections were given in 0.1 ml and flushed in with 0.1 ml isotonic saline. Administration of saline alone in these volumes had no consistent haemodynamic effects.

Results

Resting values for cardiovascular variables prior to each of the 4 experimental procedures are shown in Table 1.

Responses to i.v. and i.a. administration of ET-1

Bolus i.v. injection of ET-1 (0.5 nmol kg^{-1}) caused an initial hypotension and tachycardia, accompanied by a marked increase in hindquarters flow and vascular conductance (Figure 1). Renal flow and vascular conductance fell, but the initial response in the mesenteric vascular bed was variable (Figure 1). Thereafter, blood pressure rose and there was a bradycardia; renal flow and vascular conductance remained significantly reduced and the reductions in mesenteric flow and vascular conductance became more prominent (Figure 1). Although the transient hindquarters vasodilatation gave way to a vasoconstriction, this was not accompanied by a significant reduction in hindquarters flow (Figure 1).

The pattern and magnitude of the responses to i.a. injection of ET-1 (0.5 nmol kg^{-1}) were not significantly different from those to i.v. administration (Figure 1, Table 2). Responses to i.v. and i.a. injection of ET-1 at a dose of 0.05 nmol kg^{-1} also were not consistently different (Table 3).

Responses to i.v. and i.a. administration of big ET-1

Bolus i.v. injection of big ET-1 (0.5 nmol kg^{-1}) caused a gradual rise in blood pressure accompanied by reductions in heart rate and in renal, mesenteric and hindquarters flows and vascular conductances (Figure 1).

Injection of big ET-1 at the same dose i.a. had effects that were not significantly different from those seen with the i.v. injection (Figure 1, Table 2).

Injection of big ET-1 at a dose of 0.05 nmol kg^{-1} showed no consistent differences between the responses to the i.v. and i.a. route of administration (Table 3).

Comparision of responses to ET-1 and big ET-1 given i.v. or i.a.

With either route of administration, at a dose of 0.5 nmol kg^{-1}, big ET-1 did not elicit the initial hypotension, tachycardia or hyperaemic hindquarters vasodilatation seen with ET-1 (Figure 1). However, the subsequent pressor effects of ET-1

Table 1 Resting cardiovascular variables in the same conscious Long Evans rats ($n = 7$) prior to i.v. or i.a. administration of endothelin-1 or big endothelin-1

	Endothelin-1		*Big endothelin-1*	
	i.v.	*i.a.*	*i.v.*	*i.a.*
Heart rate (beats min^{-1})	322 ± 10	329 ± 14	316 ± 7	332 ± 10
Mean blood pressure (mmHg)	107 ± 4	103 ± 3	108 ± 4	108 ± 2
Renal Doppler shift (kHz)	6.2 ± 0.6	6.9 ± 0.6	6.0 ± 0.5	5.7 ± 0.6
Mesenteric Doppler shift (kHz)	5.1 ± 0.6	5.2 ± 0.3	4.7 ± 0.4	4.4 ± 0.4
Hindquarters Doppler shift (kHz)	3.7 ± 0.4	3.7 ± 0.4	3.8 ± 0.4	3.9 ± 0.5
Renal vascular conductance ([kHz $mmHg^{-1}$]10^3)	58 ± 6	66 ± 5	56 ± 6	53 ± 6
Mesenteric vascular conductance ([kHz $mmHg^{-1}$]10^3)	48 ± 6	50 ± 3	44 ± 5	40 ± 3
Hindquarters vascular conductance ([kHz $mmHg^{-1}$]10^3)	35 ± 5	36 ± 4	36 ± 4	36 ± 5

Values are mean ± s.e.mean.

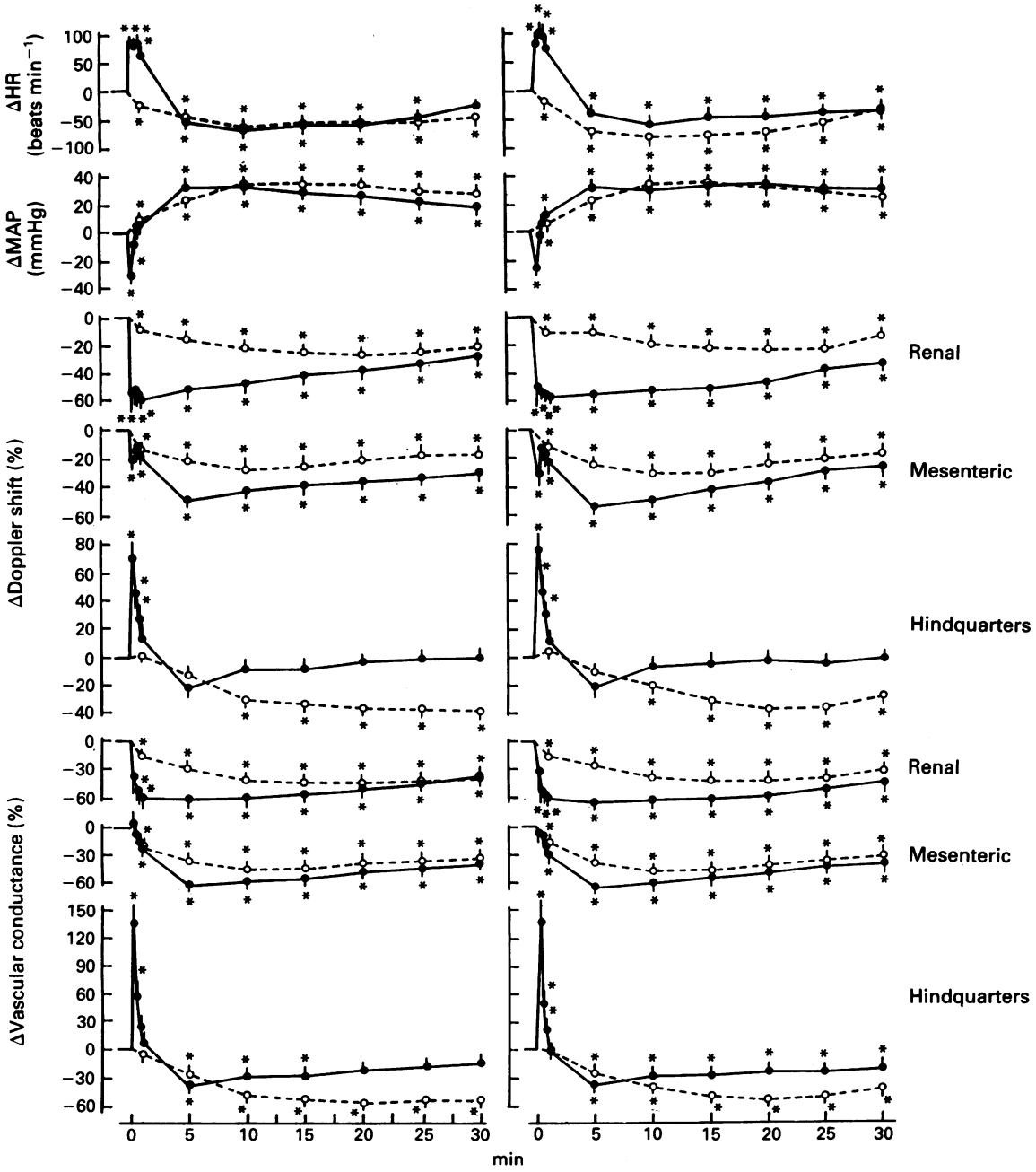


Figure 1 Cardiovascular changes in response to i.v. (left-hand panels) or i.a. (right hand panels) injections of 0.5 nmol kg^{-1} endothelin-1 (●) or big endothelin-1 (○) in the same conscious Long Evans rats ($n = 7$). Values are mean ± s.e.mean; $*P < 0.05$ versus pre-injection baseline.

Table 2 Integrated (areas under or over $curves_{0-30\ min}$) changes elicited by i.v. or i.a. injections of endothelin-1 or big-endothelin-1 (0.5 nmol kg^{-1}) in the same conscious, Long Evans rats ($n = 7$)

	Endothelin-1		*Big endothelin-1*	
	i.v.	*i.a.*	*i.v.*	*i.a.*
Heart rate (AUC, beats)	+ 258 ± 69	+ 273 ± 27	–	–
Heart rate (AOC, beats)	– 1487 ± 126	– 1279 ± 217	– 1569 ± 108	– 2015 ± 269*
Mean blood pressure (AOC, mmHg min)	– 15 ± 2	– 11 ± 1	–	–
Mean blood pressure (AUC, mmHg min)	+ 786 ± 115	+ 880 ± 165	+ 878 ± 93	+ 833 ± 103
Renal flow (AOC, % min)	– 1247 ± 218	– 1427 ± 114	– 620 ± 116	– 582 ± 104
Mesenteric flow (AOC, % min)	– 1099 ± 131	– 1184 ± 144	– 637 ± 156	– 720 ± 119
Hindquarters flow (AUC, % min)	+ 202 ± 101	+ 184 ± 48	–	–
Hindquarters flow (AOC, % min)	– 341 ± 112	– 356 ± 144	– 849 ± 109	– 783 ± 68
Renal conductance (AOC, % min)	– 1550 ± 211	– 1746 ± 139	– 1097 ± 128*	– 1041 ± 119*
Mesenteric conductance (AOC, % min)	– 1419 ± 132	– 1526 ± 172	– 1099 ± 166	– 1155 ± 120
Hindquarters conductance (AUC, % min)	+ 134 ± 73	+ 82 ± 17	–	–
Hindquarters conductance (AOC, % min)	– 758 ± 176	– 787 ± 184	– 1270 ± 88*	– 1173 ± 77*

Values are mean ± s.e.mean. The heart rate, mean blood pressure and hindquarters haemodynamics showed biphasic responses to endothelin-1 but not to big endothelin-1.
$*P < 0.05$ versus corresponding values for endothelin-1; flow changes were not compared.

Table 3 Maximum cardiovascular changes following i.v. or i.a. injection of endothelin-1 or big endothelin-1 at 0.05 nmol kg^{-1} in the same conscious Long Evans rats ($n = 4$)

	Endothelin-1		*Big endothelin-1*	
	i.v.	*i.a.*	*i.v.*	*i.a.*
Δ Heart rate (beats min^{-1})	88 ± 13	79 ± 11	–	–
Δ Heart rate (beats min^{-1})	− 11 ± 21	− 23 ± 9	8 ± 9	− 1 ± 9
Δ Blood pressure (mmHg)	− 14 ± 5	− 14 ± 2	–	–
Δ Blood pressure (mmHg)	8 ± 1	8 ± 4	4 ± 1	2 ± 1
Δ Renal conductance (%)	− 22 ± 3	− 26 ± 3	− 5 ± 1	− 5 ± 2
Δ Mesenteric conductance (%)	− 34 ± 5	− 43 ± 4	− 11 ± 2	− 11 ± 7
Δ Hindquarters conductance (%)	86 ± 27	95 ± 12	–	–
Δ Hindquarters conductance (%)	− 16 ± 2	− 15 ± 5	− 4 ± 5	− 6 ± 2

Values are mean ± s.e.mean.

and big ET-1 were the same, although ET-1 caused a significantly greater renal vasoconstriction than big ET-1, while the latter caused a significantly greater hindquarters vasoconstriction than ET-1 (Figure 1, Table 2).

Discussion

In the present work, on conscious rats, we have not been able to confirm the earlier observations of de Nucci *et al.* (1988), showing that administration of ET-1, in anaesthetized or pithed rats, had more marked effects on arterial blood pressure when the peptide was administered into the arterial side of the circulation than when it was administered into the venous side. Clearly, there were several differences between the protocols that could account for the disparate findings. We gave injections into the left carotid artery or right jugular vein, rather than into the cardiac chambers, primarily because animals do not recover well if they have had a catheter introduced into the left ventricle through the aortic valve (Gardiner, Kemp & Bennett, unpublished observations). Indeed, such a procedure could render the aortic valve incompetent, and under those conditions it is feasible that the pulmonary circulation would be affected in such a way that the pulmonary clearance of ET-1 was increased. It is also possible that anaesthesia or pithing affects lung perfusion in such a way that the pulmonary clearance of ET-1 is greater than in the conscious state, thereby accounting for the difference between our results and those of de Nucci *et al.* (1988). Since the highest dose of ET-1 we used was substantially less than the highest dose used by de Nucci *et al.* (1988), it is unlikely that our bolus injections of ET-1 were saturating pulmonary clearance mechanisms, and thereby masking their effects (Sirviö *et al.*, 1990), unless the pharmacokinetics of ET-1 differ markedly in conscious and in anaesthetized or pithed, rats. While it appears that administration of radioiodinated ET-1 in bolus doses as low as 0.2 pmol (Änggård *et al.*, 1989; Sirviö *et al.*, 1990) demonstrates selective pulmonary clearance, the functional significance of this is unclear, because ET-1 in such a low dose has no measurable haemodynamic effects *in vivo* (Gardiner, Kemp & Bennett, unpublished observations).

The haemodynamic responses to ET-1 *in vivo* involve a variety of mechanisms, including, vasoconstrictor and vasodilator processes (de Nucci *et al.*, 1988; Gardiner *et al.*, 1989; Ohlstein *et al.*, 1990), although the relative contributions of prostanoids and nitric oxide are contentious (de Nucci *et al.*, 1988; Gardiner *et al.*, 1989; 1990; Ohlstein *et al.*, 1990). de Nucci *et al.* (1988) found that the initial depressor response to ET-1 was greater when the injection was into the left ventricle than when it was into the right atrium, but it is possible that this was due to a fall in cardiac output in the former condition (consequent upon coronary vasoconstriction) in contrast to the increase in cardiac output seen with i.v. injection of ET-1 (Gardiner *et al.*, 1990). If i.a. injection of ET-1 caused more effective activation of vasodilator mechanisms than i.v. injection of ET-1, this could have influenced our ability to detect differences in the subsequent pressor and vasoconstrictor effects of ET-1. However, there was no evidence of any significant difference between the initial depressor, tachycardic or regional haemodynamic effects of the peptide administered by either route. If anything, the nadir of the depressor response to i.a. ET-1 (− 25 ± 3 mmHg) was slightly less than that to i.v. ET-1 (− 30 ± 3 mmHg), which would be consistent with more effective release of vasodilators from the lungs (de Nucci *et al.*, 1988) by the i.v. injections of ET-1.

We found that i.v. and i.a. injections of big ET-1 elicited pressor and bradycardic effects, and reductions in renal, mesenteric and hindquarters flows and vascular conductances that were not significantly different. Assuming that the regional haemodynamic effects of big ET-1 *in vivo* are due to its conversion to ET-1 (Gardiner *et al.*, 1991; 1992c) these results indicate that the pulmonary vascular bed may not play a predominant role in this process. This finding is consistent with the proposal that local, rather than systemic, conversion of big ET-1 to ET-1 is responsible for its regional haemodynamic effects (Gardiner *et al.*, 1991; 1992c).

From previous work it appears that, at a dose of 0.1 nmol kg^{-1}, i.v. injection of ET-1 has more marked pressor and haemodynamic effects than i.v. injection of big ET-1 (Gardiner *et al.*, 1992c). However, in the present work we found that, at a dose of 0.5 nmol kg^{-1}, the pressor effects of ET-1 and big ET-1 were very similar, whether injected i.v. or i.a., but the regional haemodynamic profiles differed significantly. Apart from the absence of an initial hyperaemic vasodilator response to big ET-1, which is a dose-related phenomenon and has been discussed previously (Gardiner *et al.*, 1991), it appeared that ET-1 caused more marked renal, but less marked hindquarters, vasoconstriction than did big ET-1, whereas the mesenteric vasoconstrictor effects of the two peptides were not significantly different. These findings are consistent with our previous observations, which we interpreted as showing that the conversion of big ET-1 was particularly effective in the mesenteric vascular bed, and the local conversion of big ET-1 in the hindquarters vascular bed was capable of producing substantial vasoconstriction without triggering vasodilator processes (Gardiner *et al.*, 1991).

Clearly, since we did not measure plasma levels of ET-1 or big ET-1, we cannot conclude that the pulmonary vasculature is not involved in the clearance of ET-1, or in the conversion of big ET-1 to ET-1. But we think it is fair to say that any such putative pulmonary mechanisms are not reflected in measurable differences between the haemodynamic responses to i.v. and i.a. administration of ET-1 or big ET-1 in conscious rats.

References

ÅNGGÅRD, E., GALTON, S., RAE, G., THOMAS, R., MCLOUGHLIN, L., DE NUCCI, G. & VANE, J.R. (1989). The fate of radioiodinated endothelin-1 and endothelin-3 in the rat. *J. Cardiovasc. Pharmacol.*, **13**, S46–S49.

DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9797–9800.

EMMELUTH, C. & BIE, P. (1992). Effects, release and disposal of endothelin-1 in conscious dogs. *Acta Physiol. Scand.*, **146**, 197–204.

FUKURODA, T., NOGUCHI, K., TSUCHIDA, S., NISHIKIBE, M., IKEMOTO, F., OKADA, K. & YANO, M. (1990). Inhibition of biological actions of big endothelin-1 by phosphoramidon. *Biochem. Biophys. Res. Commun.*, **172**, 390–395.

GARDINER, S.M., COMPTON, A.M. & BENNETT, T. (1990). Effects of indomethacin on the regional haemodynamic responses to low doses of endothelins and sarafotoxin. *Br. J. Pharmacol.*, **100**, 158–162.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1989). N^G-monomethyl-L-arginine does not inhibit the hindquarters vasodilator action of endothelin-1 in conscious rats. *Eur. J. Pharmacol.*, **171**, 237–240.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990). Cardiac output effects of endothelin-1, -2 and -3 and sarafotoxin S6b in conscious rats. *J. Autonom. Nerv. Syst.*, **30**, 143–148.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1991). The effects of phosphoramidon on the regional haemodynamic responses to human proendothelin [1–38] in conscious rats. *Br. J. Pharmacol.*, **103**, 2009–2015.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1992a). Inhibition by phosphoramidon of the regional haemodynamic effects of proendothelin-2 and -3 in conscious rats. *Br. J. Pharmacol.*, **103**, 2009–2015.

GARDINER, S.M., KEMP, P.A. & BENNETT, T. (1992b). Regional haemodynamic responses to i.a. and i.v. injection of endothelin-1 and proendothelin-1 (1–38) in conscious rats. *Br. J. Pharmacol.*, **107**, 414P.

GARDINER, S.M., KEMP, P.A., COMPTON, A.M. & BENNETT, T. (1992c). Coeliac haemodynamic effects of endothelin-1, endothelin-3, proendothelin-1 [1–38] and proendothelin-3 [1–41] in conscious rats. *Br. J. Pharmacol,*. **106**, 483–488.

GASIC, S., WAGNER, O.F., VIERHAPPER, H., NOWOTNY, P. & WALDHÄUSL, W. (1992). Regional haemodynamic effects and clearance of endothelin-1 in humans: renal and peripheral tissues may contribute to the overall disposal of the peptide. *J. Cardiovasc. Pharmacol.*, **19**, 176–180.

LIPPTON, H.L., HAUTH, T.A., SUMMER, W.R. & HYMAN, A.L. (1989). Endothelin produces pulmonary vasoconstriction and systemic vasodilation. *J. Appl. Physiol.*, **66**, 1008–1012.

LIPPTON, H.L., OHLSTEIN, E.H., SUMMER, W.R. & HYMAN, A.L. (1991). Analysis of responses to endothelins in the rabbit pulmonary and systemic vascular beds. *J. Appl. Physiol.*, **70**, 331–341.

MATSUMARA, Y., HISAKI, K., TAKAOKA, M. & MORIMOTO, S. (1990). Phosphoramidon, a metalloproteinase inhibitor, suppresses the hypertensive effect of big endothelin-1. *Eur. J. Pharmacol.*, **185**, 103–106.

MCMAHON, E.G., PALOMO, M.A., MOORE, W.M., MCDONALD, J.F. & STERN, M.K. (1991). Phosphoramidon blocks the pressor activity of porcine big endothelin-1-[1–39] *in vivo* and conversion of big endothelin-1-[1–39] to endothelin-1-[1–21] *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 703–707.

OHLSTEIN, E.H., VICKERY, L., SAUERMELCH, C. & WILLETTE, R.N. (1990). Vasodilation induced by endothelin: role of EDRF and prostanoids in rat hindquarters. *Am. J. Physiol.*, **259**, H1835–H1841.

PERNOW, J., HEMSÉN, A. & LUNDBERG, J.M. (1989). Tissue specific distribution, clearance and vascular effects of endothelin in the pig. *Biochem. Biophys. Res. Commun,*. **161**, 647–653.

PERNOW, J., HEMSÉN, A., HALLÉN, A. & LUNDBERG, J.M. (1990). Vascular effects, formation, clearance and release of endothelin peptides in the pig. *Neurochem. Int.*, **18**, 515–518.

RAY, S.G., MCMURRAY, J.J., MORTON, J.J. & DARGIE, H.J. (1992). Circulating endothelin is not extracted by the pulmonary circulation in man. *Chest*, **102**, 1143–1144.

RIMAR, S. & GILLIS, C.N. (1992). Differential uptake of endothelin-1 by the coronary and pulmonary circulations. *J. Appl. Physiol.*, **73**, 557–562.

SHIBA, R., YANAGISAWA, M., MIYAUCHI, T., ISHII, Y., KIMURA, S., UCHIYAMA, Y., MASAKI, T. & GOTO, K. (1989). Elimination of intravenously injected endothelin-1 from the circulation of the rat. *J. Cardiovasc. Pharmacol.*, **13**, S98–S101.

SIRVIÖ, M.-L., METSÄRINNE, K., SAIJONMAA, O. & FYHRQUIST, F. (1990). Tissue distribution and half-life of ^{125}I-endothelin in the rat: importance of pulmonary clearance. *Biochem. Biophys. Res. Commun.*, **167**, 1191–1195.

WAGNER, O.F., VIERHAPPER, H., GASIC, S., NOWOTNY, P. & WALDHÄUSL, W. (1992). Regional effects and clearance of endothelin-1 across pulmonary and splanchnic circulation. *Eur. J. Clin. Invest.*, **22**, 277–282.

(Received June 16, 1993
Revised August 10, 1993
Accepted August 12, 1993)

Br. J. Pharmacol. (1993), **110**, 1537–1543 © Macmillan Press Ltd, 1993

Relevance of the use of [^{3}H]-clonidine to identify imidazoline receptors in the rabbit brainstem

[1]G. Bricca, *J. Zhang, H. Greney, M. Dontenwill, J. Stutzmann, A. Belcourt & P. Bousquet

Laboratoire de Pharmacologie Cardiovasculaire et Rénale, CNRS URA 589, Faculté de Médecine, Université Louis Pasteur, 11 rue Humann, 67000 Strasbourg, France and *Department of Pharmacotherapy, University of Amsterdam, Academic Medical Centre, 1105 AZ Amsterdam, The Netherlands

1 [^{3}H]-clonidine binding was investigated in membranes isolated from the ventral medulla oblongata of the rabbit where clonidine produced a hypotensive effect which was not mediated by adrenoceptors. [^{3}H]-clonidine specific binding, as defined by the difference between the binding of [^{3}H]-clonidine in the presence and in the absence of 10 μM cirazoline, occurred at two sites: a high affinity site with a $K_D = 2.9 \pm 0.7$ nM and a B_{max} of 40 ± 8 fmol mg^{-1} protein and a low affinity site with a $K_D = 18.2 \pm 0.4$ nM and a B_{max} of 66 ± 14 fmol mg^{-1} protein.

2 The high affinity sites being catecholamine-sensitive were identified as α_2-adrenoceptors. The low affinity binding of [^{3}H]-clonidine was insensitive to catecholamines, as well as to other α_2-adrenoceptor specific probes, and could be inhibited with high affinity only by compounds which lowered blood pressure when directly injected in the nucleus reticularis lateralis of the ventral brainstem, or by antagonists.

3 It was concluded that in the ventral medulla of the rabbit, [^{3}H]-clonidine labelled α_2-adrenoceptors and imidazoline receptors (IRs). Only the latter were related to the hypotensive effects of clonidine and rilmenidine directly injected into the rostroventrolateral medulla oblongata (RVLM) of the rabbit. The methodological problems regarding the study of IRs with [^{3}H]-clonidine are discussed.

Keywords: Imidazoline receptor; clonidine; binding; nucleus reticularis lateralis

Introduction

The main site for the vasodepressor response to clonidine and related compounds has been located within the medullary nucleus reticularis lateralis (NRL) of the rostroventrolateral medulla (RVLM) region of various species (Bousquet *et al.*, 1981; Wolf & Mohrland, 1984; Ernsberger *et al.*, 1990). When stereotaxically injected in this region, the imidazolines proved hypotensive whereas catecholamines and phenylethylamines did not influence the arterial blood pressure whatever their selectivity for the different subtypes of α-adrenoceptors. Thus it has been suggested that the hypotensive effect of clonidine-like compounds was mediated via imidazoline receptors (IRs) (Bousquet *et al.*, 1984).

Since the existence of IRs was first proposed, numerous biochemical data have confirmed the existence of specific binding sites which could account for the properties of imidazolines. The presence of IRs was detected in the bovine and human ventrolateral medulla with [^{3}H]-*p*-aminoclonidine and [^{3}H]-clonidine (Meeley *et al.*, 1986; Bricca *et al.*, 1988) and in the rabbit kidney and forebrain (Hamilton *et al.*, 1991). Idazoxan, also an antagonist of the effect of clonidine-like drugs on IRs, has been reported to label non-catecholamine binding sites both in the brain and in various peripheral tissues of several species. Such sites were named IGRS, IRs and NAIBS (Bousquet *et al.*, 1984; Coupry *et al.*, 1987; Hamilton *et al.*, 1988; Yablonsky *et al.*, 1988; Langin & Lafontan, 1989; Michel *et al.*, 1989; Wikberg *et al.*, 1991).

Recently, we observed that the rabbit was very sensitive to the hypotensive effect of clonidine and rilmenidine, a compound structurally related to imidazolines (Feldman *et al.*, 1990). When stereotaxically injected into the rabbit NRL area, clonidine and rilmenidine exhibited potent hypotensive actions whereas noradrenaline had no influence on blood pressure, confirming that, as in the cat, the central hypotensive effect of clonidine and clonidine-like drugs was related to their interaction with IRs in the rabbit NRL area (Feldman *et al.*, 1990). Imidazoline binding sites have already been detected in the rabbit forebrain (Hamilton *et al.*, 1988; 1991; Yakubu *et al.*, 1988); but IRs have not yet been investigated in the rabbit RVLM where clonidine and related compounds induced their hypotensive effect. Moreover, clonidine and rilmenidine displayed low if no affinity for [^{3}H]-idazoxan labelled imidazoline specific binding sites (Hamilton *et al.*, 1991). This led various authors to suggest that [^{3}H]-idazoxan and [^{3}H]-clonidine labelled different classes of IRs (Tesson & Parini, 1991; Wikberg *et al.*, 1991). Therefore it was of importance to characterize IRs in the rabbit NRL region of the medulla oblongata, with [^{3}H]-clonidine, the reference hypotensive drug.

Clonidine was the prototype of the imidazoline substances inducing hypotension originating within the RVLM of the brainstem. The study of the mechanism of action of that drug allowed the development of the IR concept. Yet, there were only few studies in which this radiolabelled substance was used as a probe for the specific analysis of these receptors. Indeed, numerous binding studies have been performed with [^{3}H]-clonidine but to label α_2-adrenoceptors (U'Prichard, 1981). Previously, we used this tritiated substance in various binding studies performed on bovine and human brain membrane preparations (Bricca *et al.*, 1988; 1989a). However, because preliminary studies showed that α_2-adrenoceptors and IRs co-existed within the RVLM, we aimed to characterize precisely the properties of that binding in a species particularly sensitive to the hypotensive effects of clonidine and analogous substances. We present here the characteristics of [^{3}H]-clonidine binding in rabbit RVLM membrane preparations.

Methods

Tissues

Brains from ZYKA strain rabbits were obtained from a local slaughterhouse. The animals were killed by electric shock. The brains were rapidly removed and the brainstem isolated

[1] Author for correspondence.

by trans-section at the rostral and caudal limits of the ponto-medullary junction and the pyramidal decussatio. Samples were flash frozen in liquid nitrogen, transported on dry ice to the laboratory and stored up to 3 weeks without damage at − 20°C until membranes were prepared.

The pia mater was removed from the ventral medullary surface and the ventral part of the medulla, including the NRL region, isolated by discarding the dorsal part of the medulla.

Membrane preparation

Membranes were prepared essentially as described previously (Bricca *et al.*, 1989a) from at least 20 pooled brainstems. All steps were performed at 4°C unless otherwise specified. The tissues were homogenized for 30 s in 20 volumes (w/v) of ice-cold buffer (50 mM Tris-HCl, 5 mM EDTA, pH = 7.5) with a polytron PT12. The homogenate was centrifuged for 10 min at 500 *g*. The first sediment was discarded and the supernatant was centrifuged for 12 min at 45,000 *g*. The resulting pellets (P2) were washed twice with 50 mM ice-cold Tris-HCl buffer (pH = 7.5) and recentrifuged as above. To allow the dissociation of endogenous ligands, membranes were incubated at 25°C for 30 min between the two washes. The P2 pellets were stored for a maximum of 10 days at − 20°C before use.

Binding assay

Binding tests, run in duplicate at 25°C, were performed in a final volume of 1 ml containing 0.2 to 0.6 mg protein, [^{3}H]-clonidine and competitors in variable concentrations. The binding buffer was: 50 mM Tris-HCl, 1 mM $MgCl_2$, pH = 7.5. Kinetic experiments were carried out in the presence of 6 nM [^{3}H]-clonidine. Once equilibrium was reached, dissociation was measured at various time intervals after the addition of unlabelled clonidine to a final concentration of 10 μM. In equilibrium experiments samples were incubated for 45 min. Saturation curves were constructed with data obtained with 10 to 12 different concentrations of [^{3}H]-clonidine ranging from 0.1 to 38 nM. Competition experiments were performed with 5 to 6 nM [^{3}H]-clonidine and 9–11 different concentrations of each competitor. In all experiments involving catecholamines, 0.005% ascorbic acid was present. Non specific binding was determined with 10 μM cirazoline. In saturation experiments, two measurements of the non specific binding were performed for each concentration of [^{3}H]-clonidine, either with 10 μM (−)-noradrenaline or with 10 μM cirazoline. Whether the binding studies were performed immediately after membrane preparation or after storage of the pellets, the results were superimposable.

Protein assay

Protein concentrations were determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Calculation and statistical analysis

Data were analysed by linear regression and iterative computerized non-linear curve fitting (Munson & Rodbard, 1980; McPherson, 1985). IC_{50} and slopes were obtained by non linear curve fitting of the experimental data to a sigmoidal curve by means of the EBDA programme. Saturation curves were analysed according to Scatchard (1949) and by non-linear curve fitting with the aid of the LIGAND programme (Munson & Rodbard, 1980). Student's paired *t* test was used to compare mean K_D and B_{max} values. Kinetic constants were obtained through the KINETIC programme (McPherson, 1985).

Chemicals

[^{3}H]-clonidine (specific activity 48 to 61 Ci mmol^{-1}) was obtained from New England Nuclear. (−)-Adrenaline, (−)-noradrenaline, clonidine, dopamine, histamine, 5-hydroxytryptamine, tolazoline and yohimbine were purchased from Sigma. Rilmenidine, (±)-idazoxan and (−)-idazoxan were from Servier. Cirazoline was from LERS-Synthélabo, BDF-6143 (4-chloro-2-(2-imidazoline-2-yl-amino-isoindole)HCl) and moxonidine from Beirsdorf, UK-14304 (bromoxidine) was from Pfizer, phentolamine from Ciba-Geigy, oxymetazoline and α-methylnoradrenaline from Farmex, guanfacine from Sandoz, guanoxabenz from Roussel-Uclaf, B-HT 920 (5-allyl-2-amino-5,6,7,8-tetrahydrothiazolo-[4,5-d]azépine diHCL) from Karl Thomae GMBH and phenoxybenzamine from Röhm Pharma.

Results

[^{3}H]-clonidine binding kinetics

[^{3}H]-clonidine specific binding, determined with cirazoline, reached equilibrium within 30 min in association experiments and was stable for at least 2 h (Figure 1). The specific binding was completely reversed by addition of unlabelled clonidine. Using the KINETIC programme of McPherson (1985), the data fitted a bi-exponential model ($P < 0.01$) in both association and dissociation experiments (Table 1). The K_D values calculated for [^{3}H]-clonidine binding from kinetic constants were in good agreement with the data obtained from the equilibrium experiments (Tables 1 and 2).

Saturation of [^{3}H]-clonidine binding

[^{3}H]-clonidine specific binding was saturable whether the non specific binding was measured in the presence of 10 μM noradrenaline or cirazoline (Figure 2a). The non specific binding of 7 nM [^{3}H]-clonidine determined in the presence of either 10 μM noradrenaline or 10 μM cirazoline represented 56 ± 3% and 27 ± 4% respectively of the total binding. Cirazoline alone or a mixture of cirazoline and noradrenaline inhibited the [^{3}H]-clonidine binding to the same extent indicating that noradrenaline-sensitive sites were part of the cirazoline-sensitive population. These data confirmed that, under our experimental conditions, 10 μM cirazoline inhibited [^{3}H]-clonidine binding to both noradrenaline-sensitive and insensitive sites. [^{3}H]-clonidine binding sites sensitive to (−)-noradrenaline will be referred to as α-adrenoceptor binding and those insensitive to (−)-noradrenaline and sensitive to

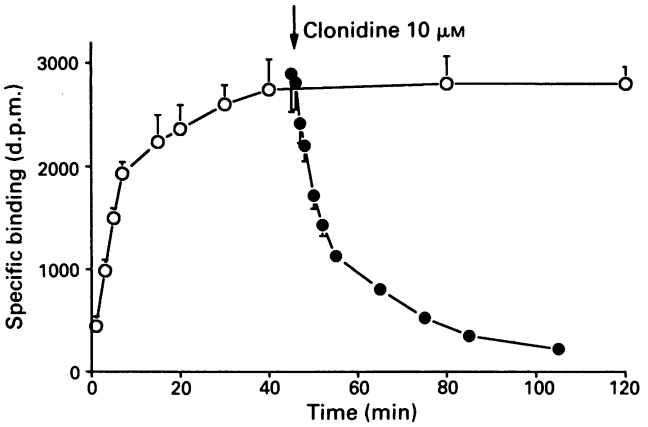


Figure 1 Kinetics of 6 nM [^{3}H]-clonidine binding to rabbit ventral medulla membranes at 25°C. Non specific binding was measured in the presence of 10 μM cirazoline subtracted from the total binding. Dissociation was obtained after 45 min equilibrium by addition of unlabelled clonidine to reach a final concentration of 10 μM. Values are means ± s.e.mean of 3 association or 4 dissociation independent experiments performed in duplicate.

Table 1 Kinetic constants of 6 nM [^{3}H]-clonidine binding to ventral medulla membranes from the rabbit brain

	Site 1	*Site 2*
k_{obs} (min^{-1})	0.098 ± 0.003	0.322 ± 0.015
k_{-1} (min^{-1})	0.036 ± 0.005	0.253 ± 0.004
K_D (nM)	3.5 ± 0.3	22 ± 0.1

Constants were calculated with the aid of a bi-exponential model computerized curve fitting procedure (McPherson, 1985). K_D were calculated according to the equation $K_D = k_{-1}/k_1$ with $k_1 = (k_{obs} - k_{-1})/[L]$. [L] being the concentration of the radioligand. Values are means ± s.e.mean of 3 association and 4 dissociation experiments.

Table 2 LIGAND analysis of data pooled from 5 experiments according to either a one- or two-site model

	One site analysis	*Two sites analysis*	
		Site 1	*Site 2*
IR + α-adrenoceptors			
K_D (nM)	6.9 ± 0.6	2.9 ± 2.9	19.8 ± 3.4
B_{max} (fmol mg^{-1} prot.)	89 ± 7	26 ± 64	94 ± 32
α-Adrenoceptors			
K_D (nM)	2.9 ± 0.7	2.9 ± 0.7	Set to
B_{max} (fmol mg^{-1} prot.)	39 ± 7	40 ± 8	zero
IR			
K_D (nM)	18.2 ± 0.4	Set to	18.2 ± 0.4
B_{max} (fmol mg^{-1} prot.)	66 ± 12	zero	66 ± 14

The specific binding was defined either as the difference between the binding of [^{3}H]-clonidine alone and the binding in the presence of 10 μM cirazoline (α-adrenoceptors ± IR), or binding of [^{3}H]-clonidine alone and binding in the presence of 10 μM noradrenaline (α-adrenoceptors), or binding of [^{3}H]-clonidine in the presence of 10 μM noradrenaline and binding in the presence of 10 μM cirazoline (IR). 'Set to zero' indicates that the data did not fit with a second site according to the computer programme.

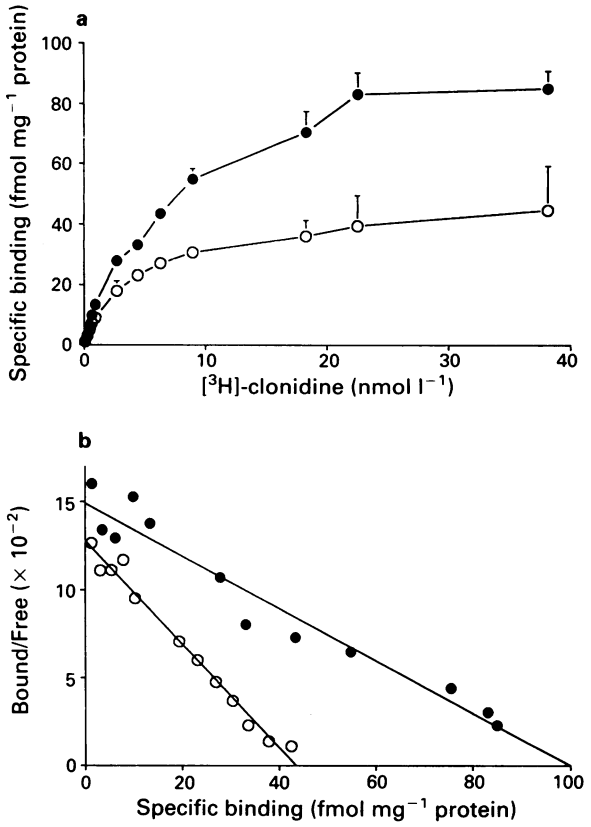


Figure 2 Saturation isotherms of [^{3}H]-clonidine specific binding to rabbit ventral medulla membranes. Specific binding was defined as the difference between binding of [^{3}H]-clonidine alone and binding in the presence of either 10 μM noradrenaline (○) or 10 μM cirazoline (●). (a) Values are means ± s.e.mean of 5 independent experiments performed in duplicate. (b) Scatchard transformation of the same data.

cirazoline defined as IRs. Thus the IRs represented the difference between [^{3}H]-clonidine binding measured in the presence of 10 μM (−)-noradrenaline and that measured in the presence of 10 μM cirazoline. When non specific binding was evaluated with (−)-noradrenaline or cirazoline, Scatchard analysis gave different K_D and B_{max} values (Figure 2b). For α-adrenoceptors, [^{3}H]-clonidine had a $K_D = 4.3 \pm 1.1$ nM for a $B_{max} = 47 \pm 6$ fmol mg^{-1} protein ($n = 5$). For cirazoline-sensitive sites (α-adrenoceptors + IRs) the K_D of [^{3}H]-clonidine was 8.3 ± 1.5 nM and the $B_{max} = 96 \pm 6$ fmol mg^{-1} protein. The differences were statistically significant ($P < 0.05$). In all cases the Hill coefficient was close to 1.

The difference in the K_D values for α-adrenoceptors and cirazoline-sensitive sites indicated that [^{3}H]-clonidine affinity was lower for IRs than for the α-adrenoceptors. To investigate further this point, a LIGAND analysis of the data pooled from five experiments was performed (Table 2).

(i) When specific binding was defined as the difference between the binding of [^{3}H]-clonidine in the presence of 10 μM cirazoline (α-adrenceptors + IRs) and in the absence of cirazoline, data matched both one- and two site models without significant statistical differences ($P = 0.09$). A one site model revealed a K_D and B_{max} in close agreement with the results of the Scatchard analysis ($K_D = 6.9 \pm 0.6$ nM, B_{max} of 89 ± 7.5 fmol mg^{-1} protein). The results obtained for a two site model matched those obtained in kinetic experiments (Table 2).

(ii) When the specific binding was defined as the difference between [^{3}H]-clonidine binding in the absence of (−)-noradrenaline and in the presence of 10 μM (−)-noradrenaline (α-adrenoceptors), the computerized iterative non-linear analysis of the binding data matched only a one site model with K_D and B_{max} close to the high affinity site data obtained with the LIGAND analysis for the total cirazoline specific binding or the Scatchard analysis of the α-adrenoceptors.

(iii) (−)-Noradrenaline-insensitive cirazoline-sensitive binding (IRs) was also analysed. The LIGAND programme matched only a one-site model with K_D and B_{max} values close to the low affinity site obtained with the LIGAND analysis of the total cirazoline specific binding.

The two sites thus defined closely matched those observed in kinetic experiments (Table 1 and 2).

Competition experiments

To check the selectivity of [^{3}H]-clonidine binding, competition experiments were performed with various compounds. IRs represented 20 to 30% of the total 5 to 6 nM [^{3}H]-clonidine cirazoline specific binding (Figure 3). Because the presence of an excess of noradrenaline usually led to a high non specific binding (about 65%), competition experiments were routinely analysed on the total specific binding (α-adrenoceptors + IRs). Substances were classified according to the maximum displacement observed at a concentration of 20 μM and three groups of compounds were revealed (Tables 3 and 4).

Group I: substances, including histamine and 5-hydroxytryptamine, totally unable to inhibit [^{3}H]-clonidine binding.

Group II: all the catecholamines and the compounds inhibiting 64 to 77% of the total [^{3}H]-clonidine specific binding with a distinct plateau in the competition curves. The

IC_{50} ranged from 6.5 ± 1 nM for (−)-adrenaline to 570 ± 190 nM for yohimbine. Substances, such as yohimbine or phenoxybenzamine, considered as α-adrenoceptor probes behaved like catecholamines (Table 3). Within this group the competition slopes were close to 1, with the exception of moxonidine, yohimbine and to a lesser extent α-methylnoradrenaline (Table 3).

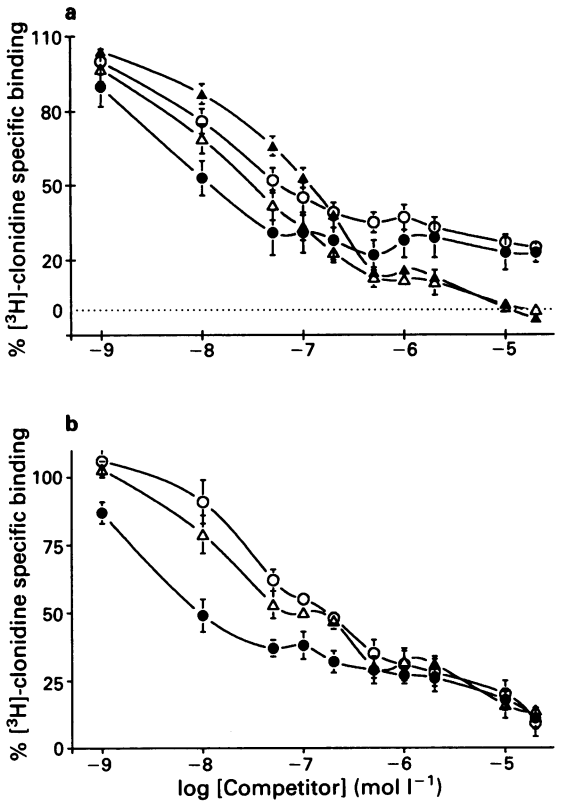


Figure 3 Competition curves between 5 to 7 nM [^{3}H]-clonidine and various compounds. Values are means ± s.e.mean of 4 to 6 independent experiments performed in duplicate. Non specific binding was defined as the binding measured in the presence of 10 μM cirazoline. In experiments with catecholamines, 0.005% ascorbic acid was present, without any effect on the [^{3}H]-clonidine binding. (a) (Δ) Clonidine; (▲) cirazoline; (●) (−)-adrenaline; (○) noradrenaline. (b) (○) Guanfacine; (Δ) phentolamine; (●) oxymetazoline.

Table 3 Maximum inhibition of [^{3}H]-clonidine specific binding observed with a 20 μM concentration of the compounds tested

	IC_{50} (nM)	*Slope*	I_{max} (%)	n
Group I				
Histamine			5 ± 5	4
5-Hydroxytryptamine			7 ± 7	4
Group II				
(−)-Adrenaline	6.5 ± 1	1.00 ± 0.10	77 ± 4	4
(−)-Noradrenaline	22 ± 6	0.80 ± 0.08	75 ± 3	5
α-Methylnoradrenaline	49 ± 15	0.72 ± 0.06	72 ± 3	5
Dopamine	643 ± 122	0.90 ± 0.08	72 ± 7	5
Phenoxybenzamine	21 ± 3	1.00 ± 0.10	64 ± 5	3
Moxonidine	81 ± 9	0.56 ± 0.03	77 ± 1	5
Yohimbine	568 ± 188	0.60 ± 0.07	68 ± 6	5

Specific binding was defined as the difference between binding of [^{3}H]-clonidine alone and binding in the presence of 10 μM cirazoline. In all cases there was a clear cut plateau of inhibition (Figure 3). Values were means ± s.e.mean of *n* independent experiments. IC_{50} and slopes were obtained by non linear curve fitting of experimental data to sigmoïdal curve with the aid of the EBDA programme. Non specific binding was considered as the maximal displacement observed with each compound.

Group III: compounds inhibiting almost completely the [^{3}H]-clonidine binding (α-adrenoceptors + IRs). This group consisted of the imidazolines and related compounds including oxazolines. Other non imidazoline substances, such as B-HT 920 or guanoxabenz, also inhibited this binding almost completely (Table 4). The order of potency of the drugs according to their IC_{50} was only possible to establish for the most abundant sites (i.e. α-adrenoceptors). Indeed, information as to the affinity of the substances for the IRs might only be obtained through comparison of the slopes of the competition curves. In contrast to group I compounds, only a few substances, (±)-idazoxan, cirazoline, ST 587, tolazoline and guanoxabenz, exhibited competition slopes close to 1 (Table 4), indicating that the affinities of these compounds for α-adrenoceptors and IRs should be of the same order of magnitude: (−)-idazoxan ≈ cirazoline > ST587 > tolazoline ≈ guanoxabenz. In all other cases, slopes differed markedly from 1 and the only information available was that the compounds were active at both sites within the concentration-range. Some substances like phentolamine, oxymetazoline and, to a lesser extent, guanfacine exhibited a maximum inhibition at the highest concentration used (20 μM), which was intermediate between those observed for group II and group III in spite of a low IC_{50} (Table 4). Competition curves obtained with these drugs were biphasic (Figure 3b). These data together with those of the two sites analysis of these curves (Table 5) confirmed that these compounds have low affinities for IRs (μM range) and high affinities for α_2-adrenoceptors (nM range).

To characterize further the low affinity noradrenaline insensitive binding, paired competition experiments were performed with cirazoline and a high [^{3}H]-clonidine concentration (20 nM) in the absence and in the presence of an excess of (−)-noradrenaline (30 μM). When the high concentration of [^{3}H]-clonidine was used, the IRs represented about 50% of the total specific binding whereas it was only 20–30% when the low concentration (6 nM) was used. The IC_{50} of cirazoline and slopes of the competition curves were 4,100 ± 1,200 nM with a slope of 0.76 ± 0.06 and 150 ± 74 nM with a slope of 0.72 ± 0.10 respectively in the absence and in the presence of

Table 4 Inhibition of 5 to 7 nM [^{3}H]-clonidine binding to membranes prepared from the ventral medulla of the rabbit brain: competition curves were established with 9 to 11 concentrations of each competitor

	IC_{50} (nM)	*Slope*	I_{max} (%)	n
Group III				
(±)-Idazoxan	85 ± 8	0.80 ± 0.26	106 ± 3	4
Cirazoline	100 ± 19	0.90 ± 0.13	103 ± 2	4
ST 587	625 ± 173	0.90 ± 0.13	101 ± 1	5
Tolazoline	1200 ± 600	0.90 ± 0.26	94 ± 3	5
Rilmenidine	113 ± 8	0.77 ± 0.11	98 ± 3	4
Guanoxabenz	1450 ± 320	1.00 ± 0.19	95 ± 3	4
BDF 6143	12 ± 3	0.50 ± 0.08	106 ± 3	5
UK-14304	22 ± 7	0.43 ± 0.11	104 ± 2	5
Clonidine	34 ± 10	0.61 ± 0.05	100 ± 2	5
B-HT 920	98 ± 15	0.66 ± 0.06	97 ± 3	4
(−)-Idazoxan	256 ± 28	0.60 ± 0.10	98 ± 4	4
Oxymetazoline	9.1 ± 3	0.50 ± 0.12	86 ± 4	6
Phentolamine	59 ± 28	0.70 ± 0.21	86 ± 2	4
Guanfacine	122 ± 29	0.54 ± 0.30	91 ± 5	6

IC_{50} and slopes, calculated by non linear curve fitting of experimental data to a sigmoid curve (EBDA programme, McPherson, 1985), were means ± s.e.mean of *n* independent experiments performed in duplicate. I_{max} represented the maximum specific inhibition observed with a 20 μM concentration of the inhibitor. Specific binding was defined as the difference between [^{3}H]-clonidine binding alone and binding measured in the presence of 10 μM cirazoline.

Table 5 Two sites analysis of competition curves for various compounds with competition slope lower than one (see Table 4)

	Large compartment			*Small compartment*	
	Size (%)	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)
BDF 6143	65	2.5	0.8	316	242.5
UK- 14304	73	4	1.3	1995	1531.0
B-HT 920	64	40	13.0	631	484.3
(−)-Idazoxan	57	63	20.5	1584	1215.6
Phentolamine	66	19.9	6.5	3981	3055.2
Guanfacine	77	50	16.3	3162	2426.7
Oxymetazoline	78	4	1.3	3162	2426.7

Two sites analysis of mean competition curves were performed with the aid of Graph Pad software. K_i were derived from IC_{50} values according to Cheng & Prusoff (1972) considering the large compartment as the α-adrenoceptor one with a K_D of clonidine of 2.9 nM and the small one being the imidazoline specific one with a K_D of 19.8 nM.

(−)-noradrenaline ($n = 6$), suggesting that cirazoline had a higher affinity for the noradrenaline-insensitive binding sites of [^{3}H]-clonidine than for α_2-adrenoceptors.

Discussion

Since the existence of IRs was proposed on the basis of the discrepancies between the mechanisms of action of substances acting on α-adrenoceptors with an imidazoline structure and those with a phenylethylamine structure (Bousquet *et al.*, 1984), a great deal of data has subsequently confirmed this dichotomy. Binding experiments have demonstrated the existence of imidazoline specific sites independent of α-adrenoceptors and pharmacological experiments have described functional properties specific for imidazolines (Meeley *et al.*, 1986; Coupry *et al.*, 1987; Ernsberger *et al.*, 1987; 1990; Convents *et al.*, 1988; Wikberg, 1988; Bricca *et al.*, 1989a; Schultz & Hasselblatt, 1989; Zonnenschein *et al.*, 1990; Göthert & Moldering, 1991; Tibiriça *et al.*, 1991). However, the biochemical data and the pharmacological properties were never available from the same tissue and/or species. We had previously shown that the rabbit was very sensitive to clonidine-like substances the hypotensive effect of which was related to the interaction with IRs in the NRL region of the ventral brainstem (Feldman *et al.*, 1990), suggesting the presence of a high concentration of IRs. Therefore, it was of interest to characterize the [^{3}H]-clonidine binding in this species in brain areas where clonidine-like substances induced their vasodepressor effect. Here, we report evidence for the existence of two distinct [^{3}H]-clonidine binding sites, α-adrenoceptors and IRs, in the rabbit RVLM the latter being supposed to be specifically associated with the hypotensive properties of clonidine and related drugs.

In equilibrium experiments, [^{3}H]-clonidine specific binding defined by noradrenaline or cirazoline was saturable, but the maximum specific binding observed under each condition was markedly different. α-Adrenoceptors were less numerous than cirazoline-sensitive sites; however, in each case, saturation was reached, indicating that [^{3}H]-clonidine binding to IRs was also saturable. [^{3}H]-clonidine binding to α_2-adrenoceptors was of high affinity whereas IRs exhibited a lower affinity for [^{3}H]-clonidine as previously suggested (Bricca *et al.*, 1989b; Hamilton *et al.*, 1991).

Both sites could only be clearly revealed in kinetic experiments and by the use of different drugs to define the non specific binding. Each time it was possible (i.e. when the competitors disclosed two compartments) the Cheng and Prusoff equation was applied; K_i values were then calculated for each of them (Table 5). These data confirmed that all of the ligands fitting with these criteria exhibited their highest affinities for the α-adrenoceptor compartment as compared to their affinities for the IR compartment.

Several results indicated that α-adrenoceptors could be identified as α_2-adrenoceptors. First, group II compounds, which inhibited binding as much as (−)-noradrenaline, included specific α-adrenoceptor probes such as phenoxybenzamine and yohimbine. The rather low potency of yohimbine for inhibition of [^{3}H]-clonidine binding to α_2-adrenoceptors found in this study had already been observed in previous studies with [^{3}H]-clonidine; this observation might be related to the fact that yohimbine is an antagonist at a receptor where clonidine is an agonist (Hornung *et al.*, 1979; Salama *et al.*, 1982). Second, IC_{50} values measured for the different drugs mainly reflected the inhibitory potency at α-adrenoceptors. The order of potencies of the compounds considered altogether was close to that expected for α_2-adrenoceptors. Although moxonidine has an imidazoline moiety, it behaved in our binding assays as a selective α-adrenoceptor competitor. It did not inhibit [^{3}H]-clonidine binding more than noradrenaline itself, thus corroborating the pharmacological data indicating that this compound was a potent α_2-adrenoceptor agent (Armah, 1988; Armah *et al.*, 1988). Moreover, in the rabbit brainstem, according to Schlicker *et al.* (1990), moxonidine decreased blood pressure by acting on presynaptic noradrenergic structures i.e. by a completely different mechanism from that of clonidine (Connor & Finch, 1981; Bousquet *et al.*, 1983). This confirmed that the imidazoline structure alone was not a sufficient requirement for interaction with the IRs. That was already suggested by several groups who described highly selective α_2-adrenoceptor properties for RX 821002, the close analogue of idazoxan (Saulnier-Blache *et al.*, 1989; Langin *et al.*, 1990; Vauquelin *et al.*, 1990).

The specific IR binding was usually inhibited by imidazoline compounds or very close analogues such as rilmenidine, an oxazoline. But in this binding also, at least one exotic drug, B-HT 920 a thiazoloazepine, devoid of the imidazoline moiety, exhibited competitive properties. Just like clonidine and rilmenidine, B-HT 920 was able to induce a hypotensive effect when microinjected in the NRL (Bousquet *et al.*, 1983; 1984; Feldman *et al.*, 1990). Thus, the ability to inhibit [^{3}H]-clonidine binding to IRs paralleled the hypotensive activity. Moreover, the sensitivity to various chemical classes (Laduron, 1988), one of the criteria required to define a receptor which the IR did not yet fulfil, has been satisfied with the ability of B-HT 920 to inhibit [^{3}H]-clonidine binding to IRs.

Yohimbine and phenoxybenzamine were unable to compete with [^{3}H]-clonidine binding to IRs. Oxymetazoline and phentolamine proved rather weak and incomplete competitors for imidazoline binding sites. This had already been observed in other binding studies on IRs in peripheral tissues such as kidney, adipocytes as in the central nervous system (Bricca *et al.*, 1989a; Langin & Lafontan, 1989; Michel *et al.*, 1989). Therefore, these compounds were probably not the most adequate tools to define non specific binding in such studies on IRs using [^{3}H]-clonidine. In the present study, we have shown that cirazoline had a higher affinity for the IRs than for α-adrenoceptors. Cirazoline was consistently the most potent agent for preventing drug binding to imidazoline specific binding sites (MacKinnon *et al.*, 1989; Parini *et al.*, 1989; Bricca *et al.*, 1989a; Brown *et al.*, 1990; Wikberg & Uhlen, 1990). Furthermore, in rat and rabbit brain or human platelets, cirazoline has been shown to have a K_i for α_2-adrenoceptors of about 50 to 300 nM consistent with the inhibition constant observed in the present study (Van Meel *et al.*, 1981; Brown *et al.*, 1990; Diamant *et al.*, 1992). In any case, under our experimental conditions, a concentration of cirazoline of 10 μM was sufficient to prevent all [^{3}H]-clonidine bindings to IRs, thus making it the most reliable compound for this purpose.

The existence of two sites labelled by [^{3}H]-clonidine was confirmed in kinetic experiments where the experimental data fitted a bi-exponential model significantly better. In addition, the K_D calculated from kinetic parameters closely matched those obtained from saturation experiments (Table 1). The kinetic experiments also demonstrated that the binding of [^{3}H]-clonidine to both compartments defined by cirazoline was reversible.

We have observed that the percentage contribution of the IRs to total binding was increased by restricting the sample more specifically to the NRL area. This point also outlines the discrete distribution of IRs within the brainstem. Although [^{3}H]-clonidine was at a higher concentration in competition experiments (5 to 7 versus 1 to 3 nM), the proportion of IRs labelled in the rabbit brainstem with [^{3}H]-clonidine or [^{3}H]-*p*-aminoclonidine was lower than that found in the human brainstem (Meeley *et al.*, 1986; Bricca *et al.*, 1989a). This may be due to species differences.

In order to correlate the binding affinity of the imidazolines to the IRs and their hypotensive potency when microinjected within the NRL, precise measurements of the affinities were required. However, under the present conditions IC_{50} values mainly reflected the competitive potency for α_2-adrenoceptors which were considerably more numerous. The only compounds for which potency at IRs could be evaluated were those with competition slopes near 1. Only 5 compounds out of the 14 competitors tested satisfied this condition. Only cirazoline and ST 587 induced hypotension when injected into the NRL area, wheras idazoxan and tolazoline were considered to be antagonists. The very weak hypotensive effect of guanoxabenz when microinjected in the NRL might be due to its low affinity for the IR.

Further hypotensive competitors with adequate Hill numbers have to be analysed to confirm the possibility of the expected correlation. A previous attempt by Ernsberger *et al.* (1990) to correlate affinity for IRs and hypotensive activity in this area included compounds such as cimetidine and imidazole acetic acid which were completely inactive in our study. Moreover, these authors gave K_i values and this was impossible in our study for the reasons mentioned above.

In conclusion, we have demonstrated that:

(1) In the rabbit, a species very sensitive to the cardiovascular effects of imidazolines, [^{3}H]-clonidine binding experiments revealed the existence of binding sites specifically sensitive to imidazolines within the RVLM region supposed to be involved in their hypotensive effect as well as the presence of classical α_2-adrenoceptors.

(2) Despite a rather low affinity for the imidazoline specific receptors, [^{3}H]-clonidine remains one of the most efficient tools to study these receptors.

(3) Since both receptors were present in the membrane preparations, the only compounds for which affinity for IRs could be approximated in the absence of selective ligands were those with competition slopes close to 1. Only a few competitors exhibited this property, namely: cirazoline, ST 587, tolazoline, idazoxan and guanoxabenz.

(4) The ability of B-HT 920 to inhibit [^{3}H]-clonidine binding to IRs, allowed this binding site to fulfill an important criterion of a receptor; that is that substances of different chemical classes with the same pharmacological properties should be able to interact with the receptor.

We are grateful to Beiersdorf, Lers-Synthélabo and Servier Laboratories for the gifts of the drugs. We also wish to thank Dr Langley, INSERM Unit 44 - Strasbourg, for careful revision of this text.

References

ARMAH, B.I. (1988). Unique presynaptic alpha$_2$-receptor selectivity and specificity of the antihypertensive agent moxonidine. *Arzneim Forsch. Drug Res.*, **38**, 1435–1442.

ARMAH, B.I., HOFFERBER, E. & STENGEL, W. (1988). General pharmacology of the novel centrally acting antihypertensive agent moxonidine. *Arzneim Forsch. Drug Res.*, **38**, 1426–1434.

BOUSQUET, P., FELDMAN, J., BLOCH, R. & SCHWARTZ, J. (1981). The nucleus reticularis lateralis a region highly sensitive to clonidine. *Eur. J. Pharmacol.*, **69**, 389–392.

BOUSQUET, P., FELDMAN, J. & SCHWARTZ, J. (1984). Central cardiovascular effects of alpha-adrenergic drugs: difference between catecholamines and imidazolines. *J. Pharmacol. Exp. Ther.*, **230**, 230–236.

BOUSQUET, P., ROUOT, B. & SCHWARTZ, J. (1983). The central alpha-adrenoceptors: some new aspects. *Trends Pharmacol. Sci.*, **4**, 206–208.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of protein utilizing the principle protein-dye binding. *Anal. Biochem.*, **72**, 248–252.

BRICCA, G., DONTENWILL,M., MOLINES, A., FELDMAN, J., BELCOURT, A. & BOUSQUET, P. (1988). Evidence for the existence of a homogenous population of imidazoline receptors in the human brainstem. *Eur. J. Pharmacol.*, **150**, 401–402.

BRICCA, G., DONTENWILL, M., MOLINES, A., FELDMAN, J., BELCOURT, A. & BOUSQUET, P. (1989a). The imidazoline preferring receptor: binding studies in bovine, rat and human brainstem. *Eur. J. Pharmacol.*, **162**, 1–9.

BRICCA, G., DONTENWILL, M., MOLINES, A., FELDMAN, J., TIBIRIÇA, E., BELCOURT, A. & BOUSQUET, P. (1989b). Rilmenidine selectivity for imidazoline receptors in human brain. *Eur. J. Pharmacol.*, **163**, 373–377.

BROWN, C.M., MACKINNON, A.C., MCGRATH, J.C., SPEDDING, M. & KILPATRICK, A.T. (1990). α_2-Adrenoceptor subtypes and imidazoline-like binding sites in the rat brain. *Br. J. Pharmacol.*, **99**, 803–809.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibitory constant (K_i) and the concentration of inhibitor which caused 50 per cent inhibition (pIC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

CONNOR, H.E. & FINCH, L. (1981). Postsynaptic spinal alpha-adrenoceptors mediate effects of intrathecal clonidine. *Eur. J. Pharmacol.*, **76**, 97–100.

CONVENTS, A., CONVENTS, D., DE BACKER, J.P., DE KEYSER, J. & VAUQUELIN, G. (1988). High affinity binding of [^{3}H]rauwolscine and [^{3}H]RX781094 to alpha$_2$-adrenergic receptors and non-stereoselective sites in human and rabbit brain cortex membranes. *Biochem. Pharmacol.*, **38**, 455–463.

COUPRY, I., PODEVIN, R.A., DAUSSE, J.P. & PARINI, A. (1987). Evidence for imidazoline binding sites in basolateral membranes from rabbit kidney. *Biochem. Biophys. Res. Commun.*, **147**, 1055–1060.

DIAMANT, S., ELDAR-GEVA, T. & ATLAS, D. (1992). Imidazoline binding sites in human placenta: evidence for heterogeneity and a search for physiological function. *Br. J. Pharmacol.*, **106**, 101–108.

ERNSBERGER, P., GIULIANO, R., WILLETTE, R.N. & REIS, D.J. (1990). Role of imidazole receptors in the vasodepressor response to clonidine analogs in the rostral ventrolateral medulla. *J. Pharmacol. Exp. Ther.*, **253**, 408–418.

ERNSBERGER, P., MEELEY, M.P., MANN, J.J. & REIS, D.J. (1987). Clonidine binds to imidazole binding sites as well as alpha$_2$-adrenoceptors in the ventrolateral medulla. *Eur. J. Pharmacol.*, **134**, 1–13.

FELDMAN, J., TIBIRIÇA, E., BRICCA, G., DONTENWILL, M., BELCOURT, A. & BOUSQUET, P. (1990). Evidence for the involvement of imidazoline receptors in the central hypotensive effect of rilmenidine in the rabbit. *Br. J. Pharmacol.*, **100**, 600–604.

GÖTHERT, M. & MOLDERING, J. (1991). Involvement of presynaptic imidazoline receptors in the alpha$_2$-adrenoceptor inhibition of noradrenaline release by imidazoline derivatives. *Naunyn-Schmied. Arch. Pharmacol.*, **342**, 271–282.

HAMILTON, C.A., REID, J.L. & YAKUBU, M.A. (1988). [^{3}H]yohimbine and [^{3}H]idazoxan bind to different sites on rabbit forebrain and kidney membranes. *Eur. J. Pharmacol.*, **146**, 345–348.

HAMILTON, C.A., YAKUBU, M.A., JARDINE, E. & REID, J.L. (1991). Imidazole binding sites in rabbit kidney and forebrain membranes. *J. Auton. Pharmacol.*, **11**, 277–283.

HORNUNG, R., PRESEK, P. & GLOSSMANN, H. (1979). Alpha-adrenoceptors in rat brain: direct identification with prazosin. *Naunyn Schmied. Arch. Pharmacol.*, **308**, 223–230.

LADURON, P.M. (1988). Stereospecificity in binding studies. A useful criterion though insufficient to prove the presence of receptors. *Biochem. Pharmacol.*, **37**, 37–40.

LANGIN, D. & LAFONTAN, M. (1989). [^{3}H]idazoxan binding at non-alpha$_2$-adrenoceptors in rabbit adipocyte membranes. *Eur. J. Pharmacol.*, **159**, 199–203.

LANGIN, D., PARIS, H., DAUZATS, M. & LAFONTAN, M. (1990). Discrimination between alpha$_2$-adrenoceptors and [^{3}H]idazoxan labelled non-adrenergic sites in rabbits fat cells. *Eur. J. Pharmacol.*, **188**, 261–272.

MACKINNON, A.C., BROWN, C.M., SPEDDING, M. & KILPATRICK, A.T. (1989). [^{3}H]idazoxan binds with on hamster adipocytes: an alpha2-adrenoceptor and a non-adrenoceptor site. *Br. J. Pharmacol.*, **98**, 1143–1150.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for the IMB PC. *J. Pharmacol. Methods*, **14**, 213–228.

MEELEY, M.P., ERNSBERGER, P.R., GRANATA, A.R. & REIS, D.J. (1986). An endogenous clonidine displacing substance from bovine brain: receptor binding and hypotensive actions in the ventrolateral medulla. *Life Sci.*, **38**, 1119–1126.

MICHEL, M.C., BRODDE, O.E., SCHNEPEL, B., BEHRENDT, J., TSCHADA, R., MOTULSKY, H.J. & INSEL, P.A. (1989). [^{3}H]-idazoxan and some other alpha$_2$-adrenergic drugs also binds with high affinity to a nonadrenergic site. *Mol. Pharmacol.*, **35**, 324–330.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.*, **107**, 220–239.

PARINI, A., COUPRY, I., GRAHAM, R.M., UZIELLI, I., ATLAS, D. & LANIER, S.M. (1989). Characterization of an imidazoline/guanidinium receptive site distinct from the alpha$_2$-adrenergic receptor. *J. Biol. Chem.*, **264**, 11874–11878.

SALAMA, A.I., LIN, L.L., REPP, L.D. & U'PRICHARD, D.C. (1982). Magnesium reduces affinities of antagonists at rat cortex alpha$_2$-adrenergic receptors labeled with [^{3}H]clonidine: evidence for heterogeneity of alpha$_2$-receptor conformations with respect to antagonists. *Life Sci.*, **30**, 1505–1511.

SAULNIER-BLACHE, J.S., CARPENE, C., LANGIN, D. & LAFONTAN, M. (1989). Imidazolinic radioligands for the identification of hamster adipocyte alpha$_2$-adrenoceptors. *Eur. J. Pharmacol.*, **171**, 145–157.

SCATCHARD, G. (1949). The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672.

SCHLICKER, E., ARMAH, B.I. & GÖTHERT, M. (1990). Central presynaptic alpha$_2$-autoreceptors are involved in the blood pressure lowering effect of moxonidine. *J. Cardiovasc. Pharmacol.*, **16**, 15–22.

SCHULZ, A. & HASSELBLATT, A. (1989). An insulin releasing property of imidazoline derivatives is not limited to compounds that block α-adrenoceptors. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 321–327.

TESSON, F. & PARINI, A. (1991). Identification of an imidazoline guanidinium receptive site in mitochondria from rabbit cerebral cortex. *Eur. J. Pharmacol. Mol. Pharmacol.*, **208**, 81–83.

TIBIRIÇA, E., FELDMAN, J., MERMET, C., GONON, F. & BOUSQUET, P. (1991). An imidazoline specific mechanism for the hypotensive effect of clonidine: a study with yohimbine and idazoxan. *J. Pharmacol. Exp. Ther.*, **256**, 606–613.

U'PRICHARD, D.C. (1981). [^{3}H]clonidine and [^{3}H]p-aminoclonidine interactions *in vitro* with central and peripheral α_2-adrenergic receptors. In *Progress in Clinical and Biological Research*, Vol. 71: *Psychopharmacology of Clonidine*. ed. Lal, H. & Fielding, S. pp. 53–74. New York: Alan R. Liss Inc.

VAN MEEL, J.C.A., DE JONGE, A., TIMMERMANS, P.B.M.W.M. & VAN ZWIETEN, P.A. (1981). Selectivity of some alpha-adrenoceptor agonists for peripheral alpha$_1$- and alpha$_2$-adrenoceptors in the normotensive rat. *J. Pharmacol. Exp. Ther.*, **219**, 760–767.

VAUQUELIN, G., DE VOS, H., DE BACKER, J.P. & EBINGER, G. (1990). Identification of alpha$_2$-adrenergic receptors in human frontal cortex membranes by binding of [^{3}H]RX821002 the 2-methoxy analog of [^{3}H]idazoxan. *Neurochem. Int.*, **17**, 537–546.

WIKBERG, J.E.S. (1988). High affinity binding of idazoxan to a non catecholaminergic binding site in the centrale nervous system: description of a putative idazoxan-receptor. *Pharmacol. Toxicol.*, **64**, 152–155.

WIKBERG, J.E.S. & UHLEN, S. (1990). Further characterization of the guinea pig cerebral cortex idazoxan receptor, solubilization distinction from the imidazoline site, and demonstration of cirazoline as an idazoxan receptor selective drug. *J. Neurochem.*, **55**, 192–203.

WIKBERG, J.E.S., UHLEN, S. & CHAJLANI, V. (1991). Medetomidine stereoisomers delineate two closely related subtypes of idazoxan (imidazoline) I receptors in the guinea pig. *Eur. J. Pharmacol.*, **193**, 335–340.

WOLFF, D.L. & MOHRLAND, J.S. (1984). Lateral reticular formation as a site for morphine and clonidine induced hypotension. *Eur. J. Pharmacol.*, **98**, 93–98.

YABLONSKY, F., RIFFAUD, J.P., LACOLLE, J.Y. & DAUSSE, J.P. (1988). Evidence for non adrenergic binding sites for [^{3}H]-idazoxan in the smooth muscle of rabbit urethra. *Eur. J. Pharmacol.*, **154**, 209–212.

YAKUBU, M.A., HAMILTON, C.A., HOWIE, C.A. & REID, J.L. (1988). Idazoxan and brain alpha$_2$-adrenoceptors in the rabbit. *Brain. Res.*, **463**, 289–295.

ZONNENSCHEIN, R., DIAMANT, S. & ATLAS, D. (1990). Imidazoline receptors in rat liver cell: a novel receptor or a subtype of alpha$_2$-adrenoceptors? *Eur. J. Pharmacol.*, **190**, 203–215.

(Received April 23, 1993
Revised August 5, 1993
Accepted August 12, 1993)

Br. J. Pharmacol. (1993), **110**, 1544–1550 © Macmillan Press Ltd, 1993

Ionotropic glutamate receptor types leading to adenosine-mediated inhibition of electrically evoked [^{3}H]-noradrenaline release in rabbit brain cortex slices

[1]Ivar von Kügelgen, Leni Späth & Klaus Starke

Pharmakologisches Institut, Hermann-Herder-Strasse 5, D-79104 Freiburg i. Br., Germany

1 Glutamate inhibits the electrically evoked release of noradrenaline in rabbit brain cortex slices; the inhibition is mediated by adenyl compounds, presumably adenosine. The aim of the present study was to identify the receptors involved in this indirect inhibitory effect of glutamate. Slices of the occipito-parietal cortex were preincubated with [^{3}H]-noradrenaline and then superfused and stimulated by trains of 6 pulses, 100 Hz.

2 The ionotropic glutamate receptor agonists α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; 10–100 μM), kainate (10–100 μM) and N-methyl-D-aspartate (NMDA; 30–300 μM) but not the metabotropic glutamate receptor agonist, 1-amino-1,3-cyclopentanedicarboxylate (ACPD; 10–100 μM) reduced the electrically evoked overflow of tritium.

3 The effects of AMPA, kainate and NMDA were attenuated or abolished by the adenosine A_1-receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as well as by adenosine deaminase but not by the α_2-adrenoceptor antagonist yohimbine, the γ-aminobutyric acid (GABA) receptor antagonists, bicuculline and 2-hydroxysaclofen and the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME).

4 The NMDA receptor antagonist, 2-amino-5-phosphonopentanoate (AP5) blocked the inhibitory effect of NMDA but not that of AMPA and kainate. The non-NMDA-receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) blocked the effect of AMPA but not of kainate and NMDA.

5 In addition to decreasing the electrically evoked overflow of tritium, AMPA, kainate and NMDA but not ACPD caused a steep but transient rise of basal tritium efflux. This immediate releasing effect was not significantly changed by DPCPX, adenosine deaminase, yohimbine, bicuculline, 2-hydroxysaclofen and L-NAME (except that L-NAME enhanced the effect of kainate). AP5 and CNQX antagonized the immediate releasing effects in the same way that they antagonized the inhibition by AMPA, kainate and NMDA of the electrically evoked overflow of tritium.

6 It is concluded that AMPA, kainate and NMDA, like glutamate, reduce the electrically evoked release of noradrenaline by releasing adenosine or an adenine nucleotide which is then degraded to adenosine. Activation of each of the three ionotropic glutamate receptors, AMPA, kainate and NMDA receptors, but not activation of metabotropic glutamate receptors can initiate this indirect inhibitory effect on the release of noradrenaline (as well as the known noradrenaline releasing effect).

Keywords: α-Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA); N-methyl-D-aspartate (NMDA); kainate; endogenous adenosine; ionotropic glutamate receptors; metabotropic glutamate receptors; presynaptic adenosine A_1-receptors; noradrenaline release; rabbit brain cortex

Introduction

Glutamate and other excitatory amino acids (EAA) release noradrenaline from cerebral axons (man: Fink *et al.*, 1992; rat: Nelson *et al.*, 1980; Jones *et al.*, 1987; Vezzani *et al.*, 1987; Fink *et al.*, 1989; Blandina *et al.*, 1992; Fink & Göthert, 1992; Pittaluga & Raiteri, 1992; Wang *et al.*, 1992; mouse: Zhao *et al.*, 1990). At least part of the effect is direct at the terminal noradrenergic axons since it is also observed in synaptosomal preparations (Fink & Göthert, 1992; Pittaluga & Raiteri, 1992; Wang *et al.*, 1992). It seems that activation of any of the three types of inotropic glutamate receptor, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors, will elicit the response (see references above).

We recently observed a second effect of glutamate on cerebral noradrenergic terminal axons: inhibition of the electrically evoked release of [^{3}H]-noradrenaline (rabbit brain cortex slices; von Kügelgen *et al.*, 1992a). The effect was indirect: it was abolished by the A_1-purinoceptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as well as by addition of the enzyme adenosine deaminase, indicating that glutamate initially released adenosine (or an adenine nucleotide which was degraded to adenosine) and adenosine then caused presynaptic, A_1-purinoceptor-mediated inhibition (von Kügelgen *et al.*, 1992a). Release by EAA of adenyl compounds in brain cortex has been shown directly (guinea-pig: Pull & McIlwain, 1975; rat: Jhamandas & Dumbrille, 1980; Perkins & Stone, 1983; Hoehn & White, 1989). In the present experiments, the type of glutamate receptor involved in the indirect inhibitory effect of glutamate was examined. The selective ionotropic glutamate receptor agonists AMPA, kainate and NMDA were used, as well as the agonist at metabotropic glutamate receptors 1-amino-1,3-cyclopentanedicarboxylate (ACPD), and the antagonists 2-amino-5-phosphonopentanoate (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (for reviews see Collingridge & Lester, 1989; Schoepp *et al.*, 1990; Watkins *et al.*, 1990). Some of these results have been reported to the Deutsche Gesellschaft für Pharmakologie und Toxikologie (von Kügelgen *et al.*, 1992b).

Methods

Rabbits of either sex and weighing 1.9–2.6 kg were killed by cervical dislocation and exsanguination. The brain was quick-

[1] Author for correspondence.

ly removed and chilled. Two slabs were cut from the occipito-parietal cortex, parallel to the surface. The superficial slab, 0.3 mm thick, consisted of pia mater, lamina I and in some cases part of lamina II; the second, deeper slab, 0.4 mm thick, consisted of laminae II–IV and in some cases parts of laminae I or V (Reichenbacher *et al.*, 1982). Unless stated otherwise, the superficial slab was discarded and round slices, 3.5 mm diameter, were punched from the second slab. Six to eight slices were preincubated at 37°C for 30 min in 2 ml medium containing (−)-[^{3}H]-noradrenaline 0.1 μM. In some experiments, 3.5 mm diameter slices were also prepared from the superficial slab, and slices from occipital and parietal areas and from the superficial and the deeper layer were incubated with [^{3}H]-noradrenaline separately. Following the labelling incubation, the slices were washed three times with 3 ml [^{3}H]-noradrenaline-free medium. One slice was transferred to each of six 0.16 ml superfusion chambers where it was held by a polypropylene mesh between platinum wire electrodes 6 mm apart. The slices were superfused with [^{3}H]-noradrenaline-free medium for 116 min at a rate of 1 ml min^{-1} at 37°C. A Stimulator I (Hugo Sachs Elektronik, March-Hugstetten, Germany) operating in the constant voltage mode was used for electrical field stimulation. Three periods of stimulation were applied (rectangular pulses of 2 ms width and 12.5 V cm^{-1}, yielding a current strength of 18 mA). The first was delivered after 20 min of superfusion and consisted of 18 pulses at 1 Hz. It was not used for determination of tritium outflow. The following two periods were delivered after 59 (S_1) and 98 (S_2) min of superfusion; each consisted of a train of 6 pulses at 100 Hz. The collection of successive 3 min superfusate samples began after 50 min of superfusion, i.e., 9 min before S_1. Glutamate receptor agonists or their solvent (water) were added to the medium between S_1 and S_2 (24 min before S_2 unless stated otherwise) and kept for the remainder of the experiment; the delay from addition to arrival at tissue was about 60 s. Other drugs were added to the medium throughout superfusion. After superfusion, each slice was solubilized in 0.5 ml Soluene-350 (Canberra Packard, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized slices by liquid scintillation counting.

Unless stated otherwise, the medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03. It was saturated with 5% CO_2 in O_2. The pH was adjusted to 7.4 with NaOH 1 M. The medium used for superfusion but not that used for preincubation contained desipramine 1 μM in order to block uptake$_1$. In some experiments, incubation and superfusion media were Mg^{2+}-free.

The outflow of tritium was expressed as fractional rate (min^{-1}), i.e., (tritium outflow per 3 min)/[3 × (tritium present in the slice at the onset of the respective collection period)]. The electrically evoked overflow of tritium was calculated as the difference between the total overflow (during the 12 min after electrical stimulation) and the estimated basal outflow; the basal outflow was assumed to decline linearly from the 3 min interval before, to the interval 12–15 min after, electrical stimulation. The transient overflow of tritium evoked by glutamate receptor agonists themselves, when added between S_1 and S_2, was calculated in an analogous manner: as the difference between the total overflow (from addition to the 3 min interval before S_2) and the estimated basal outflow; the basal outflow was assumed to decline linearly from the 3 min interval before addition to the 3 min interval before S_2. The differences (total minus basal; Bq) were expressed as a percentage of the tritium content (Bq) of the slice at the onset of electrical stimulation or glutamate receptor agonist addition, respectively. For further evaluation of basal tritium efflux, ratios were calculated of the fractional rate of outflow in the 3 min interval before S_2 and the 3 min interval before S_1 (b_2/b_1). For further evaluation of the electrically evoked overflow, ratios were calculated of the overflow elicited by S_2 and the overflow elicited by S_1 (S_2/S_1). S_2/S_1 ratios obtained in individual experiments with a glutamate receptor agonist were calculated as a percentage of the average S_2/S_1 ratio in the appropriate control group.

Materials

The following drugs were used: (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 1.94 or 2.11 TBq $mmol^{-1}$ (Du Pont, Dreieich, Germany), (±)-*cis*-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD; IUPAC nomenclature; see the Editorial in Schoepp *et al.*, 1990), (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid HBr (AMPA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-hydroxysaclofen (Research Biochemicals, Natick, MA, U.S.A.), yohimbine HCl (Roth, Karslruhe, Germany), adenosine deaminase type VII from calf intestinal mucosa (EC 3.5.4.4), (±)-2-amino-5-phosphonopentanoic acid (AP5), (−)-bicuculline methiodide, desipramine HCl, kainic acid, N-methyl-D-aspartic acid (NMDA), N^G-nitro-L-arginine methyl ester HCl (L-NAME) (Sigma, Deisenhofen, Germany). Solutions of drugs were prepared with either distilled water or (2-hydroxysaclofen) the KH_2PO_4- and $NaHCO_3$-containing stock solution of the medium or (CNQX and DPCPX) dimethyl sulphoxide (Roth, Karlsruhe, Germany, final concentration about 0.4 mM). Dimethyl sulphoxide was the solvent for CNQX and DPCPX only, but was added in all experiments throughout superfusion to make them directly comparable.

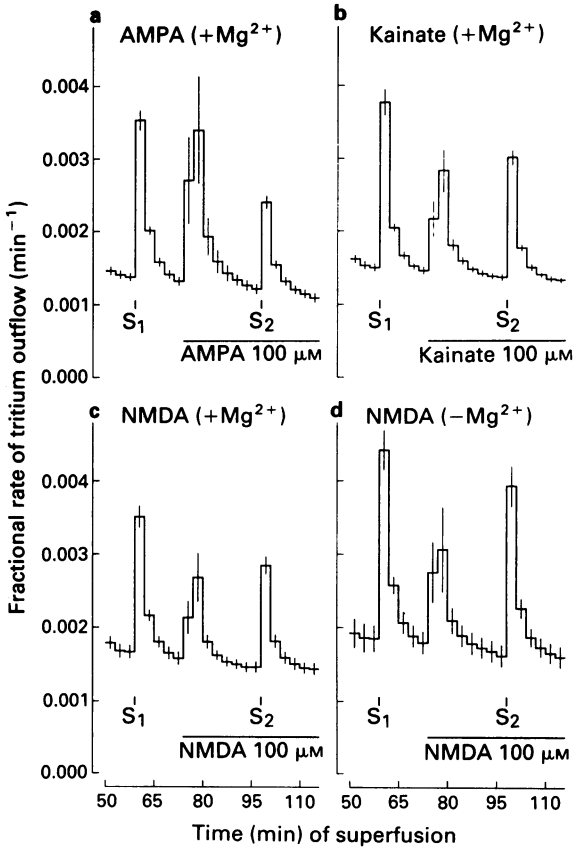


Figure 1 Time course of tritium outflow from rabbit brain cortex slices and effects of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; a), kainate (b) and N-methyl-D-aspartate (NMDA; c and d). After preincubation with [^{3}H]-noradrenaline, slices were superfused and stimulated twice (S_1 and S_2; 6 pulses/100 Hz). In some experiments, incubation and superfusion media were Mg^{2+}-free (d) instead of containing the normal 1.2 mM Mg^{2+} (a-c). Glutamate receptor agonists were added as indicated. Mean ± s.e.mean of 9 (a), 21 (b), 14 (c) and 6 (d) experiments. Abscissae, min of superfusion.

Table 1 Basal tritium outflow (b_1), electrically evoked tritium overflow (S_1), and effects of drugs

Drugs added throughout superfusion	b_1 (min^{-1})	S_1 (% of tissue tritium	n
	Magnesium 1.2 mM		
–	0.00173 ± 0.00003[a]	0.760 ± 0.020[b]	195
DPCPX 10 nM	0.00200 ± 0.00004**	1.252 ± 0.032**	108
Adenosine deaminase 3 u ml^{-1}	0.00240 ± 0.00008**	1.480 ± 0.077**	18
Yohimbine 1 μM	0.00182 ± 0.00009	1.024 ± 0.071**	30
Bicuculline 50 μM	0.00135 ± 0.00004**	0.898 ± 0.044	18
2-Hydroxysaclofen 50 μM	0.00143 ± 0.00005*	0.847 ± 0.050	17
L-NAME 100 μM	0.00135 ± 0.00005**	1.021 ± 0.078*	20
AP5 100 μM	0.00153 ± 0.00005	0.864 ± 0.056	24
CNQX 30 μM	0.00203 ± 0.00023	0.938 ± 0.059*	35
CNQX 100 μM	0.00154 ± 0.00007	1.416 ± 0.068**	18
	Magnesium absent		
–	0.00176 ± 0.00003	1.035 ± 0.027	77
DPCPX 10 nM	0.00198 ± 0.00006**	1.450 ± 0.051**	29

After preincubation with [^{3}H]-noradrenaline, cortical slices were superfused with medium containing the drugs indicated (and in addition desipramine 1 μM which is not noted since it was always present throughout superfusion). S_1 was applied after 59 min of superfusion and consisted of 6 pulses/100 Hz. b_1 refers to the 3-min period immediately before S_1. AP5, 2-amino-5-phosphonopentanoate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; L-NAME, N^G-nitro-L-arginine methyl ester. Means ± s.e.mean of *n* experiments.
[a]Corresponding to 14.0 ± 0.3 Bq min^{-1}.
[b]Corresponding to 61.6 ± 1.9 Bq.
Significant differences from corresponding experiments without the drug: *$P<0.05$ and **$P<0.01$.

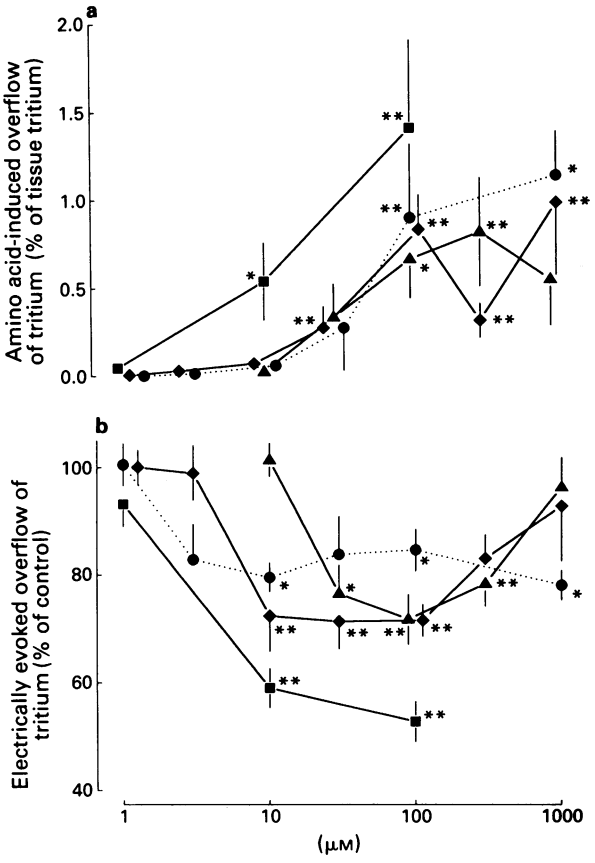


Figure 2 Glutamate receptor agonist-induced overflow of tritium (a) and inhibition of electrically evoked tritium overflow (b). Slices were stimulated twice (S_1 and S_2). α-Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA, ■), kainate (◆) or N-methyl-D-aspartate (NMDA, ▲ and ●) was added 24 min before S_2. In some experiments with NMDA, incubation and superfusion media were Mg^{2+}-free (●····●). Abscissae, concentration of glutamate receptor agonists. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means ± s.e.mean of 4–21 experiments. Significant differences from control: *$P<0.05$ and **$P<0.01$.

Statistics

Means ± s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test. $P<0.05$ or lower was the significance criterion. For multiple comparisons with the same control, P levels were adjusted according to Bonferroni. *n* is the number of brain slices.

Results

Electrical stimulation by six pulses delivered at 100 Hz markedly increased the outflow of tritium (Figure 1). Values for the magnitude of the basal outflow of tritium before S_1 and for the overflow elicited by S_1 are summarized in Table 1. In the absence of drugs (except desipramine 1 μM which was in the superfusion medium in all experiments; magnesium present), the fractional rate of tritium efflux immediately before S_1 averaged 0.00173 min^{-1}, and stimulation by six pulses/100 Hz caused an average overflow (S_1) of 0.760% of the tritium content of the tissue. Drug-induced changes will be mentioned in the appropriate sections below.

In control experiments in which the medium was drug-free (except for desipramine; magnesium present) and solvent (water) was added before S_2, the b_2/b_1 ratio was 0.87 ± 0.01 and the S_2/S_1 ratio 1.06 ± 0.01 ($n = 33$). The average b_2/b_1 ratio also was slightly below unity, and the S_2/S_1 ratio close to unity, when solvent was added before S_2 in any other group defined in Table 1 (not shown).

Effects of glutamate receptor agonists

When added 24 min before S_2 at a concentration of 100 μM, each of the three agonists after which the ionotropic glutamate receptors are named – AMPA, kainate and NMDA – elicited two effects (Figure 1). First, there was an immediate steep rise in tritium outflow. The increase was transient, and the efflux of tritium returned to baseline well before S_2 so that b_2/b_1 ratios did not differ from the corresponding control (solvent before S_2). This will be called the immediate releasing effect. The second effect was a decrease of the overflow elicited by electrical stimulation (S_2). In the case of NMDA, both effects were obtained in the presence (Figure 1c) as well as in the absence of Mg^{2+} (Figure 1d).

Concentration-response curves are shown in Figure 2. As can be seen from the error bars, the immediate releasing effect (Figure 2a) was very variable, more variable than the inhibition of the electrically evoked overflow of tritium (Figure 2b). Concentration-response curves for the inhibitory effect of kainate, and of NMDA in the presence of Mg^{2+}, were bell-shaped (Figure 2b). Kainate, and NMDA in the absence of Mg^{2+}, were more potent in inhibiting the electrically evoked overflow than in eliciting the immediate release: marked inhibition was obtained at 10 μM, a concentration at which there was not even a tendency for the releasing effect. AMPA, and NMDA in the presence of Mg^{2+}, produced release and inhibition of electrically evoked overflow at the same concentrations. AMPA was the most potent agonist with respect to either effect.

The question was studied whether the variability of the immediate releasing effect of NMDA (Figure 2) was due to the use of slices from different parts of the occipito-parietal cortex. Slices from the occipital area on the one hand, and the parietal area on the other, were studied separately. Moreover, slices from the outer 0.3 mm cortical layer which normally was discarded were examined in comparison with the standard slices from 0.3–0.7 mm below the surface. No difference was found: NMDA 100 μM, in Mg^{2+}-free medium, elicited increases in tritium outflow similar in magnitude and variability, irrespective of the area and layer chosen ($n = 6-8$ for each group; not shown).

In some experiments carried out in the presence of Mg^{2+}, NMDA 100 μM was added 3 or 12 min instead of the usual 24 min before S_2. The electrically evoked overflow of tritium was evaluated only if the immediate releasing effect of NMDA was so small that b_2/b_1 in the individual slice was below 1.1 (and b_2/b_1 ratios on average did not differ significantly from control). The overflow of tritium evoked by S_2 was significantly ($P<0.01$) reduced to 59.9 ± 6.8% of control after 3 min ($n = 5$) and to 56.7 ± 4.7% after 12 min of contact with NMDA ($n = 5$), values not significantly different from the 71.7 ± 4.7% (Figure 2b) obtained after 24 min of contact with NMDA 100 μM ($n = 14$).

The selective agonist at metabotropic glutamate receptors, ACPD, when added 24 min before S_2 at concentrations of 10 and 100 μM, caused no immediate release and did not change the b_2/b_1 ratio. The electrically evoked overflow of tritium (S_2) also remained unchanged ($n = 3-5$).

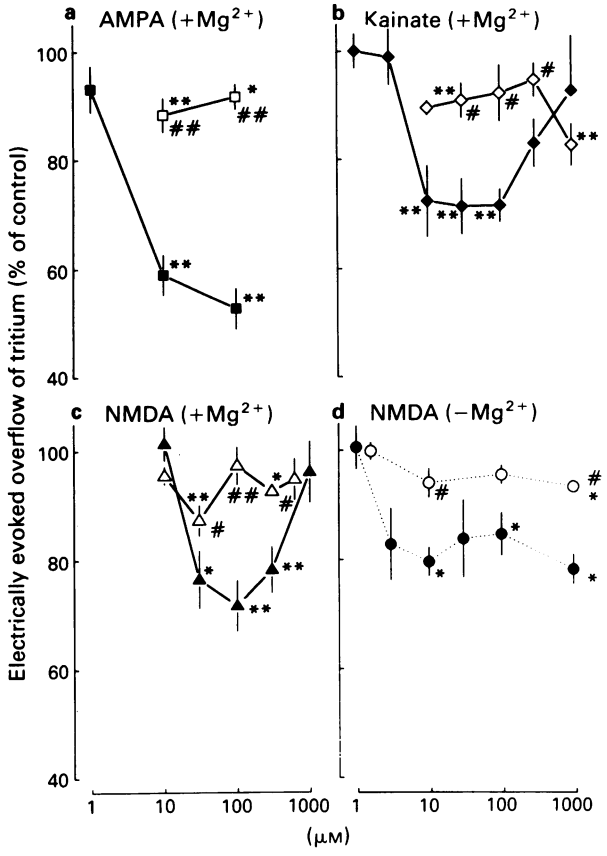


Figure 3 Interaction of glutamate receptor agonists with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on electrically evoked tritium overflow. Slices were stimulated twice (S_1 and S_2). α-Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; a), kainate (b) or N-methyl-D-aspartate (NMDA; c and d) was added 24 min before S_2. In some experiments, incubation and superfusion media were Mg^{2+}-free (d) instead of containing the normal 1.2 mM Mg^{2+} (a-c). Filled symbols represent experiments in which the medium was DPCPX-free (from Figure 2). Open symbols represent experiments in which the medium contained DPCPX 10 nM throughout superfusion. Abscissae, concentration of glutamate receptor agonists. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means ± s.e.mean of 4–21 experiments. Significant differences from control (no agonist): $*P<0.05$ and $**P<0.01$. Significant differences from experiments without DPCPX: $\#P<0.05$ and $\#\#P<0.01$.

Possible mediators

In a search for possible mediators of the inhibitory effects of AMPA, kainate and NMDA, their interaction with other compounds was examined that were added throughout superfusion. The interaction with the adenosine A_1-receptor antagonist, DPCPX (Bruns *et al.*, 1987; Lohse *et al.*, 1987) was studied most extensively. DPCPX greatly attenuated or abol-

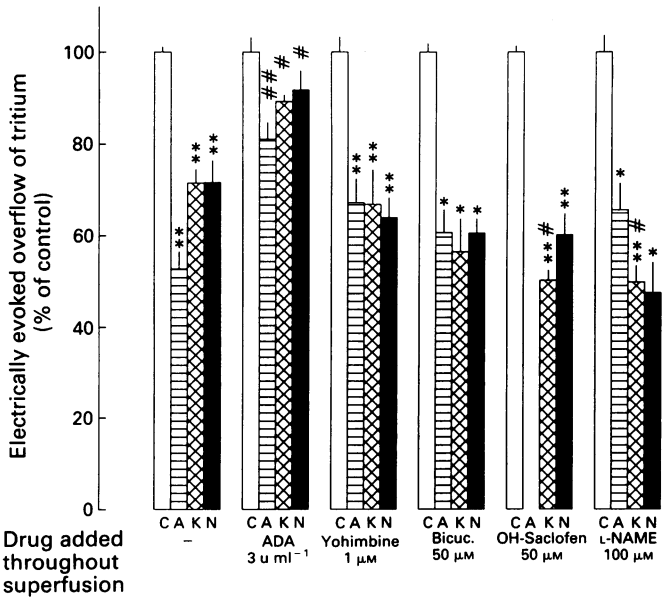


Figure 4 Interaction of glutamate receptor agonists with adenosine deaminase (ADA), yohimbine, bicuculline (Bicuc), 2-hydroxysaclofen (OH-Saclofen) and N^G-nitro-L-arginine methyl ester (L-NAME) on electrically evoked tritium overflow. Slices were stimulated twice (S_1 and S_2). α-Amino-3-hydroxy-5-methylisoxazole-4-propionate 100 μM (A; horizontally striped columns), kainate 100 μM (K; crosshatched columns), N-methyl-D-aspartate 100 μM (N; solid columns) or solvent (C, control; open columns) was added 24 min before S_2. Other drugs were present throughout superfusion; experiments without these interacting drugs are from Figure 2. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means ± s.e.mean of 4–33 experiments. Significant differences from C, $*P<0.05$ and $**P<0.01$. Significant differences from experiments without interacting drugs: $\#P<0.05$ and $\#\#P<0.01$.

ished the depression, by a wide range of concentrations of any of the three agonists, of the electrically evoked overflow of tritium (Figure 3). The inhibition by AMPA, kainate and NMDA 100 μM was also abolished by adenosine deaminase (Figure 4). In contrast, no attenuation of the inhibition was observed in the presence of the α_2-adrenoceptor antagonist yohimbine, the γ-aminobutyric acid$_A$ ($GABA_A$) receptor antagonist bicuculline, the $GABA_B$ receptor antagonist 2-hydroxysaclofen (Kerr et al., 1988) and the inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME; Rees et al., 1990) (Figure 4). The immediate releasing effects of AMPA, kainate and NMDA were not changed by any of the interacting drugs, except for a significant ($P<0.05$) increase of the effect of kainate 100 μM by L-NAME and a marked but statistically non-significant reduction of the effect of NMDA 100 μM by DPCPX (not shown).

The interacting drugs caused changes in b_1 and S_1 values (Table 1). For example, DPCPX, adenosine deaminase, yohimbine and L-NAME increased the evoked overflow of tritium (S_1).

Receptors involved

The receptors involved in the inhibitory effects of AMPA, kainate and NMDA were further studied by means of the antagonists AP5, which is selective for NMDA receptors (Davies et al., 1981; Evans et al., 1982), and CNQX, which selectively blocks non-NMDA receptors (Fletcher et al., 1988; Honoré et al., 1988). The agonists were tested at a concentration of 100 μM (Figure 5). AP5 100 μM abolished the effect of NMDA but failed to alter the effects of AMPA and kainate. CNQX 30 and 100 μM, on the other hand, significantly attenuated the inhibition produced by AMPA but did not change the inhibition produced by NMDA and kainate (Figure 5). The same pattern was found for the immediate releasing effects of the agonists: AP5 100 μM selectively abolished the effect of NMDA, whereas CNQX 30 and 100 μM selectively abolished the effect of AMPA (not shown).

The only change in b_1 and S_1 values was an increase by CNQX of the electrically evoked overflow of tritium (S_1; Table 1).

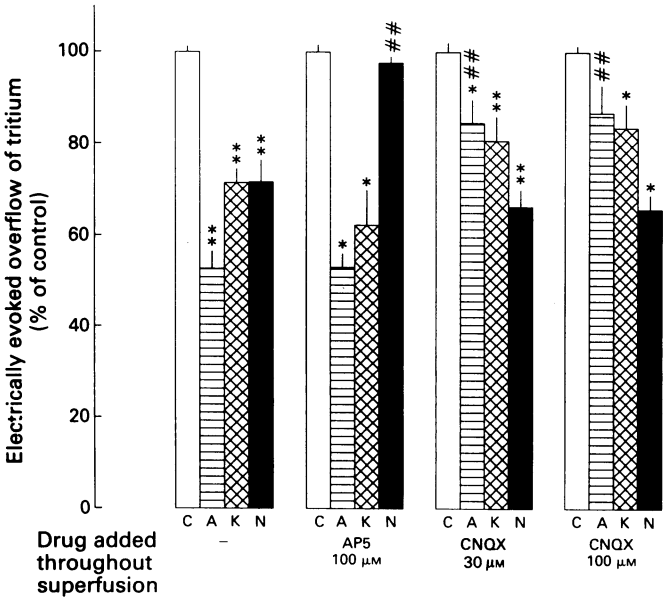


Figure 5 Interaction of glutamate receptor agonists with 2-amino-5-phosphonopentanoate (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on electrically evoked tritium overflow. Slices were stimulated twice (S_1 and S_2). α-Amino-3-hydroxy-5-methylisoxazole-4-propionate 100 μM (A; horizontally striped columns), kainate 100 μM (K; crosshatched columns), N-methyl-D-aspartate 100 μM (N; solid columns) or solvent (C, control; open columns) was added 24 min before S_2. AP5 and CNQX were present throughout superfusion; experiments without AP5 and CNQX are from Figure 2. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means ± s.e.mean of 4–33 experiments. Significant differences from C, $*P<0.05$ and $**P<0.01$. Significant differences from experiments without AP5 and CNQX: $\#\#P<0.01$.

Discussion

Under the conditions used, the electrically evoked overflow of tritium represents an action potential-induced, neuronal release of [^{3}H]-noradrenaline (see Reichenbacher et al., 1982). Similarly, the immediate increase in tritium outflow elicited by AMPA, kainate and NMDA certainly reflected a glutamate receptor-mediated release of [^{3}H]-noradrenaline (see Introduction). Brief high-frequency pulse trains were used for electric stimulation in order to minimize the presynaptic α_2-autoinhibition of the release of [^{3}H]-noradrenaline (Singer, 1988) which may cloud effects mediated by other presynaptic receptors (Allgaier et al., 1987; Limberger et al., 1988). Yohimbine nevertheless increased the release of [^{3}H]-noradrenaline elicited by S_1 (Table 1). However, the increase was slight. Moreover, differences between S_1 (and b_1) values must be viewed with caution since this kind of study is planned to compared S_2/S_1 (and b_2/b_1) ratios; conclusions from minor changes in S_1 (b_1) should be drawn only when there is additional evidence; the increase caused by DPCPX and adenosine deaminase, for example (Table 1), is a real change, also obtained when these compounds are added before S_2 and reflecting removal of an inhibition by endogenous adenosine (von Kügelgen et al., 1992a,c). Desipramine was present throughout superfusion in all experiments for two reasons: to increase the signal measured, i.e. electrically evoked overflow of tritium, by inhibition of [^{3}H]-noradrenaline re-uptake; and to rule out the possibility that drug effects were due to changes in this re-uptake.

AMPA, kainate and NMDA all reduced the electrically evoked release of [^{3}H]-noradrenaline. Apart from this basic fact which confirms and extends previous findings with glutamate (von Kügelgen et al., 1992a), the present study allows us to draw two main conclusions: that, as in the case of glutamate, the inhibition was due to adenosine; and that it can be elicited by activation of each of the three types of ionotropic glutamate receptor, AMPA, kainate and NMDA receptors, but not by activation of metabotropic glutamate receptors.

In support of the first conclusion, both the A_1 antagonist DPCPX and adenosine deaminase attenuated and in most cases even abolished the inhibitory effects of AMPA, kainate and NMDA. The blockade occurred without simultaneous suppression of the immediate releasing effect of AMPA, kainate and NMDA and, hence, was not due to some antagonism at glutamate receptors. The interaction is exactly analogous to the interaction of DPCPX and adenosine deaminase with glutamate (von Kügelgen et al., 1992a), indicating that, as far as the role of adenosine is concerned, the mechanisms of action are identical. None of the release-inhibiting effects was attenuated by yohimbine, bicuculline, 2-hydroxysaclofen or L-NAME, indicating that neither an activation of presynaptic α_2-adrenoceptors nor release of GABA or an induction of NO synthesis was involved.

The involvement of AMPA receptors in the effect of AMPA, of kainate receptors in the effects of kainate, and of NMDA receptors in the effect of NMDA is supported by the fact that the agonists produced inhibition at concentrations (10–30 μM) at which they elicit other effects through these respective receptors, most interestingly perhaps the immediate release of noradrenaline (Jones et al., 1987; Hoehn et al., 1990; Fink et al., 1989; 1992; Pittaluga & Raiteri, 1992; and see below). Moreover, only the inhibition of NMDA but not

the inhibition by AMPA and kainate was blocked by the selective NMDA receptor antagonist, AP5. Conversely, only the inhibition by AMPA but not the inhibition by NMDA and kainate was significantly attenuated by CNQX, the ionotropic glutamate receptor affinity of which declines in the order AMPA > kainate >> NMDA (Fletcher *et al.*, 1988; Honoré *et al.*, 1988). Due to lack of an appropriate antagonist, selective blockade of the effect of kainate could not be demonstrated. However, the fact that CNQX attenuated the effect of AMPA but not of kainate, although the concentration of AMPA and kainate chosen (100 μM) occupied a similar position on each agonist's concentration-response curve (Figure 2b), is best explained by an action of kainate at a separate, i.e. the kainate, receptor.

Our receptor characterization refers directly to the glutamate receptors involved in inhibition of [^{3}H]-noradrenaline release, but indirectly it also shows that AMPA, kainate and NMDA receptors, when activated, all elicit release of adenosine (or an adenine nucleotide which is then degraded to adenosine). In agreement with this conclusion from rabbit brain slices it has been demonstrated directly that activation of any of the three ionotropic glutamate receptor releases adenosine in rat brain cortex slices (Hoehn & White, 1990; Craig & White, 1993). There are additional parallels. The release by NMDA of adenosine from rat brain cortex slices, like the inhibition by NMDA of the release of [^{3}H]-noradrenaline from rabbit brain cortex slices, was observed both in the absence and in the presence of Mg^{2+} (Hoehn *et al.*, 1990, and Figure 2b). In rat brain cortex slices in the absence of Mg^{2+}, NMDA was more potent in releasing adenosine than [^{3}H]-noradrenaline (Hoehn *et al.*, 1990), and similarly in rabbit brain cortex slices in the absence of Mg^{2+}, NMDA was more potent in inhibiting electrically evoked [^{3}H]-noradrenaline release than in releasing [^{3}H]-noradrenaline (Figure 2). The possibility of a release of adenosine following activation of metabotropic glutamate receptors has not been studied previously; the lack of effect of ACPD in our experiments suggests that such release does not occur.

For reasons we still do not understand, glutamate itself failed to release [^{3}H]-noradrenaline from rabbit cortical slices (von Kügelgen *et al.*, 1992a). AMPA, kainate and NMDA, on the other hand, all caused an immediate transient release in the rabbit. In terms of antagonism, the receptors involved behaved like those mediating the inhibition of electrically evoked release, indicating that, as in other species (see Introduction), all three ionotropic glutamate receptors can elicit the immediate releasing effect. The noradrenaline releasing effect of NMDA was blocked by Mg^{2+} in human, rat and mouse brain slices (Vezzani *et al.*, 1987; Fink *et al.*, 1989; 1992; Hoehn *et al.*, 1990; Zhao *et al.*, 1990) but not in rabbit brain slices (Figure 2a), possibly because the rabbit noradrenergic terminal axons were slightly depolarized under our conditions. It should be noted that the immediate releasing effect was not responsible for the subsequent inhibition of electrically evoked release: in the case of kainate, and of NMDA in the absence of Mg^{2+}, the inhibition occurred at concentrations below the [^{3}H]-noradrenaline-releasing threshold, and DPCPX as well as adenosine deaminase abolished the inhibition but not the releasing effect. The metabotropic receptor agonist, ACPD, once again did not share the effect of the ionotropic receptor agonists.

Glutamate, acting on NMDA receptors, and adenosine inhibited the same ω-conotoxin-sensitive, dihydropyridine-resistant, N-type Ca^{2+} current in rat hippocampal neurones (Chernesvkaya *et al.*, 1991). One explanation, not discussed by the authors, would be that the effect of glutamate was mediated by adenosine. Inhibition of N-type Ca^{2+} channels is a possible mechanism triggered by activation of prejunctional A_1-purinoceptors (see Fredholm & Hu, 1993). The Ca^{2+} channel effect (Chernesvkaya *et al.*, 1991) and the release-inhibiting effect (present study), hence, may be serial events following the activation of ionotropic EAA receptors, release of adenosine and activation by adenosine of prejunctional A_1-receptors.

We thank Dr R.B. Illing (Unit for Morphological Brain Research, HNO-Klinik, Freiburg, Germany) for histological analysis of the brain slices. The study was supported by the Jung-Stiftung, Hamburg, and the Deutsche Forschungsgemeinschaft (SFB 325).

References

ALLGAIER, C., HERTTING, G. & VON KÜGELGEN, O. (1987). The adenosine receptor-mediated inhibition of noradrenaline release possibly involves a N-protein and is increased by α_2-autoreceptor blockade. *Br. J. Pharmacol.*, **90**, 403–412.

BLANDINA, P., JOHNSON, D., WALCOTT, J. & GOLDFARB, J. (1992). Release of endogenous norepinephrine from rat hypothalamus by stimulation of N-methyl-D-aspartic acid receptors. *J. Pharmacol. Exp. Ther.*, **263**, 61–68.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 59–63.

CHERNEVSKAYA, N.I., OBUKHOV, A.G. & KRISHTAL, O.A. (1991). NMDA receptor agonists selectively block N-type calcium channels in hippocampal neurons. *Nature*, **349**, 418–420.

COLLINGRIDGE, G.L. & LESTER, R.A.J. (1989). Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.*, **40**, 143–210.

CRAIG, C.G. & WHITE, T.D. (1993). N-Methy-D-aspartate- and non-N-methyl-D-aspartate-evoked adenosine release from rat cortical slices: distinct purinergic sources and mechanisms of release. *J. Neurochem.*, **60**, 1073–1080.

DAVIES, J., FRANCIS, A.A., JONES, A.W. & WATKINS, J.C. (1981). 2-Amino-5-phosphonovalerate (2APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci. Lett.*, **21**, 77–81.

EVANS, R.H., FRANCIS, A.A., JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1982). The effects of a series of ω-phosphonic α-carboxylic amino acids on electrically evoked and excitant amino acid-induced responses in isolated spinal cord preparations. *Br. J. Pharmacol.*, **75**, 65–75.

FINK, K. & GÖTHERT, M. (1992). Presynaptic site of action underlying the ethanol-induced inhibition of norepinephrine release evoked by stimulation of N-methyl-D-aspartate (NMDA) receptors in rat cerebral cortex. *Brain Res.*, **572**, 27–32.

FINK, K., GÖTHERT, M., MOLDERINGS, G. & SCHLICKER, E. (1989). N-Methyl-D-aspartate (NMDA) receptor-mediated stimulation of noradrenaline release, but not release of other neurotransmitters, in the rat brain cortex: receptor location, characterization and desensitization. *Naunyn-Schmied. Arch. Pharmacol.*, **339**, 514–521.

FINK, K., SCHULTHEIß, R. & GÖTHERT, M. (1992). Stimulation of noradrenaline release in human cerebral cortex mediated by N-methyl-D-aspartate (NMDA) and non-NMDA receptors. *Br. J. Pharmacol.*, **106**, 67–72.

FLETCHER, E.J., MARTIN, D., ARAM, J.A., LODGE, D. & HONORÉ, T. (1988). Quinoxalinediones selectively block quisqualate and kainate receptors and synaptic events in rat neocortex and hippocampus and frog spinal cord *in vitro*. *Br. J. Pharmacol.*, **95**, 585–597.

FREDHOLM, B.B. & HU, P.S. (1993). Effect of an intracellular calcium chelator on the regulation of electrically evoked [^{3}H]-noradrenaline release from rat hippocampal slices. *Br. J. Pharmacol.*, **108**, 126–131.

HOEHN, K., CRAIG, C.G. & WHITE, T.D. (1990). A comparison of N-methyl-D-aspartate-evoked release of adenosine and [^{3}H]norepinephrine from rat cortical slices. *J. Pharmacol. Exp. Ther.*, **255**, 174–181.

HOEHN, K. & WHITE, T.D. (1989). Evoked release of endogenous adenosine from rat cortical slices by K^+ and glutamate. *Brain Res.*, **478**, 149–151.

HOEHN, K. & WHITE, T.D. (1990). N-Methyl-D-aspartate, kainate and quisqualate release endogenous adenosine from rat cortical slices. *Neuroscience*, **39**, 441–450.

HONORÉ, T., DAVIES, S.N., DREJER, J., FLETCHER, E.J., JACOBSEN, P., LODGE, D. & NIELSEN, F.E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science*, **241**, 701–703.

JHAMANDAS, K. & DUMBRILLE, A. (1980). Regional release of [^{3}H]adenosine derivatives from rat brain in vivo: effect of excitatory amino acids, opiate agonists, and benzodiazepines. *Can. J. Physiol. Pharmacol.*, **58**, 1262–1278.

JONES, S.M., SNELL, L.D. & JOHNSON, K.M. (1987). Phencyclidine selectively inhibits N-methyl-D-aspartate-induced hippocampal [^{3}H]norepinephrine release. *J. Pharmacol. Exp. Ther.*, **240**, 492–497.

KERR, D.I.B., ONG, J., JOHNSTON, G.A.R., ABBENANTE, J. & PRAGER, R.H. (1988). 2-Hydroxy-saclofen: an improved antagonist at central and peripheral $GABA_B$ receptors. *Neurosci. Lett.*, **92**, 92–96.

LIMBERGER, N., SPÄTH, L. & STARKE, K. (1988). Presynaptic α_2-adrenoceptor, opioid κ-receptor and adenosine A_1-receptor interactions on noradrenaline release in rabbit brain cortex. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 53–61.

LOHSE, M.J., KLOTZ, K.N., LINDENBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **336**, 204–210.

NELSON, M.F., ZACZEK, R. & COYLE, J.T. (1980). Effects of sustained seizures produced by intrahippocampal injection of kainic acid on noradrenergic neurons: evidence for local control of norepinephrine release. *J. Pharmacol. Exp. Ther.*, **214**, 694–702.

PERKINS, M.N. & STONE, T.W. (1983). In vivo release of [^{3}H]-purines by quinolinic acid and related compounds. *Br. J. Pharmacol.*, **80**, 263–267.

PITTALUGA, A. & RAITERI, M. (1992). N-Methyl-D-aspartic acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. I. Location on axon terminals and pharmacological characterization. *J. Pharmacol. Exp. Ther.*, **260**, 232–237.

PULL, I. & MCILLWAIN, H. (1975). Actions of neurohumoral agents and cerebral metabolites on output of adenine derivatives from superfused tissues of the brain. *J. Neurochem.*, **24**, 695–700.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

REICHENBACHER, D., REIMANN, W. & STARKE, K. (1982). α-Adrenoceptor-mediated inhibition of noradrenaline release in rabbit brain cortex slices. *Naunyn-Schmied. Arch. Pharmacol.*, **319**, 71–77.

SCHOEPP, D., BOCKAERT, J. & SLADECZEK, F. (1990). Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. *Trends Pharmacol. Sci.*, **11**, 508–515.

SINGER, E.A. (1988). Transmitter release from brain slices elicited by single pulses: a powerful method to study presynaptic mechanisms. *Trends Pharmacol. Sci.*, **9**, 274–276.

VEZZANI, A., WU, H.Q. & SAMANIN, R. (1987). [^{3}H]Norepinephrine release from hippocampal slices is an in vitro biochemical tool for investigating the pharmacological properties of excitatory amino acid receptors. *J. Neurochem.*, **49**, 1438–1442.

VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992a). Adenosine but not an adenine nucleotide mediates tonic purinergic inhibition, as well as inhibition by glutamate, of noradrenaline release in rabbit brain cortex slices. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 677–684.

VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992b). A purinergic mechanism is involved in the inhibitory effects of glutamate, N-methyl-D-aspartate and kainic acid but not of methoxamine on the electrically evoked release of noradrenaline in rabbit brain cortex. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, R60.

VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992c). Stable adenine nucleotides inhibit [^{3}H]-noradrenaline release in rabbit brain cortex slices by direct action at presynaptic adenosine A_1-receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 187–196.

WANG, J.K.T., ANDREWS, H. & THUKRAL, V. (1992). Presynaptic glutamate receptors regulate noradrenaline release from isolated nerve terminals. *J. Neurochem.*, **58**, 204–211.

WATKINS, J.C., KROGSGAARD-LARSEN, P. & HONORÉ, T. (1990). Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharmacol. Sci.*, **11**, 25–33.

ZHAO, X.H., KITAMURA, Y. & NOMURA, Y. (1990). Involvement of glutamate receptor subtypes in L-[^{3}H]noradrenaline release from cerebral cortical and hippocampal slices of mice. *Neurochem. Int.*, **16**, 11–16.

(Received June 18, 1993
Revised August 11, 1993
Accepted August 13, 1993)

Br. J. Pharmacol. (1993), **110**, 1551–1555 © Macmillan Press Ltd, 1993

Effects of selective antagonism of β-adrenoceptor sub-types on responses to isoprenaline in rat distal colon *in vitro*

[1]Allan MacDonald & Michelle Lamont

Department of Biological Sciences, Glasgow Caledonian University, City Campus, Cowcaddens Road, Glasgow G4 0BA

1 The effects of the β_1- and β_2-adrenoceptor selective antagonists, CGP 20712A and ICI 118551 respectively, on responses to isoprenaline-induced relaxation of rat distal colon were investigated in order to determine the contributions of these subtypes to relaxation. In addition, the properties of ICI D7114, a novel putative stimulant of atypical β-adrenoceptors, were investigated. Our preliminary experiments with ICI D7114 showed that this compound lacked agonist activity in rat distal colon and in fact antagonized responses to isoprenaline. We therefore studied the antagonism of isoprenaline by ICI D7114 in more detail.

2 Longitudinal segments of rat distal colon were suspended in Krebs solution at 37°C for isometric recording. The Krebs solution contained EDTA (23 μM) and prazosin (0.1 μM) and was gassed with 95/5% O_2/CO_2. After an initial equilibration period, reproducible contractions to a submaximal concentration of methacholine (1 μM) were obtained before carrying out a concentration-response curve (CRC) to isoprenaline in a non-cumulative manner. Four consecutive CRCs to isoprenaline were carried out in each tissue with a 1 h interval between each curve. Antagonists were present in increasing concentrations during the intervals between CRCs. Control tissues received no antagonists to allow estimation of the magnitude of time-dependent changes.

3 Isoprenaline produced a concentration-dependent inhibition of methacholine-induced contractions. CRCs to isoprenaline were reproducible with no significant time-dependent changes. Propranolol produced no shift of the isoprenaline CRC at 0.01 μM and a 5 fold shift at 0.1 μM. No further shift was observed with 1 μM. CGP 20712A had no effect on the CRC to isoprenaline at 0.1, 1 and 3 μM. ICI 118551 produced little or no shift at 0.1 μM and a six fold shift with 1 μM. No further shift was observed with 3 μM. ICI D7114 produced a concentration-dependent parallel rightward shift of the CRC to isoprenaline. Schild analysis gave a slope close to unity and a mean pA_2 value of 7.29 for ICI D7114.

4 The results with propranolol and β_1- and β_2-adrenoceptor antagonists confirm the mainly atypical nature of β-adrenoceptors in rat distal colon. There may also be a small contribution from β_2-adrenoceptors in the response to isoprenaline but β_1-adrenoceptors are absent. ICI D7114 has no agonist activity and behaves as a relatively high affinity reversible competitive antagonist of atypical β-adrenoceptors in this preparation.

Keywords: β-Adrenoceptor; β_1-adrenoceptor; β_2-adrenoceptor; β_3-adrenoceptor; atypical β-adrenoceptor; rat distal colon; isoprenaline; smooth muscle

Introduction

β-Adrenoceptors were originally subclassified by Lands *et al.* (1967a,b) into β_1- and β_2-subtypes on the basis of agonist potency. More recently, pharmacological studies have suggested the existence of atypical β-adrenoceptors, or β_3-adrenoceptors, characterized by resistance to classical β-adrenoceptor antagonists and by selective stimulation by novel synthetic agonists such as BRL 37344 (reviewed by Zaagsma & Nahorski, 1990).

In the gastrointestinal tract β-adrenoceptors are well known to mediate relaxation of smooth muscle. In the study by Lands *et al.* (1967b) the β-adrenoceptor mediating relaxation in rabbit jejunum was classified as a β_1-adrenoceptor. Later studies showed that gastrointestinal tissues may also relax via β_2-adrenoceptors e.g. rabbit colon (Sim & Lim, 1983) or via a mixture of β_1- and β_2-adrenoceptors e.g. rat gastric fundus (Lefebvre *et al.*, 1984). In rat colon a mixture of β_1- and β_2-adrenoceptors was also found, with the β_1-adrenoceptors located on enteric neurones and the β_2-adrenoceptors located post-junctionally, on the smooth muscle (Ek, 1985). Experiments with BRL 37344, the atypical β-adrenoceptor agonist, also suggested the presence of atypical β-adrenoceptors, or β_3-adrenoceptors, mediating relaxation in several gut preparations, for example, guinea-pig ileum (Bond & Clarke, 1988), rat distal colon (McLaughlin & MacDonald, 1990) and rat gastric fundus (McLaughlin & MacDonald, 1991).

Our own previous studies in rat distal colon showed that β-adrenoceptor-mediated relaxation to isoprenaline was poorly antagonized by propranolol and that the selective adipocyte β-adrenoceptor stimulant, BRL 37344 was also a potent relaxant agent. In the present study we investigated the effects of the selective β_1- and β_2-adrenoceptor antagonists CGP 20712A (Dooley *et al.*, 1986) and ICI 118551 (O'Donnell & Wanstall, 1980) on isoprenaline-induced relaxation of rat distal colon in order to determine the contributions of different subtypes to relaxation. In addition, the properties of ICI D7114, a novel putative stimulant of atypical β-adrenoceptors (Holloway *et al.*, 1991; 1992) were investigated. Our preliminary experiments with ICI D7114 showed that this compound lacked agonist activity in rat distal colon and in fact antagonized responses to isoprenaline. We therefore studied the antagonism of isoprenaline by ICI D7114 in more detail.

A preliminary account of this work has been presented to the British Pharmacological Society (MacDonald & Lamont, 1992).

Methods

Male Wistar rats (200–300 g) were killed by a blow to the head and cervical dislocation. The distal 6 cm of the colon

[1] Author for correspondence.

was removed and immediately placed in Krebs physiological saline solution (PSS), at room temperature. The colon segments were cleared of any faecal material by gentle squeezing with the fingers and then cut into 3 cm segments. The segments were suspended in organ baths containing 30 ml Krebs solution, at 37°C, bubbled continuously with 95% O_2 and 5% CO_2, under an initial tension of 1 g. The composition of the Krebs solution was as follows (mM): NaCl 118, $CaCl_2$ 2.5, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2. and glucose 11.1. The Krebs solution contained EDTA (23 μM) and prazosin (0.1 μM). Tissues were allowed to equilibrate for at least 40 min before experimental procedures were begun. Reproducible contractions to a submaximal concentration of methacholine (1 μM) were obtained before carrying out a concentration-response curve (CRC) to isoprenaline in a non-cumulative manner. Four CRCs were carried out in each tissue, with a 1 h interval between CRCs, thus allowing a 1 h equilibration time for antagonists which were added at the end of each CRC. The concentration of antagonist was increased after each consecutive CRC. Control tissues received no antagonist to allow estimation of the magnitude of time-dependent changes.

Statistical analysis

Results are expressed as means ± s.e.mean with the number of observations, n, in parentheses with the exception of the regression line slope which is expressed as slope ± 95% confidence limits. Statistical significance between two data sets was tested by a paired t test. A probability level of $P<0.05$ was considered to be statistically significant.

Schild plot

A Schild plot (Arunlakshana & Schild, 1959) for the antagonism of isoprenaline by ICI D7114 was constructed as follows. Agonist concentration ratios (CRs) were obtained from individual EC_{50} values with and without antagonist. The plot of log (CR − 1) versus log [antagonist] (Schild plot) was analysed by linear regression. Antagonism was considered to be simple competitive in nature since the slope of the regression line was not significantly different from unity. A mean pA_2 value was obtained from individual estimates from the equation:

$$pA_2 = \log(CR - 1) - \log[\text{antagonist}]$$

after verifying that there was no significant regression of pA_2 on antagonist concentration (McKay, 1978).

Drugs used

The following drugs were dissolved in distilled water: acetyl-β-methyl choline (methacholine) chloride (Sigma); CGP 207-12A (1-[2-((3-carbamoyl-4-hydroxy) phenoxy) ethylamino]-3-[4- (1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol methanesulphonate) (gifted by ICI Pharmaceuticals); ICI D7114 ((S)-4-[2-hydroxy-3-phenoxypropylaminoetoxy]-N-(2-methoxyethyl)phenoxyacetamide) (gifted by ICI Pharmaceuticals); ICI 118551 (erythro-(±)-1(7-methylindan-4-yloxy) -3-isopropylaminobutan-2-ol) (gifted by ICI Pharmaceuticals); (−)-isoprenaline (+) bitartrate (Sigma); prazosin hydrochloride (synthesized by Reckitt & Colman plc); (±)-propranolol hydrochloride (Sigma).

Results

Isoprenaline produced a concentration-dependent inhibition of methacholine-induced contractions (Figures 1 and 2). CRCs to isoprenaline were reproducible, there being little or no change in the sensitivity to isoprenaline with four consecutive CRCs, separated by an interval of 1 h (Figure 2a). In subsequent experiments therefore, antagonists were present during the intervals between curves in progressively increasing concentrations. Tissues with no antagonist present were also studied and served as time controls.

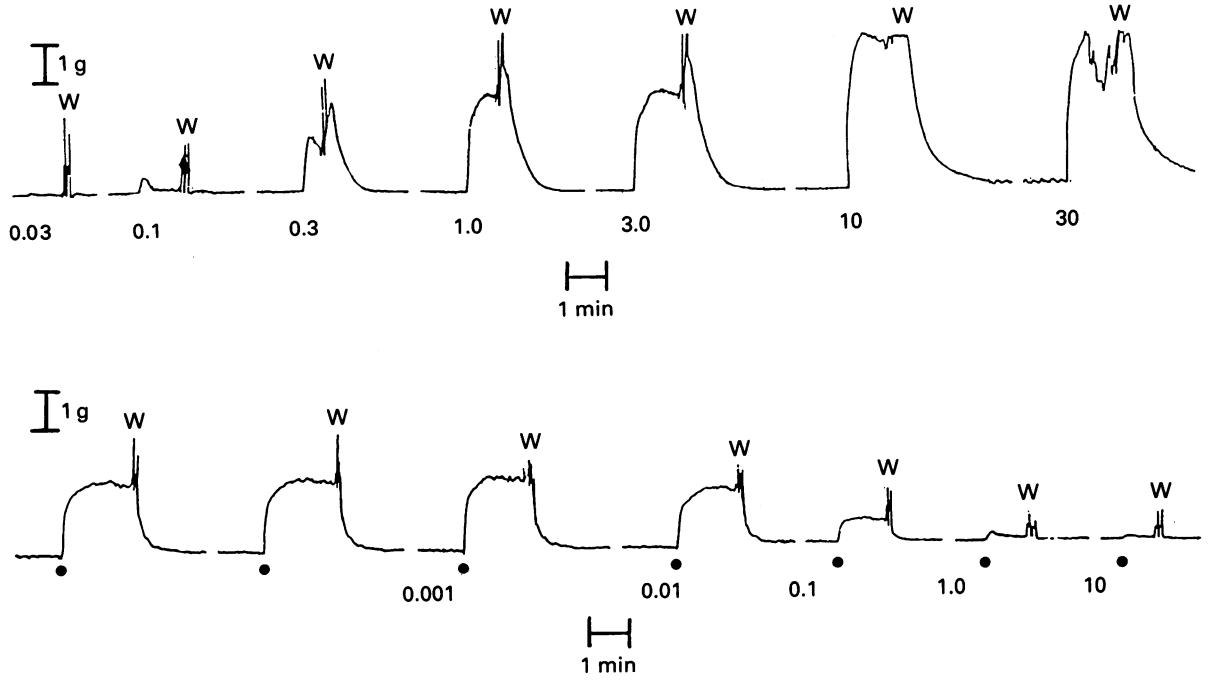


Figure 1 Representative tracings of the effects of methacholine and isoprenaline on rat distal colon *in vitro*. The upper trace shows the concentration-response to methacholine. Values shown are concentration (μM). Methacholine was added to the organ baths at approximately 3 min intervals. The tissues were washed (W) after the contractions had peaked. The lower trace shows the effect of isoprenaline on contractions to methacholine. After obtaining reproducible contractions to repeated administration of methacholine (1 μM) (●), increasing concentrations of isoprenaline were added 1 min before methacholine. Values are concentrations of isoprenaline (μM).

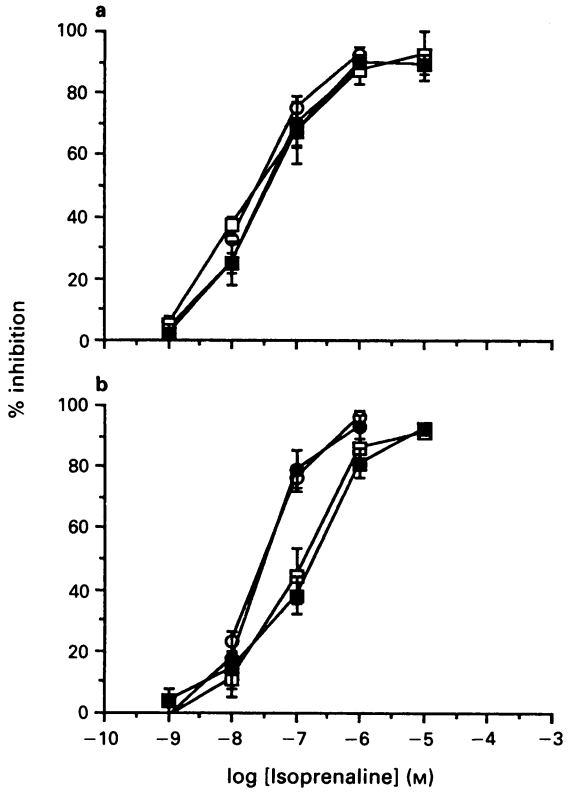


Figure 2 Concentration-response curves (CRCs) to isoprenaline in rat distal colon. (a) Time controls; (○) first, (●) second, (□) third and (■) fourth CRCs to isoprenaline, constructed at hourly intervals. (b) Effect of propranolol: (○) control, (●) propranolol 0.01 μM, (□) 0.1 μM, (■) 1.0 μM. Values are means ± s.e.mean of 5–8 experiments.

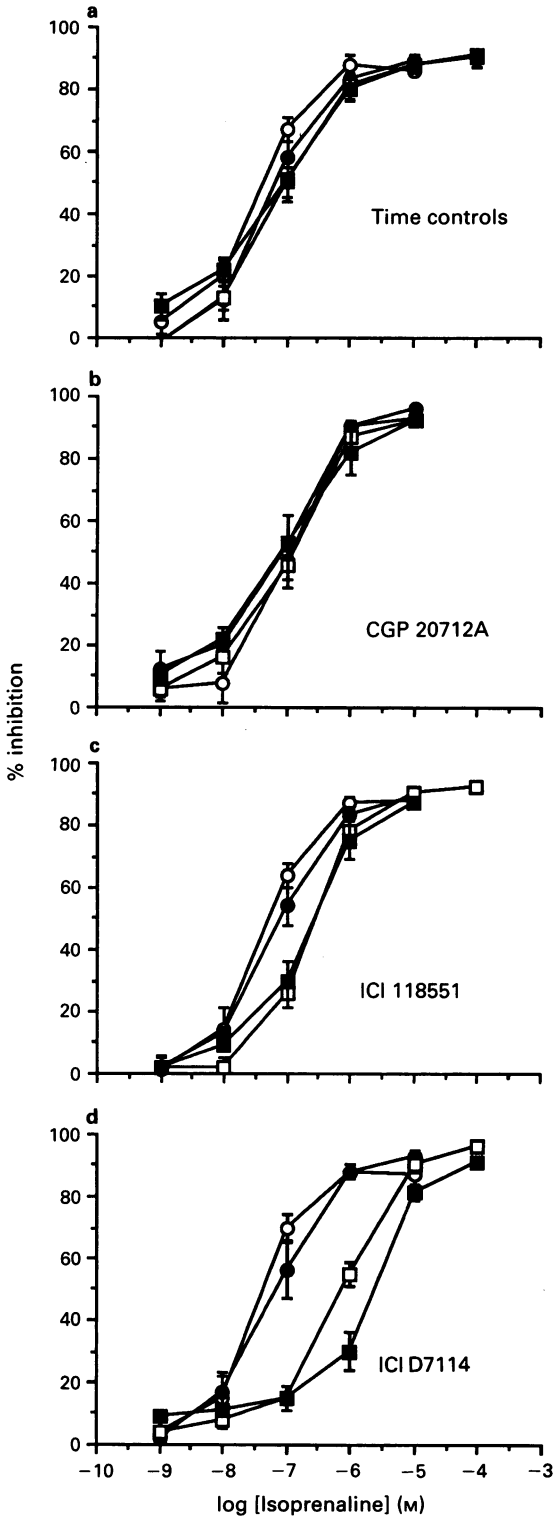


Figure 3 Concentration-response curves (CRCs) to isoprenaline in rat distal colon. (a) Time controls; (○) first, (●) second, (□) third and (■) fourth CRCs to isoprenaline, constructed at hourly intervals. (b) Effect of CGP 20712A: (○) control, (●) CGP 20712A 0.1 μM, (□) 1.0 μM, (■) 3.0 μM. (c) Effect of ICI 118551: (○) control, (●) ICI 118551 0.1 μM, (□) 1.0 μM, (■) 3.0 μM. (d) Effect of ICI D7114: (○) control, (●) ICI D7114 0.1 μM, (□) 1.0 μM, (■) 3.0 μM. Values are means ± s.e.mean of 4–5 experiments.

Propranolol had no effect on the isoprenaline CRC at a concentration of 0.01 μM and produced an approximately 5 fold shift at 0.1 μM (Figure 2b). Increasing the propranolol concentration to 1 μM produced no further shift of the isoprenaline CRC.

CGP 20712A had no effect on the isoprenaline CRC at concentrations of 0.1, 1 and 3 μM (Figure 3b).

ICI 118551 had no effect on the isoprenaline CRC at 0.1 μM, produced an approximately 6 fold shift at 1 μM and produced no further shift of the isoprenaline CRC at 3 μM (Figure 3c).

ICI D7114 (0.1, 1 and 3 μM) produced a concentration-dependent, parallel, rightward shift of the isoprenaline concentration-response curve, consistent with reversible competitive antagonism (Figure 3d). A Schild regression gave a straight line with a slope of 0.99 ± 0.29 (± 95% confidence limits, $n = 14$) supporting reversible competitive antagonism of isoprenaline by ICI D7114 (Figure 4). A mean pA_2 value of 7.29 ± 0.29 was calculated. Any possible agonist effects of ICI D7114 were investigated by comparing the sizes of the control response to methacholine in the presence of different concentrations of ICI D7114. No changes in the sizes of the control responses either in the time controls or in the tissues treated with ICI D7114 were seen (Figure 5) confirming a lack of agonist activity of ICI D7114 in this preparation.

Discussion

The results confirm previous observations that responses to isoprenaline in rat distal colon are poorly antagonized by propranolol (McLaughlin & MacDonald, 1990), although in this study there was a small propranolol-sensitive component to the response (5 fold shift with 0.1 and 1 μM propranolol). It is difficult to determine whether the effect of propranolol is due to blockade of β_1 and/or β_2-adrenoceptors or whether propranolol has some weak blocking activity at atypical β-

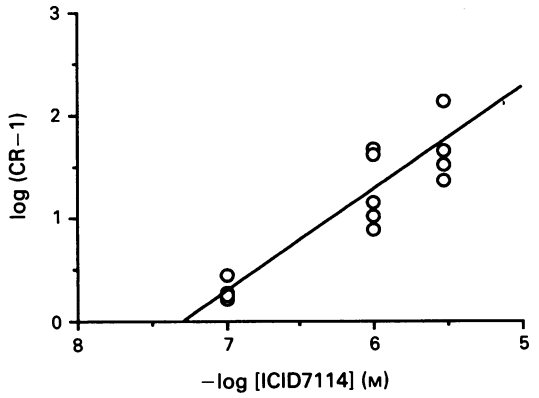


Figure 4 Schild plot for antagonism of isoprenaline by ICI D7114. The data are taken from experiments shown in Figure 3 (d).

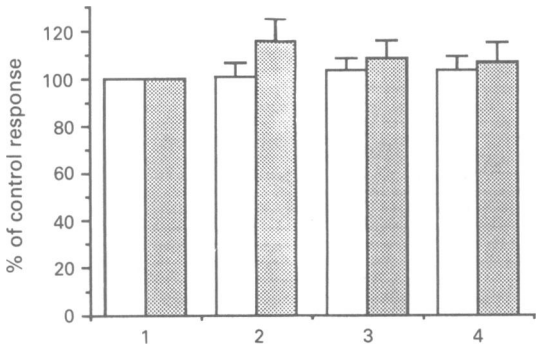


Figure 5 Effects of ICI D7114 on responses to methacholine in rat distal colon *in vitro*. Responses to methacholine are expressed as a % of the control responses for the first concentration-response curve (CRC) for the time controls (open columns) and the ICI D7114-treated preparations (stippled columns). Open columns show the time controls for the first (1), second (2), third (3) and fourth (4) CRCs. Stippled columns show control responses to methacholine in the presence of ICI D7114: (1) control, no ICI D7114, (2) 0.1 μM, (3) 1 μM, (4) 3 μM. Values are means ± s.e.mean of 5 experiments.

adrenoceptors in this preparation. However, the fact that raising the concentration of propranolol from 0.1 to 1 μM produced no further antagonism of responses would argue against a weak competitive antagonism of atypical β-adrenoceptors and would suggest instead that propranolol is removing a small contribution to the isoprenaline response from classical β-adrenoceptors.

The lack of effect of the selective β_1-adrenoceptor antagonist, CGP 20712A, on isoprenaline suggests that β_1-adrenoceptors do not contribute to the inhibitory effect of isoprenaline in this preparation. Inhibition of spontaneous activity via β_1-adrenoceptor activation has been previously reported in rat *proximal* colon (Ek, 1985). However in this case the β_1-adrenoceptors were located on enteric neurones.

The selective β_2-adrenoceptor antagonist, ICI 118551, at a concentration of 1 μM produced an approximately 6 fold shift of the isoprenaline CRC but raising the concentrations to 3 μM produced no further shift. These results might suggest that ICI 118551 is blocking a small proportion of β_2-adrenoceptors which contribute to the isoprenaline response. Ek (1985) also reported the presence of inhibitory post-junctional β_2-adrenoceptors on smooth muscle in rat proximal colon.

The shift, and pattern of shift, of the isoprenaline CRC produced by ICI 118551 was similar to that produced by propranolol, suggesting that ICI 118551 and propranolol may be blocking the same population of β_2-adrenoceptors. This is not entirely consistent with the results, however, since the concentration of propranolol producing a five fold shift was ten times lower than that of ICI 118551 producing a similar shift, whereas the reported pK_Bs for β_2-adrenoceptors are similar, 8.3–8.6 for propranolol (Wilson *et al.*, 1984) and 8.7 for ICI 118551 (O'Donnell & Wanstall, 1980). An alternative explanation is that propranolol and ICI 118551 are acting with weak affinity at atypical β-adrenoceptors. Both propranolol and ICI 118551 antagonized responses to isoprenaline in rat adipocytes with pA_2 values of 6.6 and 5.8 respectively (Wilson *et al.*, 1984) so that some blocking activity at atypical β-adrenoceptors might be expected at the concentrations used. However, as discussed earlier, the fact that raising the concentrations of the antagonists produced no further shifts would not be consistent with weak competitive antagonism at the atypical β-adrenoceptor. Since neither explanation is fully consistent with the findings, it may be that the results are due to a combination of effects: a weak affinity of both propranolol and ICI 118551 at the atypical β-adrenoceptor plus the presence of a small population of β_2-adrenoceptors.

The novel putative stimulant of atypical β-adrenoceptors, ICI D7114 (Holloway *et al.*, 1991), lacked agonist activity in this preparation and antagonized responses to isoprenaline. Since it is established that the β-adrenoceptors in rat distal colon are mainly atypical in nature, it would appear that ICI D7114 is behaving as an atypical β-adrenoceptor antagonist in this preparation. In addition, Schild analysis showed that ICI D7114 behaved as a reversible competitive antagonist at the atypical β-adrenoceptors with relatively high affinity (pA_2 value of 7.29) and may therefore prove to be a useful tool in elucidating the properties of these receptors. ICI D7114 has previously been reported to act as an agonist at atypical β-adrenoceptors in guinea-pig ileum but as an antagonist at atypical β-adrenoceptors in rat small intestine (Growcott *et al.*, 1992a,b). The difference in efficacy was attributed to differences in receptor reserve rather than to subtypes of the atypical receptor. The present results suggest that the rat colon is similar to the rat ileum although the pA_2 value for the antagonism of isoprenaline obtained in the present study in rat colon (7.29) was higher than that obtained by Growcott *et al.* (1992b) in rat ileum (6.4).

In conclusion, the results with β-adrenoceptor antagonists confirm the mainly atypical nature of β-adrenoceptors in rat distal colon. There may also be a small contribution from β_2-adrenoceptors in the response to isoprenaline but β_1-adrenoceptors are absent. ICI D7114 behaves as a relatively high affinity reversible competitive antagonist of atypical β-adrenoceptors in this preparation.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BOND, R.A. & CLARKE, D.E. (1988). Agonist and antagonist characterization of a putative adrenoceptor with distinct pharmacological properties from the α- and β-subtypes. *Br. J. Pharmacol.*, **95**, 723–734.

DOOLEY, D.J., BITTIGER, H. & REYMANN, N.C. (1986). CGP 20712 A: a useful tool for quantitating β_1- and β_2-adrenoceptors. *Eur. J. Pharmacol.*, **130**, 137–139.

EK, B. (1985). Studies on mechanisms for beta-adrenoceptor mediation inhibition of colon motility. *Acta. Physiol. Scand.*, **125**, suppl. 546, 5–39.

GROWCOTT, J.W., HOLLOWAY, B., WILSON, C., MAINWARING, S. & GREEN, M. (1992a). The novel brown adipocyte stimulant, ICI D7114, reveals possible differences in atypical β-adrenoceptor reserve. *Br. J. Pharmacol.*, **105**, 231P.

GROWCOTT, J.W., GREEN, M. & HOLLOWAY, B. (1992b). Atypical β-adrenoceptor antagonist profile of ICI D7114 in guinea-pig and rat isolated ileum. *Br. J. Pharmacol.*, **107**, 372P.

HOLLOWAY, B.R., HOWE, R., RAO, B.S., STRIBLING, D., MAYERS, R.M., BRISCOE, M.G. & JACKSON, J.M. (1991). ICI D7114 a novel selective β-adrenoceptor agonist selectively stimulates brown fat and increases whole-body oxygen consumption. *Br. J. Pharmacol.*, **104**, 97–104.

HOLLOWAY, B.R., HOWE, R., RAO, B.S. & STRIBLING, D. (1992). ICI D7114: a novel selective adrenoceptor agonist of brown fat and thermogenesis. *Am. J. Clin. Nutr.*, **55**, 262S–264S.

LANDS, A.M., ARNOLD, A., MCAULIFF, J.P., LUDUENA, F.P. & BROWN JUN, T.G. (1967a). Differentiation of receptor systems activated by sympathomimetic amines. *Nature*, **214**, 597–598.

LANDS, A.M., LUDUENA, F.P. & BUZZO, H.J. (1967b). Differentiation of receptors responsive to isoproterenol. *Life Sci.*, **6**, 2241–2249.

LEFEBVRE, R.A., VERPLANKEN, P.A. & BOGAERT, M.G. (1984). Pharmacological characterization of the post-junctional β-adrenoceptors in the rat gastric fundus. *Eur. J. Pharmacol.*, **106**, 1–9.

MACDONALD, A. & LAMONT, M. (1992). Relative contributions of β-adrenoceptor sub-types in isoprenaline-induced relaxation of rat distal colon. *Br. J. Pharmacol.*, **107**, 24P.

MCKAY, D. (1978). How should values of pA_2 and affinity constants for competitive antagonists be estimated? *J. Pharm. Pharmacol.*, **30**, 312–313.

MCLAUGHLIN, D.P. & MACDONALD, A. (1990). Evidence for the existence of 'atypical' β-adrenoceptors (β_3-adrenoceptors) mediating relaxation in the rat distal colon *in vitro*. *Br. J. Pharmacol.*, **101**, 569–574.

MCLAUGHLIN, D.P. & MACDONALD, A. (1991). Characterization of catecholamine-mediated relaxations in rat isolated gastric fundus: evidence for an atypical β-adrenoceptor. *Br. J. Pharmacol.*, **103**, 1351–1356.

O'DONNELL, S.R. & WANSTALL, J.C. (1980). Evidence that ICI 118,551 is a potent, highly $beta_2$-selective adrenoceptor antagonist and can be used to characterize beta-adrenoceptor populations in tissues. *Life Sci.*, **27**, 671–677.

SIM, M.K. & LIM, J.M.E. (1983). Adrenergic receptor-mediated response of the rabbit small and large intestine. *Jpn. J. Pharmacol.*, **33**, 409–413.

WILSON, C., WILSON, S., PIERCY, V., SENNITT, M.V. & ARCH, J.R.S. (1984). The rat lipolytic β-adrenoceptor: studies using novel β-adrenoceptor agonists. *Eur. J. Pharmacol.*, **100**, 309–319.

ZAAGSMA, J. & NAHORSKI, S.R. (1990). Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical 'β_3-adrenoceptor'. *Trends Pharmacol. Sci.*, **11**, 3–7.

(Received June 16, 1993
Revised August 10, 1993
Accepted August 16, 1993)

Br. J. Pharmacol. (1993), **110**, 1556–1564 © Macmillan Press Ltd, 1993

Antagonism of levcromakalim by imidazoline- and guanidine-derivatives in rat portal vein: involvement of the delayed rectifier

Timothy Ibbotson, Gillian Edwards & [1]Arthur H. Weston

Smooth Muscle Research Group, Department of Physiological Sciences, University of Manchester, Manchester M13 9PT

1 In rat whole portal veins, guanabenz (100 nM to 10 μM) and antazoline (100 nM to 100 μM) each increased the amplitude, frequency and duration of spontaneous contractions. In addition, guanabenz (30 μM) and antazoline (30 μM) each antagonized the ability of levcromakalim (3 nM to 10 μM) to inhibit the spontaneous contractions of this tissue.

2 Whole-cell voltage-clamp recordings were made from freshly-isolated rat portal vein cells dispersed by a collagenase/pronase enzyme treatment. The ability of several agents (antazoline, cirazoline, clonidine, guanabenz and phentolamine, each containing an imidazoline or guanidine moiety), to modulate potassium (K) currents and to inhibit the actions of levcromakalim was investigated.

3 Antazoline, cirazoline, clonidine, guanabenz and phentolamine (each at a concentration of 30 μM) had little effect on control non-inactivating currents but inhibited the delayed-rectifier current, $I_{K(V)}$.

4 Levcromakalim (1 μM) induced a non-inactivating current, $I_{K(ATP)}$, and also inhibited the delayed rectifier current, $I_{K(V)}$.

5 Glibenclamide (1 μM) had no effect on control delayed rectifier or non-inactivating currents, but it inhibited the simultaneous induction of $I_{K(ATP)}$ and reduction of $I_{K(V)}$ produced by levcromakalim (1 μM).

6 Antazoline, cirazoline, clonidine and guanabenz (each at a concentration of 30 μM) prevented the induction of $I_{K(ATP)}$ by levcromakalim (1 μM). Phentolamine (30 μM) and clonidine (30 μM) each inhibited the $I_{K(ATP)}$ generated by levcromakalim (1 μM).

7 It is concluded that a variety of agents which possess either an imidazoline (antazoline, cirazoline, clonidine and phentolamine) or a guanidine (guanabenz) moiety within their structure inhibit the delayed rectifier current, $I_{K(V)}$. This action may thus be mediated via a so-called non-adrenoceptor imidazoline binding site. Furthermore, the ability of these ligands to inhibit $I_{K(V)}$ and to antagonize both the induction of $I_{K(ATP)}$ and the vasorelaxation produced by levcromakalim is consistent with the view that the channel (K_{ATP}) which underlies $I_{K(ATP)}$ is a voltage-insensitive state of the delayed rectifier K-channel (K_V).

Keywords: Levcromakalim; clonidine; phentolamine; guanabenz; cirazoline; antazoline; rat portal vein; voltage-clamp; non-adrenoceptor imidazoline binding site; potassium channels

Introduction

Studies in this laboratory have shown that the structurally-dissimilar potassium (K)-channel openers, levcromakalim, P1060 and aprikalim each induce an ATP-sensitive current, $I_{K(ATP)}$, and simultaneously inhibit the delayed rectifier current, $I_{K(V)}$, in vascular smooth muscle (Noack *et al.*, 1992a,b,c; Ibbotson *et al.*, 1993; Criddle *et al.*, 1994). The induction of $I_{K(ATP)}$ by the K-channel openers is inhibited by phentolamine (Ibbotson *et al.*, 1993), an imidazoline derivative which has previously been shown to inhibit the relaxant effects of K-channel openers in smooth muscle (McPherson & Angus, 1989; Murray *et al.*, 1989) and to block the ATP-sensitive K-channel (K_{ATP}) in the pancreatic β-cell (Plant & Henquin, 1990).

Several agents which possess an imidazoline or guanidine moiety are known to antagonize the relaxant effects of the K-channel openers (McPherson & Angus, 1989; Murray *et al.*, 1989; Noack *et al.*, 1992b; Schwietert *et al.*, 1992). In addition they also enhance spontaneous mechanical activity in rat portal vein (Noack *et al.*, 1992b; Schwietert *et al.*, 1992). This effect may be related to inhibition of $I_{K(V)}$, evidence for which has been obtained both in pancreatic β-cells and in vascular smooth muscle (Jonas *et al.*, 1992; Noack *et al.*, 1992b; Ibbotson *et al.*, 1993). In ligand-binding studies using liver and brain membrane preparations, these derivatives bind to a so-called imidazoline-guanidine binding site which exhibits characteristics similar to those of the α_2-adrenoceptor (Parini *et al.*, 1989). However, the site does not recognize the catecholamines adrenaline and noradrenaline but it does bind the α_2-adrenoceptor antagonist, idazoxan, a finding which has given rise to the alternative designations, non-adrenoceptor idazoxan binding site (NAIBS, Zonnenschein *et al.*, 1990; Atlas, 1991) or non-adrenoceptor imidazoline binding site (Hussain *et al.*, 1993).

In the present study, we have investigated the effects of several ligands (Figure 1) at the non-adrenoceptor imidazoline binding site on the modulation of whole-cell K-currents by levcromakalim in single cells from rat portal vein. By this approach, together with measurement of mechanical changes in whole tissues, it was hoped to learn more about the ability of these agents to inhibit not only $I_{K(V)}$ in this tissue but also the induction of $I_{K(ATP)}$ by the K-channel openers.

Methods

Experiments were performed on portal veins removed from male Sprague-Dawley rats (100–250 g), previously killed by stunning and cervical dislocation.

Whole-tissue mechanical studies

Whole portal veins were mounted under 10 mN tension for isometric recording. The tissues were allowed to equilibrate

[1] Author for correspondence.

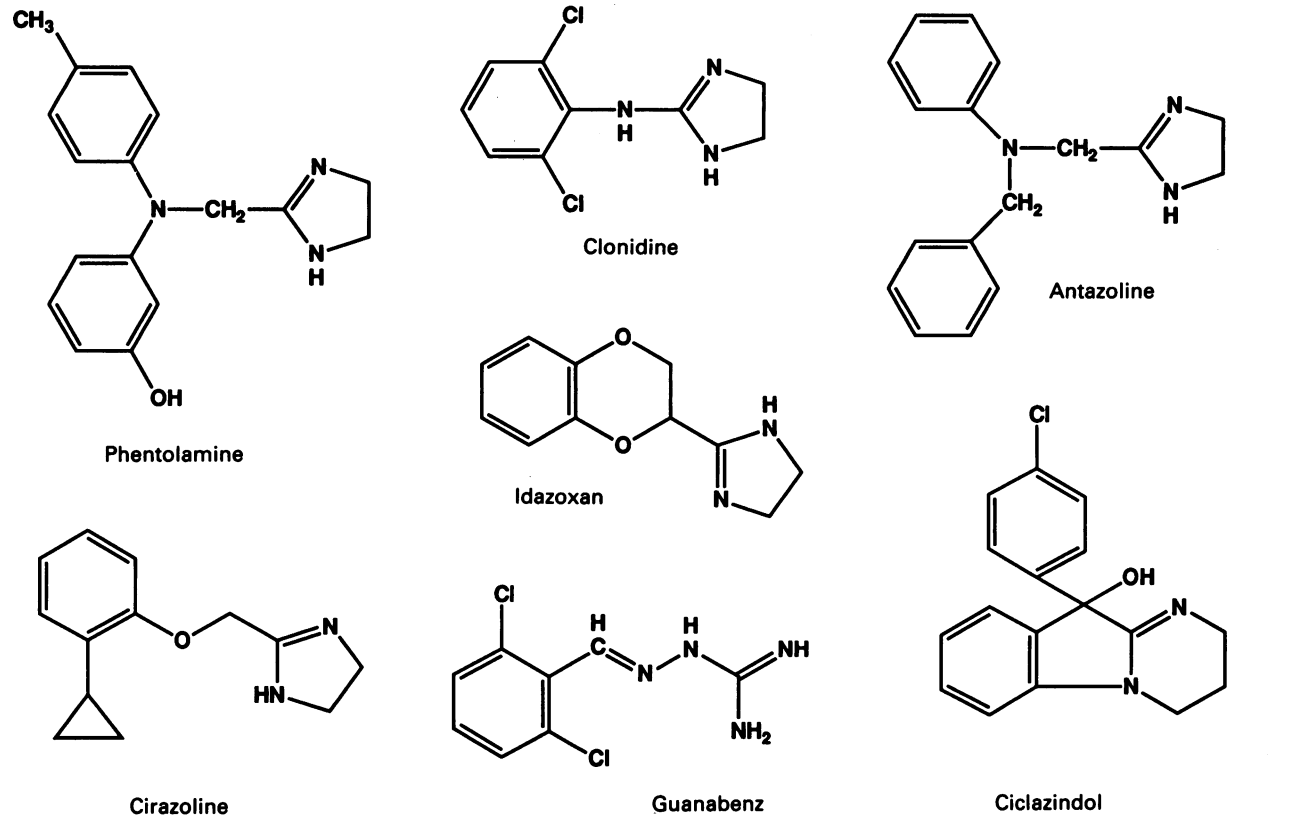


Figure 1 Structural formulae of some of the agents mentioned in the text and used in the present study.

in physiological salt solution (see Drugs and solutions) for 1 h before they were exposed at 6 min intervals to increasing concentrations of levcromakalim, antazoline or guanabenz added cumulatively. The sensitivity of the stimulatory effects of guanabenz to the α_2-adrenoceptor-selective antagonist, RX 821002, was determined by preincubating the relevant tissues with the α-adrenoceptor antagonist for 20 min before starting concentration-response protocols. Antagonism of the relaxant effect of levcromakalim was determined by preincubating the tissues with antazoline or guanabenz for 20 min before the cumulative addition of levcromakalim in the continuing presence of either antazoline or guanabenz. The control dose-response relationship for levcromakalim was determined in physiological salt solution containing noradrenaline (1 μM) which increased spontaneous mechanical activity to levels not different from those obtained in the presence or either guanabenz or antazoline.

Mechanical responses of the portal vein were recorded and this activity was integrated with respect to time by use of an Apple Macintosh computer in conjunction with Maclab hardware (Maclab 8) and software (Chart, version 2.5) (Analog Digital Instruments).

Single cell electrophysiology

Isolation of cells Cells were prepared as previously described (Ibbotson *et al.*, 1993). Briefly, portal veins were dissected into a nominally calcium-free physiological salt solution (Klöckner & Isenberg, 1985) and cleaned of extraneous connective tissue under a dissecting microscope. The vein was opened along its longitudinal axis and placed in a collagenase solution originally described for the separation of guinea-pig bladder smooth muscle cells (Klöckner & Isenberg, 1985). The tissue was agitated for 35 min in the enzyme solution at 37°C, washed with the same solution free of enzyme and cut into 4 segments. These segments were then triturated by use of a wide bore, smooth tipped pipette in Kraftbrühe (KB-medium; Klöckner & Isenberg, 1985). Cells were stored at 4°C in KB-medium and used within 9 h of separation. All experiments were performed at room temperature (22–26°C). Patch pipettes were made from Pyrex glass (H15/10, Jencons, U.K.) and had a resistance of 2–4 MOhm when filled with internal solution.

Generation of K-currents The whole-cell configuration of the patch clamp technique (Hamill *et al.*, 1981) was employed using an Axopatch-1C amplifier (Axon Instruments). The settling time of the system was less than 500 μs. Voltage commands and data acquisition were performed on-line with an AT-compatible computer equipped with an Axon TL-1 interface. For cell stimulation and for recording and analysing data, the pClamp 5.5 programme was used (Axon Instruments). Data were stored on a digital audio tape recorder (Sony) and the evoked membrane currents were monitored continuously on a Gould Windograf recorder. The data described were not corrected by linear leak subtraction.

The bath containing the isolated cells was perfused with a Ca^{2+}-free physiological salt solution at approximately 0.7 ml min^{-1} by means of a peristaltic pump (LKB Microperpex); a second identical pump was used to remove the solution from the recording chamber. The effects of drugs were examined by adding appropriate quantities into the reservoir perfusing the bath so that responses could be examined under equilibrium conditions. The ability of agents to modify responses to levcromakalim was determined either by pre-applying the agent to cells and then examining the responses to levcromakalim or by testing the ability of the antagonist to reverse the effects of levcromakalim. Results are expressed as the mean ± s.e.mean.

Drugs and solutions

For the single cell electrophysiological studies, the composition of the solutions used was as follows (mM): pipette

(internal) NaCl 5, KCl 120, $MgCl_2$ 1.2, K_2HPO_4 1.2, glucose 11, HEPES 10, EGTA 1.2, oxalacetic acid 5, sodium pyruvate 2, sodium succinate 5, pH 7.3; bath (external) NaCl 125, KCl 4.8, $MgCl_2$ 3.7, KH_2PO_4 1.2, glucose 11, HEPES 10, EGTA 1, pH 7.3 aerated with O_2.

The enzyme solution comprised (mM): KCl 130, $CaCl_2$ 0.05, taurine 20, pyruvate 4, creatine 5, HEPES 10, collagenase (1 mg ml^{-1}; Sigma Type 8), pronase (0.2 mg ml^{-1}; Calbiochem), fatty acid-free albumin (1 mg ml^{-1}; Sigma). The pH was adjusted to 7.4 with methanesulphonic acid (Sigma).

The KB solution comprised (mM): KCl 85, KH_2PO_4 30, $MgSO_4$ 5, Na_2ATP 5, K-pyruvate 5, creatine 5, taurine 20, β-OH butyrate 5, fatty acid free albumin 1 mg ml^{-1}, pH adjusted to 7.2 with KOH.

The physiological salt solution used for the whole-tissue mechanical studies comprised (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1. This solution was gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C.

The following substances were used: levcromakalim (Pfizer), glibenclamide, phentolamine hydrochloride, noradrenaline bitartrate, antazoline, clonidine, guanabenz (all Sigma), cirazoline (Zeneca), RX 821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline: Reckitt & Coleman).

Data analysis

In the whole tissue studies, the significance of differences between the mean log IC_{50} value of levcromakalim in physiological salt solution containing noradrenaline (1 μM) and those mean log IC_{50} values obtained in the presence of either antazoline or guanabenz was determined by Student's *t* test. To determine the significance of drug treatment on current-voltage relationships, a 2-way, within subject (repeated measures) ANOVA test was used. Both tests were applied using software (Statistica v.3.0a: Statsoft) and *P* values less than 0.05 were assumed to indicate significance.

Results

Mechanical changes in whole tissues

In the present investigation we selected an imidazoline (antazoline) and a guanidine (guanabenz) for detailed mechanical studies. In these experiments antazoline (300 nM–10 μM) produced a concentration-dependent increase in the spontaneous myogenic activity of rat portal veins (Figure 2a,c). This was characterized by an increase in the magnitude and frequency of contractions and a disruption of the previously regular pattern of phasic activity. The ability of levcromakalim to inhibit spontaneous mechanical activity was then examined in the continuing presence of antazoline (30 μM). The equivalent control dose-response relationship to levcromakalim was determined with noradrenaline (1 μM) in the physiological salt solution. This produced an approximately 500% increase in the integrated mechanical activity of the vein, a change identical to that seen with antazoline (Figure 2b). Under these conditions, the inhibitory effects of levcromakalim were significantly antagonized in the presence of antazoline (Figure 2d; $P<0.05$).

In parallel experiments, guanabenz (0.1–100 μM) produced an increase in total mechanical activity similar in magnitude to that seen with antazoline. At relatively low concentrations of guanabenz (up to 10 μM), this effect was partially inhibited by the α_2-adrenoceptor antagonist, RX 821002 (1 μM, data not shown), indicating that a component of the guanabenz-induced increase in spontaneous activity was mediated via an α_2-adrenoceptor. However, responses to higher concentra-

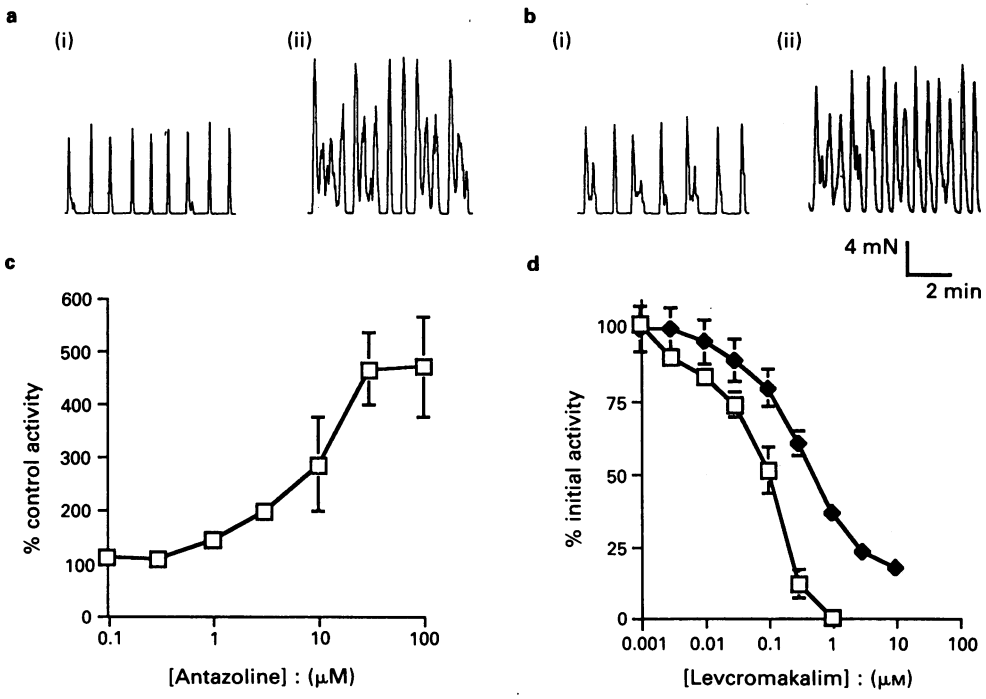


Figure 2 The effect of antazoline on the spontaneous contractions in rat portal vein and its antagonism of the inhibitory effect of levcromakalim. (a) and (b) show the pattern of spontaneous mechanical activity in two different veins before (i) or 15 min after (ii) the application of 30 μM antazoline (a) or 1 μM noradrenaline (b). This concentration of noradrenaline was selected to evoke an increase in mechanical activity equivalent to that caused by 30 μM antazoline. (c) The concentration-dependent increase in mechanical activity produced by antazoline (□), is expressed as a percentage of the initial control activity. (d) The antagonism by antazoline (30 μM; ◆) of the inhibitory effect of levcromakalim on spontaneous mechanical activity is compared to the relaxant effect of levcromakalim on tissues treated with noradrenaline (1 μM; □). Data are expressed as a percentage of the activity in the presence of either noradrenaline (□) or antazoline (◆) immediately before the addition of levcromakalim. Each point represents the mean ± s.e.mean ($n=4$).

tions of guanabenz (30–100 μM) wer largely unaffected by RX 821002. In the presence of guanabenz (30 μM), the levcromakalim dose-response relationship was shifted significantly ($P<0.05$) to the right of that obtained in the presence of noradrenaline (1 μM).

Single-cell electrophysiology

The control currents recorded were similar to those reported in previous studies (Noack *et al.*, 1992a,b,c; Ibbotson *et al.*, 1993). When cells were clamped at − 90 mV and then stepped to test potentials between − 80 mV and − 40 mV in 10 mV increments, a basal non-inactivating current with a reversal potential of approximately − 30 mV was observed and this was designated I_{NI}. Thus, I_{NI} was not a pure K-current and was probably carried by cation channels. In addition, when stepping to test potentials more positive than − 40 mV a slowly activating, slowly inactivating delayed-rectifier K-current ($I_{K(V)}$) (previously designated I_{TO}; Noack *et al.*, 1992a,b,c) was obtained. When cells were held at − 10 mV and stepped to test potentials in the range − 80 mV to + 30 mV, only the non-inactivating currents, I_{NI}, were observed due to inactivation of $I_{K(V)}$ (see Figure 3a,(ii)). Digital subtraction of the currents available when stepping to test potentials from − 10 mV (non-inactivating currents) from those currents elicited when stepping to identical test potentials from − 90 mV (total currents) yielded absolute values for $I_{K(V)}$ (see Figure 3a(i)). For a further description of these currents see Edwards *et al.* (1993) and Ibbotson *et al.* (1993).

Inhibition of levcromakalim-modulated K-currents by phentolamine, clonidine and glibenclamide

The concentrations of antazoline, cirazoline, clonidine and guanabenz used in these studies were selected on the basis of initial dose-ranging experiments. They represent approximate EC_{50} concentrations for the effects of these agents on whole-cell currents. These concentrations were in the same range as those required to increase mechanical activity and to antagonize the effects of levcromakalim in whole veins (see earlier results).

Addition of levcromakalim (1 μM) to the extracellular bathing solution induced the non-inactivating K-current $I_{K(ATP)}$. When cells were clamped at − 10 mV, this was characterized by a slow increase in the holding current at this potential, with a marked enhancement in current noise (Figure 4; see also Noack *et al.*, 1992a,c). With the holding potentials and stepping protocols described in Methods, the induction of $I_{K(ATP)}$ was seen as an increase in the non-inactivating current component ($P<0.05$, Figure 3). Simultaneously, levcromakalim inhibited the delayed rectifier current $I_{K(V)}$, an effect which we have also previously reported ($P<0.05$, Figure 3; see also Noack *et al.*, 1992a,c).

The levcromakalim-induced generation of $I_{K(ATP)}$ was inhibited by subsequent exposure to phentolamine (30 μM) ($P<0.05$, Figures 4 and 5) or to clonidine (30 μM) ($P<0.05$, Figures 3 and 5). However, over the same time-scale, the inhibition of $I_{K(V)}$ by levcromakalim (1 μM) was enhanced by the subsequent addition of phentolamine ($P<0.05$, Figure 5) or clonidine ($P<0.05$, Figures 3 and 5).

When glibenclamide (1 μM) was applied after exposure to levcromakalim, $I_{K(ATP)}$ was fully inhibited ($P<0.05$) and, over the same time-scale, the inhibition of $I_{K(V)}$ by levcromakalim was partially reversed ($P<0.05$, Figure 6).

Effects of antazoline, cirazoline and guanabenz on whole-cell K-currents

To obtain more information about the ability of these agents to enhance the inhibitory action of levcromakalim on $I_{K(V)}$, the effects of antazoline, cirazoline and guanabenz were

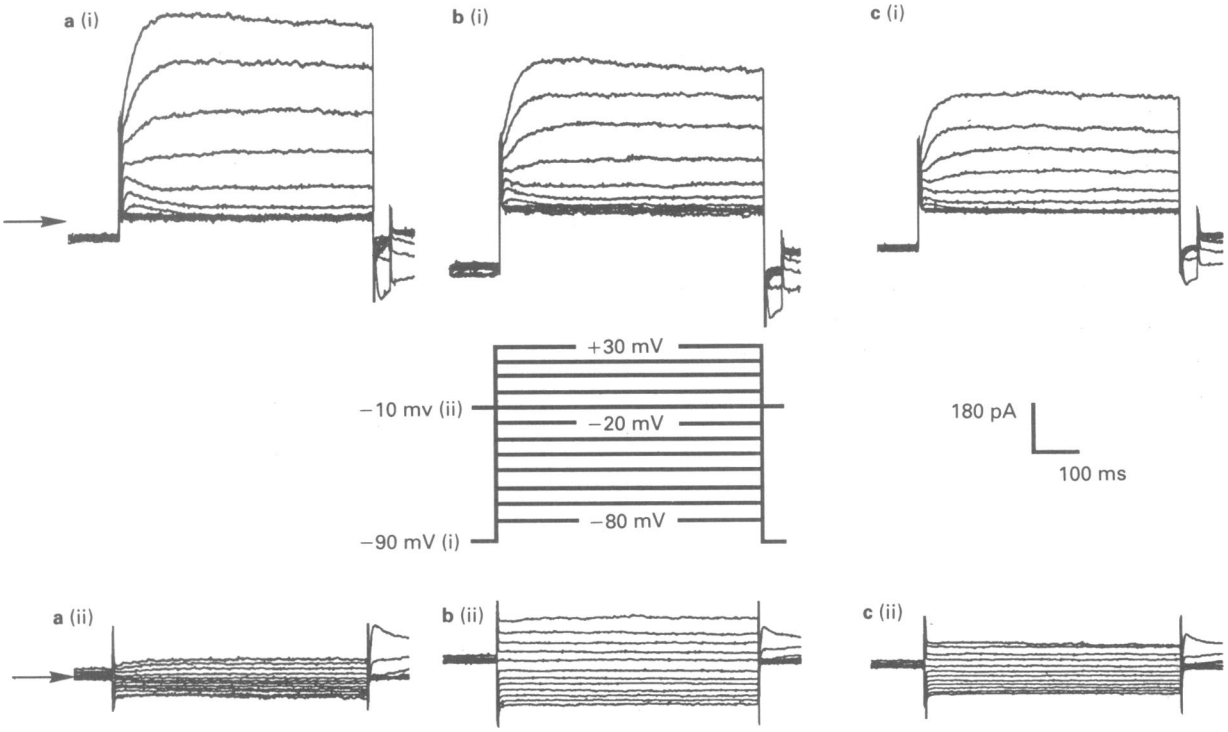


Figure 3 Typical effect of clonidine (30 μM) on the changes in whole-cell currents produced by levcromakalim (1 μM) in a single, isolated portal vein cell of the rat under whole-cell voltage-clamp conditions: (a(i)) control delayed rectifier currents, $I_{K(V)}$; holding potential − 90 mV; (a(ii)) control non-inactivating currents, holding potential − 10 mV. In the presence of levcromakalim (1 μM), $I_{K(V)}$ was reduced (b(i)) and $I_{K(ATP)}$ became apparent (b(ii)). On subsequent exposure to clonidine (30 μM) in the continuing presence of levcromakalim (1 μM), $I_{K(V)}$ was further reduced (c(i)) and $I_{K(ATP)}$ was markedly inhibited (c(ii)). The arrows indicate the zero current level. The families of current traces were generated using the stepping protocol illustrated and the traces of $I_{K(V)}$ were obtained by digital subtraction of the non-inactivating current (holding potential − 10 mV) obtained at a given step potential from the total current (holding potential − 90 mV) at the same step potential.

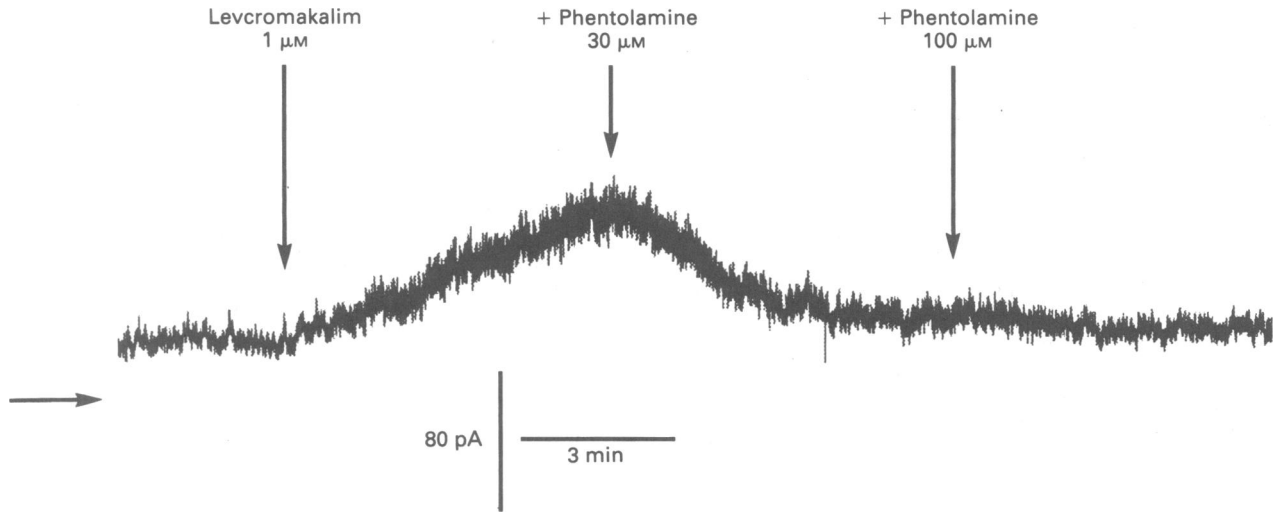


Figure 4 Typical development of $I_{K(ATP)}$ in a freshly-isolated single cell from rat portal vein following exposure to levcromakalim and the inhibition of this current by phentolamine. With a holding potential of −10 mV, levcromakalim (1 μM) induced an outward K-current $I_{K(ATP)}$. Exposure to phentolamine (30 μM) during the rising phase of $I_{K(ATP)}$ markedly inhibited this current; subsequent application of 100 μM phentolamine further inhibited $I_{K(ATP)}$. Note the characteristic increase in current noise during the development of $I_{K(ATP)}$ and the decrease in both current noise and magnitude following exposure to phentolamine. The horizontal arrow indicates the zero current level.

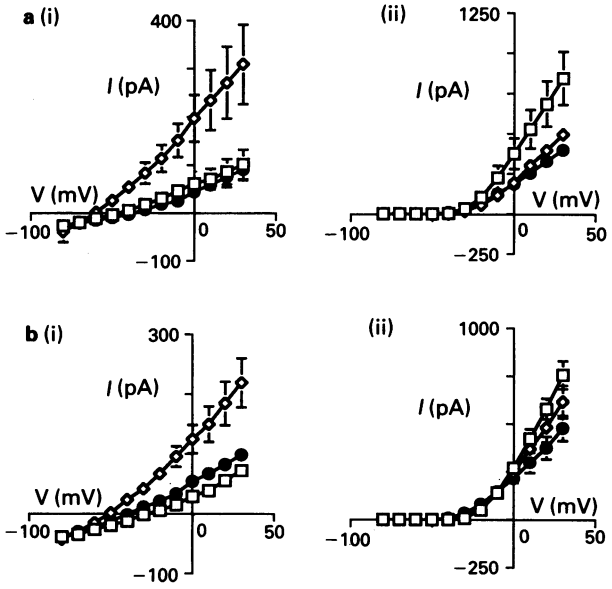


Figure 5 The effects of phentolamine (a) and clonidine (b) on the changes in (i) non-inactivating and (ii) inactivating (delayed rectifier, $I_{K(V)}$) currents produced by levcromakalim. Experiments were carried out on freshly-isolated cells from rat portal vein under whole-cell voltage-clamp conditions: (□) represents control currents before application of any drug. Levcromakalim (1 μM, ◇) induced a non-inactivating current, $I_{K(ATP)}$ (a(i), b(i)) and simultaneously decreased the magnitude of $I_{K(V)}$ (◇, a(ii), b(ii)). Subsequent application of phentolamine (30 μM, ●) or clonidine (30 μM, ●) reversed the induction of $I_{K(ATP)}$ (a(i) and b(i), respectively) but not the inhibition of $I_{K(V)}$ by levcromakalim (a(ii) and b(ii), respectively). Each point represents the mean value derived from 3 observations ± s.e.mean.

examined prior to exposure to levcromakalim. Using the holding potentials and stepping protocols described in Methods, addition of guanabenz (30 μM) or cirazoline (30 μM) to the extracellular bathing solution inhibited the delayed rectifier current, $I_{K(V)}$ ($P<0.05$), with only a slight reduction in the control non-inactivating currents (Figures 7 and 8). Similar results were obtained with phentolamine (30 μM; see also Ibbotson *et al.*, 1993) and with antazoline (30 μM) ($P<0.05$, data not shown).

Pre-exposure (15 min) of cells to guanabenz (30 μM) or to cirazoline (30 μM) prevented the induction of $I_{K(ATP)}$ by levcromakalim (1 μM) ($P<0.05$, Figures 7 and 8). However, the accompanying inhibition of $I_{K(V)}$ by levcromakalim was enhanced by these agents ($P<0.05$, Figures 7 and 8). Qualitatively similar effects were observed upon pre-exposure of cells to antazoline (30 μM) or clonidine (30 μM) followed by application of levcromakalim (1 μM; data not shown).

Cirazoline, clonidine and guanabenz can all be regarded as agonists at α-adrenoceptors and thus the effects of noradrenaline on whole-cell currents were examined. Noradrenaline (10 μM) had no effect on either I_{NI} or $I_{K(V)}$ ($P>0.05$, data not shown).

Discussion

Several compounds which possess an imidazoline or guanidine moiety are known to antagonize the relaxant effects of K-channel openers in a variety of smooth muscles (McPherson & Angus, 1989; Murray *et al.*, 1989; Noack *et al.*, 1992b; Okumura *et al.*, 1992; Schwietert *et al.*, 1992). Unlike glibenclamide, which also antagonizes the K-channel openers, the agents which contain an imidazoline or guanidine grouping also enhance spontaneous mechanical activity in smooth muscle (Buckingham *et al.*, 1989; Noack *et al.*, 1992b; Okumura *et al.*, 1992; Schwietert *et al.*, 1992). Recent experiments have shown that three such drugs, namely ciclazindol, clonidine and phentolamine, inhibit the delayed rectifier K-current, $I_{K(V)}$, in vascular smooth muscle or in pancreatic β-cells (Plant *et al.*, 1991; Noack *et al.*, 1992b; Ibbotson *et al.*, 1993). Thus the experiments of the present study were designed to elucidate whether a variety of agents containing an imidazoline or a guanidine moiety could inhibit both $I_{K(V)}$ and the current induced by the K-channel openers, $I_{K(ATP)}$.

Effects of antazoline and guanabenz on mechanical activity in whole portal veins

In the present study, both antazoline and guanabenz produced a concentration-dependent increase in the amplitude

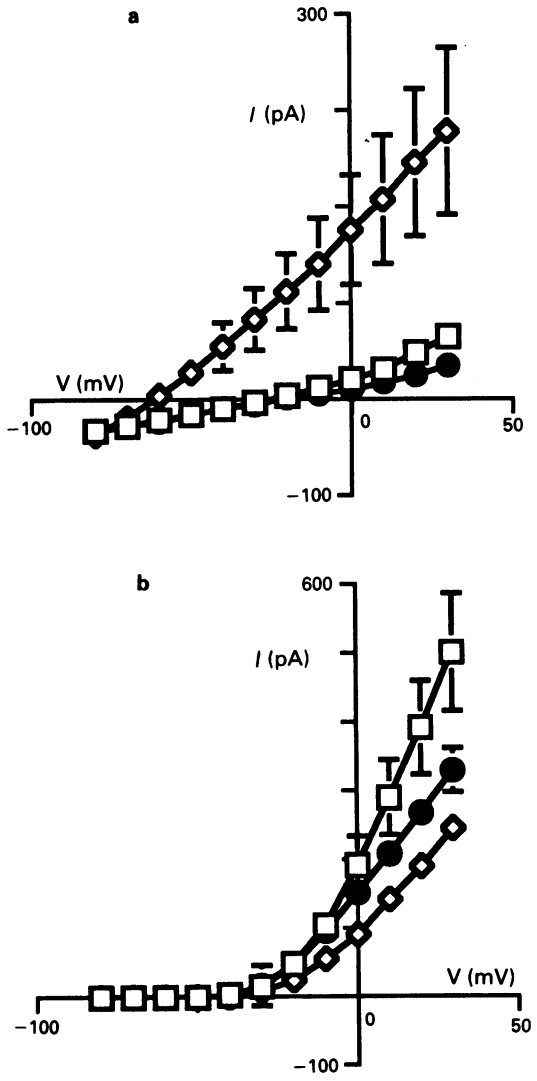


Figure 6 The effects of glibenclamide on the changes in (a) non-inactivating and (b) inactivating (delayed rectifier, $I_{K(V)}$) currents produced by levcromakalim. Experiments were carried out on freshly-isolated cells from rat portal vein under whole-cell voltage-clamp conditions; (□) represents control currents before application of any drug. Levcromakalim (1 μM, ◇) induced a non-inactivating current, $I_{K(ATP)}$ (a) and simultaneously inhibited the delayed rectifier current ($I_{K(V)}$) (b). After 15 min exposure to levcromakalim, glibenclamide (1 μM, ●) inhibited the induced $I_{K(ATP)}$ (a) and partially reversed the inhibition of $I_{K(V)}$ by levcromakalim. Each point represents the mean value derived from 3 observations ± s.e.mean.

and duration of spontaneous contractions in whole veins. These results thus confirm the findings of Schwietert *et al.* (1992) with antazoline in rat portal vein and extend them to the guanidine derivative, guanabenz. This agent is normally regarded as an α_2-adrenoceptor agonist, and thus the observed increase in mechanical activity could have resulted from an interaction with this adrenoceptor sub-type. Indeed, the guanabenz concentration-effect curve for stimulation of myogenic activity was partially depressed by RX 821002, a selective α_2-adrenoceptor antagonist (Stillings *et al.*, 1985; present study). However, the mechanical stimulation produced by relatively high concentrations of guanabenz (30 to 100 μM) was little affected by RX 821002, suggesting that over this concentration range the guanidine derivative was acting predominantly at a site other than the α_2-adrenoceptor. The ability of both antazoline and guanabenz to enhance spontaneous activity in the portal vein is similar to that exhibited by the imidazo-isoindole derivative, ciclazindol, an inhibitor of $I_{K(V)}$ in rat portal vein (Noack *et al.*, 1992b). Furthermore, both antazoline and guanabenz inhibited the mechano-inhibitory effects of levcromakalim on whole veins. This action is also shared by ciclazindol, an inhibitor of $I_{K(ATP)}$ (Noack *et al.*, 1992b).

Effects of antazoline, cirazoline, clonidine, guanabenz and phentolamine on $I_{K(V)}$ and $I_{K(ATP)}$

A striking feature of the present investigation was the ability of antazoline, cirazoline, clonidine, guanabenz and phentolamine to inhibit the delayed rectifier current, $I_{K(V)}$. Such an action almost certainly underlies the observed increase in the magnitude of spontaneous contractions in portal vein produced by antazoline and by guanabenz. Although not reported in this paper, both clonidine (in the presence of RX 821002) and phentolamine also increase the spontaneous mechanical activity of rat portal vein (M. Miller, personal communication).

In addition to their ability to inhibit $I_{K(V)}$, guanabenz and all the tested imidazolines inhibited the non-inactivating K-current induced by levcromakalim. In vascular smooth muscle, this current ($I_{K(ATP)}$) appears to be carried by ATP-sensitive K-channels with an underlying unitary conductance in the range 10 to 20 pS (quasi-physiological conditions: Noack *et al.*, 1992a,c; Bolton *et al.*, 1993; Ibbotson *et al.*, 1993; Langton *et al.*, 1993; Criddle *et al.*, 1994). Thus the results of the present study are consistent with the ability of phentolamine to inhibit $I_{K(ATP)}$ in pancreatic β-cells (Plant & Henquin, 1990) and to antagonize the induction of this current in portal vein by aprikalim and P1060 (Ibbotson *et al.*, 1993).

Is K_{ATP} a substate of K_V?

Beech & Bolton (1989) considered the possibility that cromakalim might remove the voltage-sensitivity of K_V and thus convert $I_{K(V)}$ into a non-inactivating current. However, this view was rejected because glibenclamide, which inhibited the cromakalim-induced current, had essentially no effect on $I_{K(V)}$ (Beech & Bolton, 1989). Since then, all investigators have taken the view that the K-channel openers interact with the so-called ATP-sensitive K-channel, K_{ATP} (see reviews by Ashcroft & Ashcroft, 1990; Escande & Cavero, 1992; Edwards & Weston, 1993).

However, we have recently proposed that K_{ATP} is not a separate entity but is, instead, a partially dephosphorylated form of K_V (Edwards *et al.*, 1993). The basis of this view is that procedures designed to reduce channel phosphorylation decrease $I_{K(V)}$ and simultaneously increase a non-inactivating K-current. Such changes are glibenclamide-sensitive and are thus identical to those of the K-channel openers (Edwards *et al.*, 1993). The possibility that an inactivating current (i.e. $I_{K(V)}$) could be converted into a non-inactivating one (i.e. $I_{K(ATP)}$) is not a novel concept. Some fifteen years ago, Fink & Wettwer (1978) showed that metabolic exhaustion of frog skeletal muscle converted $I_{K(V)}$ into a non-inactivating K-current. They concluded that under these conditions, K_V lost its property of inactivation and that its voltage-sensitivity was altered. In addition, modification of Na-channel gating by loss of inactivation is the mechanism by which many sodium channel openers such as batrachotoxin and N-bromoacetamide are believed to act (see Hille, 1992).

The proposal that K_{ATP} and K_V are different states of the same channel (Edwards *et al.*, 1993) is supported by the findings of the present study that the imidazoline- and guanidine-containing compounds inhibit both $I_{K(ATP)}$ and $I_{K(V)}$. Other agents such as phencyclidine, quinidine, 4-aminopyridine, tedisamil, yohimbine and ciclazindol, many of which are apparently chemically-distinct from the imidazolines and guanidines, similarly inhibit both $I_{K(ATP)}$ and $I_{K(V)}$ (Beech & Bolton, 1989; Plant & Henquin, 1990; Bray & Quast, 1992; Noack *et al.*, 1992b; Pfründer & Kreye, 1992;

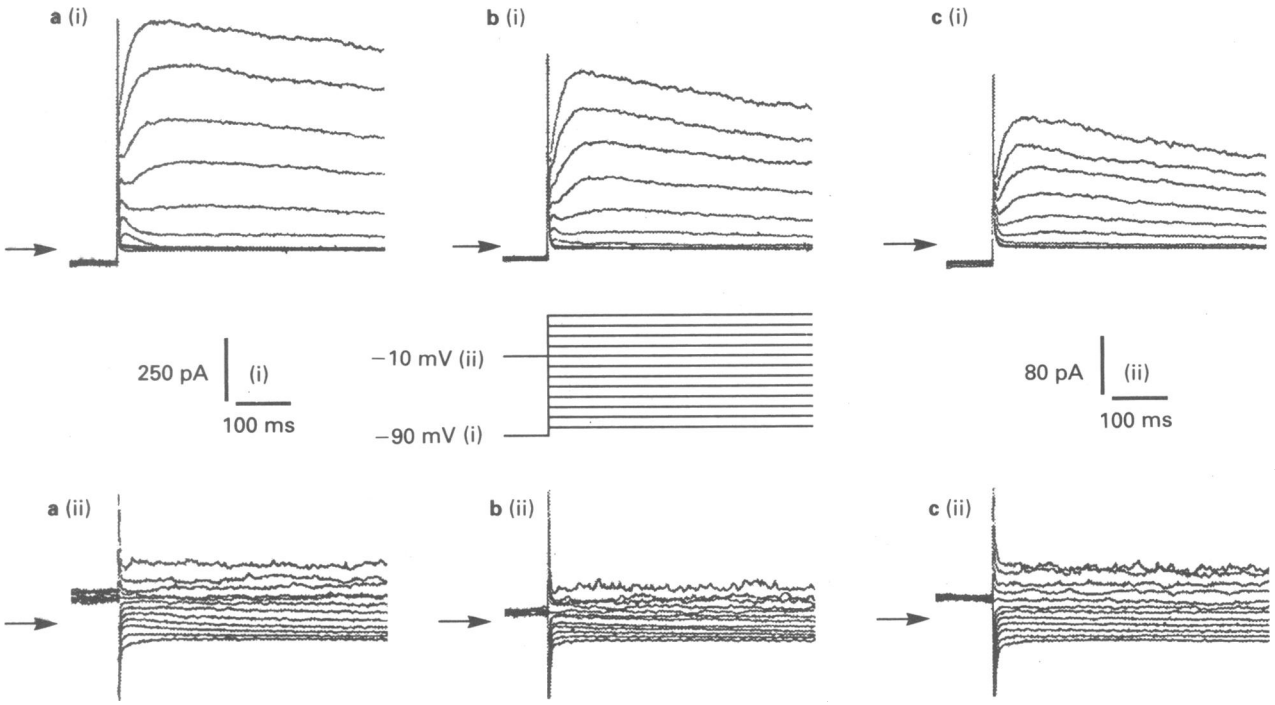


Figure 7 Typical effect of pre-exposure to guanabenz (30 μM) on the changes in the mean whole-cell currents produced by levcromakalim in isolated portal vein cells of rat under whole-cell voltage-clamp conditions. (a(i)) Control delayed rectifier currents, $I_{K(V)}$, holding potential − 90 mV; (a(ii)) control non-inactivating currents, holding potential − 10 mV. Exposure to guanabenz (30 μM) inhibited $I_{K(V)}$ (b(i)) and the evoked non-inactivating currents (b(ii)). In the continuing presence of guanabenz, levcromakalim (1 μM) produced further inhibition of $I_{K(V)}$ (c(i)) but development of $I_{K(ATP)}$ (c(ii)) was markedly inhibited (compare with Figure 3). The arrows indicate the zero current level. Each trace represents the computer-derived mean from 3 different cells.

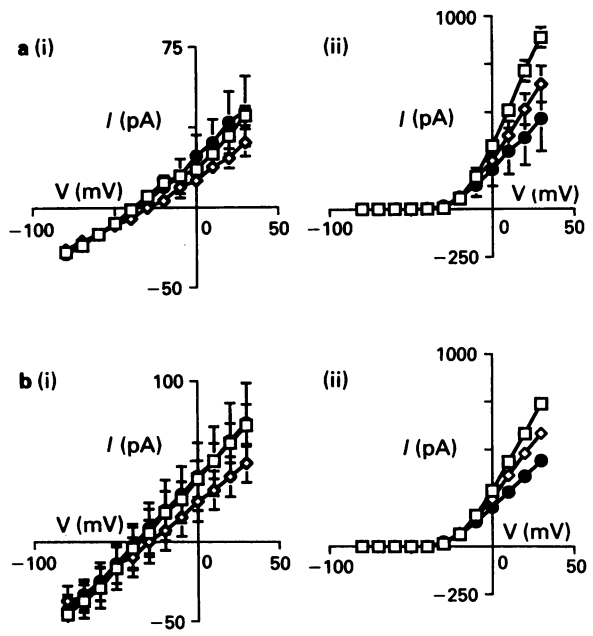


Figure 8 The effects of pre-exposure to guanabenz and cirazoline on the changes in (i) non-inactivating and (ii) inactivating (delayed rectifier, $I_{K(V)}$) currents produced by levcromakalim. Experiments were carried out on freshly-isolated cells from rat portal vein under whole-cell voltage-clamp conditions: (□) represents control currents before application of any drug. After 15 min exposure to guanabenz (30 μM, ◇, a(i)) or cirazoline (30 μM, ◇, b(i)), levcromakalim (1 μM, ●) failed to induce $I_{K(ATP)}$ (compare with Figure 5). Similar concentrations of guanabenz (◇, a(ii)) and cirazoline (◇, b(ii)) inhibited $I_{K(V)}$ and this effect was enhanced by levcromakalim (1 μM, ●). Each point represents the mean value derived from 3 observations ± s.e.mean.

Schwietert *et al.*, 1992). The mechanism involved in the inhibitory effects of these drugs on K-currents and on their ability to antagonize the K-channel openers is not known. However, they could simply inhibit $K_{(V)}$ and thus reduce the number of delayed rectifier channels available for conversion to $K_{(ATP)}$. Alternatively, they could inhibit both states of the channel and these possibilities are the subject of an on-going study.

Are the effects of antazoline, cirazoline, clonidine, guanabenz and phentolamine on K-currents exerted via a non-adrenoceptor imidazoline binding site?

Cirazoline, guanabenz, phentolamine and clonidine all exhibit affinity for the non-adrenoceptor imidazoline binding site identified in the membrane fraction of rabbit urethral smooth muscle (Yablonsky & Dausse, 1991). These authors reported that the K_i values for the binding of these agents to this site were 2.9 nM, 4.4 nM and 1,265 nM, respectively. The affinity of antazoline was not examined. In the present study, these agents were approximately equipotent in their ability to inhibit $I_{K(V)}$ and the induction of $I_{K(ATP)}$. Thus the binding data of Yablonsky & Dausse (1991) do not strongly support the idea that the electrophysiological effects of cirazoline, guanabenz, phentolamine and clonidine could involve a non-adrenoceptor imidazoline binding site.

In spite of this, the common feature which links guanabenz (a guanidine α_2-adrenoceptor agonist), antazoline (an H_1-antagonist), cirazoline (an α_1-adrenoceptor agonist), clonidine (an imidazoline α_2-adrenoceptor agonist) and phentolamine (an α_1- and α_2-adrenoceptor antagonist) is the possession of an imidazoline or a guanidine moiety. It is thus possible that the effects of these agents are, in fact, mediated via a non-adrenoceptor imidazoline binding site, the existence of which on the plasmalemma has been reported (Zonnenschein *et al.*, 1990; Yablonsky & Dausse, 1991). The possibility that the

non-adrenoceptor imidazoline binding site could play a role in K-channel modulation is consistent with the views of other workers (Atlas, 1991; Michel & Ernsberger, 1992). A similar conclusion was recently reported following a study of transmitter release from peripheral sympathetic neurones (Smith & Weston, 1993) and further studies are in progress to clarify the possible functional role of the non-adrenoceptor imidazoline binding site in the periphery.

Effects of glibenclamide

Of all the compounds tested, only glibenclamide failed to inhibit $I_{K(V)}$. However, glibenclamide-binding is reduced under phosphorylating conditions (Schwanstecher *et al.*, 1991), a property which would be consistent with its lack of inhibitory effect on K_V because of its relatively low affinity for the channel in the delayed rectifier (phosphorylated) state (Edwards *et al.*, 1993). Glibenclamide is able to inhibit completely the K-channel opener-stimulated current, $I_{K(ATP)}$, but it only partially antagonizes the inhibition of $I_{K(V)}$ by these agents (present study; Ibbotson *et al.*, 1993). At this stage we cannot offer a definitive explanation for this apparent paradox. However, glibenclamide could act as a combined ATP-generator and phosphorylating agent and the ability of this sulphonylurea to stimulate the production of glycolytically-derived ATP has indeed been described (Caro, 1990). In our experimental conditions, substrates for ATP production are present in both the bath (glucose) and pipette (glucose, pyruvate, succinate and oxalacetate) solutions. Thus, both the inhibition of $I_{K(ATP)}$ and reversal of the K-channel opener-induced inhibition of $I_{K(V)}$ by glibenclamide could be attributed to the combined effects of an increase in the intracellular ATP concentration and in channel phosphorylation. This would both inhibit $K_{(ATP)}$ *per se* via its ATP-regulatory site (Ashcroft & Ashcroft, 1990; Edwards & Weston, 1993) and produce additional inhibition by initiating conversion of the partially-dephosphorylated channel (K_{ATP}) to its fully-phosphorylated and voltage-dependent state, K_V. Thus, $I_{K(ATP)}$ could be totally inhibited before the inhibition of $I_{K(V)}$ had been fully reversed.

To explain the inhibitory effects of glibenclamide in isolated patches (see Ashcroft & Ashcroft, 1990; Edwards & Weston, 1993), such speculation requires that enzymes for both glycolysis and phosphorylation are closely associated with the plasma membrane. Recent findings suggest that this is the case in smooth muscle (glycolysis) and in the insulinoma HIT cell line (protein kinases) (Paul *et al.*, 1989; F.M. Ashcroft, personal communication).

Conclusions

In isolated portal vein cells, several agents containing an imidazoline or guanidine moiety inhibited both the delayed rectifier current, $I_{K(V)}$, and the current, $I_{K(ATP)}$, induced by levcromakalim. These actions correlated with the effects of these agents on mechanical activity in whole portal veins. The results suggest that antazoline, cirazoline, clonidine, guanabenz and phentolamine could exert their effects by interaction with a smooth muscle non-adrenoceptor imidazoline binding site which is functionally-coupled to inhibition of K-channels. The data also strengthen the recent suggestion (Edwards *et al.*, 1993) that the so-called ATP-sensitive K-channel, K_{ATP}, is not a discrete entity but is a sub-state of the delayed rectifier K-channel, K_V.

T.I. is supported by an MRC Collaborative Award in conjunction with Pfizer Central Research. G.E. is supported by Pfizer Central Research. The authors wish to thank Drs T. Grant (Zeneca Pharmaceuticals), M.G. Wyllie (Pfizer Central Research) and P.W. Dettmar (Reckitt & Coleman) for the kind gifts of cirazoline, levcromakalim and RX 821002, respectively.

References

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP-sensitive K-channels. *Cellular Signalling*, **2**, 197–214.

ATLAS, D. (1991). Clonidine-displacing substance (CDS) and its putative imidazoline receptor – new leads for further divergence of α_2-adrenergic receptor activity. *Biochem. Pharmacol.*, **41**, 1541–1549.

BEECH, D.J. & BOLTON, T.B. (1989). Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98**, 851–864.

BOLTON, T.B., ZHANG, H. & BEECH, D.J. (1993). Evidence that nucleotide-dependent K^+ channels are the site of action of levcromakalim in smooth muscle cells isolated from the rabbit portal vein. *J. Physiol.*, **467**, 312P.

BRAY, K. & QUAST, U. (1992). Differential inhibition by tedisamil (KC-8857) and glibenclamide of the responses to cromakalim and minoxidil sulphate in rat isolated aorta. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 244–250.

BUCKINGHAM, R.E., HAMILTON, T.C., HOWLETT, D.R., MOOTOO, S. & WILSON, C. (1989). Inhibition by glibenclamide of the vasorelaxant action of cromakalim in the rat. *Br. J. Pharmacol.*, **97**, 57–64.

CARO, J.F. (1990). Effects of glyburide on carbohydrate metabolism on insulin action in the liver. *Am. J. Med.*, **89** (Suppl 2A), 17S–25S.

CRIDDLE, D., GREENWOOD, I.A. & WESTON, A.H. (1994). Levcromakalim-induced relaxation of membrane potassium currents, intracellular calcium and mechanical activity in rat mesenteric artery. *Naunyn-Schmied. Arch. Pharmacol.*, (in press).

EDWARDS, G., IBBOTSON, T. & WESTON, A.H. (1993). Levcromakalim may induce a voltage-independent K-current in rat portal veins by modifying the gating properties of the delayed rectifier. *Br. J. Pharmacol.*, (in press).

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 597–631.

ESCANDE, D. & CAVERO, I. (1992). K^+ channel openers and natural cardioprotection. *Trends Pharmacol. Sci.*, **13**, 269–272.

FINK, R. & WETTWER, E. (1978). Modified K-channel gating by exhaustion and the block by internally applied TEA^+ and 4-aminopyridine in muscle. *Pflügers Arch.*, **374**, 289–292.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HILLE, B. (1992). Modifiers of gating. In *Ionic Channels of Excitable Membranes*. 2nd edition. pp. 445–471. Sunderland, Massachusetts: Sinauer Associates Inc.

HUSSAIN, J.F., KENDALL, D.A. & WILSON, V.G. (1993). Species selective binding of [^{3}H]-idazoxan binding to α_2-adrenoceptors and non-adrenoceptor, imidazoline binding sites in the central nervous system. *Br. J. Pharmacol.*, **109**, 831–837.

IBBOTSON, T., EDWARDS, G., NOACK, TH. & WESTON, A.H. (1993). Effects of P1060 and aprikalim on whole-cell currents in rat portal vein: inhibition by glibenclamide and phentolamine. *Br. J. Pharmacol.*, **108**, 991–998.

JONAS, J.C., PLANT, T.D. & HENQUIN, J.C. (1992). Imidazoline antagonists of α_2-adrenoceptors increase insulin release in vitro by inhibiting ATP-sensitive K^+ channels in pancreatic β-cells. *Br. J. Pharmacol.*, **107**, 8–14.

KLÖCKNER, U. & ISENBERG, G. (1985). Action potentials and net membrane currents of isolated smooth muscle cells (urinary bladder of the guinea-pig). *Pflügers Arch.*, **405**, 329–339.

LANGTON, P.D., CLAPP, L.H., DART, C., GURNEY, A.M. & STANDEN, N.B. (1993). Whole cell K^+ current activated by lemakalim in isolated myocytes from rabbit pulmonary artery: estimate of unitary conductance and density by noise analysis. *J. Physiol.*, **459**, 254P.

MCPHERSON, G.A. & ANGUS, J.A. (1989). Phentolamine and structurally-related compounds selectively antagonise the vascular actions of the K^+ channel opener, cromakalim. *Br. J. Pharmacol.*, **97**, 941–949.

MICHEL, M.C. & ERNSBERGER, P. (1992). Keeping an eye on the I site: imidazoline-preferring receptors. *Trends Pharmacol. Sci.*, **13**, 369–370.

MURRAY, M.A., BOYLE, J.P. & SMALL, R.C. (1989). Cromakalim-induced relaxation of guinea-pig isolated trachealis: antagonism by glibenclamide and by phentolamine. *Br. J. Pharmacol.*, **98**, 865–874.

NOACK, TH., DEITMER, P., EDWARDS, G. & WESTON, A.H. (1992a). Characterization of potassium currents modulated by BRL 38227 in rat portal vein. *Br. J. Pharmacol.*, **106**, 717–726.

NOACK, TH., EDWARDS, G., DEITMER, P., GREENGRASS, P., MORITA, T., ANDERSSON, P.-O., CRIDDLE, D., WYLLIE, M.G. & WESTON, A.H. (1992b). The involvement of potassium channels in the action of ciclazindol in rat portal vein. *Br. J. Pharmacol.*, **106**, 17–24.

NOACK, TH., EDWARDS, G., DEITMER, P. & WESTON, A.H. (1992c). Potassium channel modulation in rat portal vein by ATP depletion: a comparison with the effects of levcromakalim (BRL 38227). *Br. J. Pharmacol.*, **107**, 945–955.

OKUMURA, K., ICHIHARA, K. & NAGASAKA, M. (1992). Effects of imidazoline-related compounds in the mechanical response to nicorandil in the rat portal vein. *Eur. J. Pharmacol.*, **215**, 253–257.

PARINI, A., COUPRY, I., GRAHAM, R.M., UZIELLI, I., ATLAS, D. & LANIER, S.M. (1989). Characterization of an imidazoline/guanidinium receptive site distinct from the α_2-adrenergic receptor. *J. Biol. Chem.*, **264**, 11874–11878.

PAUL, R.J., HARDIN, C.D., RAEYMAEKERS, L., WUYTACK, F. & CASTEELS, R. (1989). Preferential support of Ca^{2+} uptake in smooth muscle plasma membrane vesicles by an endogenous glycolytic cascade. *FASEB J.*, **3**, 2298–2301.

PFRÜNDER, D. & KREYE, V.A.W. (1992). Tedisamil inhibits the delayed rectifier K^+ current in single smooth muscle cells of the guinea-pig portal vein. *Pflügers Arch.*, **421**, 22–25.

PLANT, T.D. & HENQUIN, J.C. (1990). Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **101**, 115–120.

PLANT, T.D., JONAS, J.-C. & HENQUIN, J.C. (1991). Clonidine inhibits ATP-sensitive potassium channels in mouse pancreatic β-cells. *Br. J. Pharmacol.*, **104**, 385–390.

SCHWIETERT, R., WILHELM, D., WILFFERT, B. & VAN ZWIETEN, P.A. (1992). The effect of some α-adrenoceptor antagonists on spontaneous myogenic activity in the rat portal vein and the putative involvement of ATP-sensitive K^+ channels. *Eur. J. Pharmacol.*, **211**, 87–95.

SCHWANSTECHER, M., LÖSER, S., RIETZE, I. & PANTEN, U. (1991). Phosphate and thiophosphate group donating adenine and guanine nucleotides inhibit glibenclamide binding to membranes from pancreatic islets. *Naunyn-Schmied. Arch. Pharmacol.*, **343**, 83–89.

SMITH, R.D. & WESTON, A.H. (1993). The effects of UK 14,304 and guanabenz on ^{3}H-noradrenaline release in the rat anococcygeus muscle. *J. Physiol.*, **467**, 302P.

STILLINGS, M.R., CHAPLEO, C.B., BUTLER, R.C.M., DAVIS, J.A., ENGLAND, C.D., MYERS, P.L., TWEDDLE, N., WELBOURN, A.P., DOXEY, J.C. & SMITH, J.P.C. (1985). α-Adrenoceptor reagents. 3. Synthesis of some 2-substituted 1,4-benzodioxans as selective presynaptic α-adrenoceptor antagonists. *J. Med. Chem.*, **28**, 1054–1062.

YABLONSKY, F. & DAUSSE, J.P. (1991). Non-adrenergic binding sites for the 'α_2-antagonist' [^{3}H]idazoxan in the rabbit urethral smooth muscle. *Biochem. Pharmacol.*, **41**, 701–707.

ZONNENSCHEIN, R., DIAMANT, S. & ATLAS, D. (1990). Imidazoline receptors in rat liver cells – a novel receptor or a subtype of alpha-2-adrenoceptors. *Eur. J. Pharmacol.*, **190**, 203–215.

(Received June 14, 1993
Revised August 2, 1993
Accepted August 16, 1993)

Br. J. Pharmacol. (1993), **110**, 1565–1571 © Macmillan Press Ltd, 1993

The effect of defibrotide on thromboembolism in the pulmonary vasculature of mice and rabbits and in the cerebral vasculature of rabbits

*‡W. Paul, †‡P. Gresele, †‡S. Momi, **G. Bianchi & [1]*‡C.P. Page

*Department of Pharmacology, King's College, University of London, Manresa Road, London SW3 6LX; †Istituto di Medicina Interna e di Medicina Vascolare, Universita di Perugia, via Enrico dal Pozzo, 06100 Perugia, Italy; **CRINOS S p A, 22079 Villa Guardia, Como, Italy and ‡International Inter-University Centre on Thrombosis and Haemostasis

1 Administration of bovine thrombin (100 u kg^{-1}) into the carotid artery of rabbits induces a sustained accumulation of ^{111}Indium-labelled platelets within the cranial vasculature over the subsequent 3 h.

2 Intracarotid (i.c.) administration of defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} for 1 h) prior to i.c. thrombin (100 u kg^{-1}) significantly reduces the ability of thrombin to induce cranial thromboembolism in rabbits.

3 Intravenous (i.v.) administration of thrombin (20 u kg^{-1}) in rabbits induces a reversible accumulation of radiolabelled platelets into the thoracic circulation which is significantly reduced by i.v. administration of defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} for 1 h) prior to i.v. thrombin. In contrast, platelet accumulation in response to adenosine diphosphate (ADP; 20 μg kg^{-1}, i.v.) or platelet activating factor (PAF; 50 ng kg^{-1}, i.v.) is not significantly affected by this treatment.

4 Intravenous administration of the nitric oxide (NO)-synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 10 mg kg^{-1}) potentiates platelet accumulation induced by low dose thrombin (10 u kg^{-1}, i.v.) within the pulmonary vasculature of rabbits. The potentiated response is significantly abrogated following pretreatment with defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} for 1 h, i.v.).

5 Intravenous injection of human thrombin (1250 u kg^{-1}) to mice induces death within the majority of animals which is significantly reduced by pretreatment with defibrotide (150–175 mg kg^{-1}, i.v.). In contrast, death induced by i.v. collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}) is not significantly affected by defibrotide pretreatment.

6 The inhibitory effect of defibrotide in mice is abolished following concomitant treatment with the inhibitor of fribrinolysis, tranexamic acid (100 mg kg^{-1}, i.v.), but is unaffected following treatment with the cyclo-oxygenase inhibitor, aspirin (300 mg kg^{-1}, i.p.).

7 The protective effect of defibrotide against thrombin-induced thromboembolism in the mouse is potentiated by recombinant tissue-plasminogen activator (rt-PA; 1 mg kg^{-1}, i.v.) or unfractionated heparin (10 u kg^{-1}, i.v.) administration.

8 The results suggest that defibrotide may possess antithrombotic activity on thrombin-induced thromboembolism which, at least in the mouse, may be partially mediated via induction of the fibrinolytic pathway.

Keywords: ^{111}-Indium; platelets; defibrotide; thrombosis; fibrinolysis

Introduction

Defibrotide, a single stranded polydeoxyribonucleotide obtained by controlled depolymerisation of mammalian DNA, is a cluster of chains of different length with a mean molecular weight ranging between 15 and 30 kDa. This substance displays a multiple pharmacological profile in animals, by being profibrinolytic (Pescador *et al.*, 1983), anti-thrombotic (Niada *et al.*, 1986) and by preventing post-ischaemic organ failure in experimental models (Thiemermann *et al.*, 1989; Berti *et al.*, 1991). Defibrotide has also been reported to possess some beneficial effects in patients with thrombotic disorders (Coccheri *et al.*, 1988; Strano *et al.*, 1991). Defibrotide exhibits a number of pharmacological effects on the vascular endothelium and on circulating blood elements. It favourably modulates the release of tissue plasminogen activator (t-PA) and its inhibitor(s) (plasminogen activator inhibitor, PAI) from the vascular wall (Nunziata *et al.*, 1991), enhances the release of prostaglandins E_2 and I_2 from isolated organ preparations (Bianchi & Berti, 1992) and inhibits activation and adherence of polymorphonuclear leukocytes (Di Perri & Laghi Pasini, 1988).

We have recently described an automated isotope monitoring system to investigate the behaviour of platelets and blood elements *in vivo* in a variety of circumstances (May *et al.*, 1990; 1992). In studies in the rabbit, it was shown that intravenous (i.v.) or intracarotid (i.c.) injection of any one of a variety of platelet agonists caused transient, reversible accumulation of platelets within the pulmonary vasculature with a concomitant decrease in platelets in the vascular beds of the hind limb or head (May *et al.*, 1990). Among the agents tested, thrombin was unique in its ability to produce a significant, sustained accumulation of platelets in the cerebral vasculature following its injection directly into a carotid artery (May *et al.*, 1990). Platelet accumulation within the cranial vasculature was accompanied by increased localization of radiolabelled fibrinogen (May *et al.*, 1990). These changes were not merely reflections of alterations in local blood flow or blood pooling, since they were not seen in experiments using radiolabelled red blood cells or serum albumin (May *et al.*, 1990). Interestingly, the aggregation of platelets within the pulmonary (but not the cerebral) circulation could be increased by pretreating animals with N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO)-synthase (May *et al.*, 1991), suggesting that the

[1] Author for correspondence.

control of the interaction of blood elements with the vessel wall may vary in different vascular beds. While established fibrinolytic agents such as urokinase, eminase or recombinant tissue-plasminogen activator (rt-PA) could effectively reverse thrombin-induced embolisation within the cerebral vasculature, defibrotide only weakly affected this response (May *et al.*, 1992), an observation which conflicted with previously reported data showing that defibrotide was able to reduce the size of pre-formed thrombi (Fumagalli *et al.*, 1989). The reason for this discrepancy is not clear and remains to be explained. The aim of the present study was to understand better the mechanism of the anti-thrombotic activity of defibrotide. Therefore, in addition to investigating the effects of defibrotide pretreatment on thrombin-induced embolisation within the cerebral and pulmonary vasculature of the rabbit, we have also investigated thrombin-induced pulmonary embolism in the mouse, a convenient method for elucidating the site(s) at which drugs act to prevent thrombus formation (Gresele *et al.*, 1990a,b; Momi *et al.*, 1992).

Methods

Experimental animals

The study was carried out on rabbits (New Zealand White; Froxfield Farms, Petersfield, Hants, UK and Charles River, Milan, Italy) of either sex weighing 1.8–2.8 kg or male Swiss CD-1 mice (Charles River, Calco, Italy) weighing 20–25 g. Animals received a standard diet and water *ad libitum*.

Preparation of rabbits

Neuroleptic analgesia was induced in rabbits with a combination of diazepam (5 mg kg^{-1}, i.p.) followed 10 min later by fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1} (Hypnorm; 0.4 ml kg^{-1}, i.m.). Neuroleptic analgesia was maintained by i.m. injections of Hypnorm (0.1 ml kg^{-1}) as necessary (approximately 30 min intervals). The left common carotid artery was cannulated in the direction of blood flow for intracarotid bolus injections and infusions. Intravenous bolus injection and infusions were made into a marginal ear vein.

^{111}Indium labelling of rabbit platelets

Full details of the protocol for isolation and radiolabelling of rabbit platelets with ^{111}indium-oxine have been described elsewhere (May *et al.*, 1990). Briefly, 9 ml blood obtained from a marginal ear vein was collected into 1 ml of 3.8% (w/v) trisodium citrate and centrifuged at 200 *g* (10 min) to obtain platelet-rich plasma (PRP). The platelets were then washed by centrifugation (640 *g*, 10 min) in a calcium- and magnesium-free Tyrode solution containing prostaglandin E_1 (PGE_1) 300 ng ml^{-1} (CFTP). After removing the supernatant, the surface of the platelet pellet was carefully rinsed with 1 ml CFTP. The platelets were gently resuspended in 1 ml CFTP and incubated for 1.5 min at 37°C with ^{111}indium-oxine (25–50 μCi). After a further centrifugation (640 *g*, 10 min) to remove any excess, unbound ^{111}indium, the platelet pellet was carefully rinsed and then resuspended in 2 ml CFTP. The labelled platelets were administered (1 ml/recipient) to anaesthetized rabbits via a marginal ear vein.

Monitoring of radiolabelled platelets in vivo

Radioactivity associated with ^{111}indium-labelled platelets was continuously monitored by means of one inch crystal scintillation detectors placed over the thorax (to measure changes in the pulmonary circulation) and head (to measure changes in the cerebral circulation). Counts were estimated with a multi-channel spectrometer (Nuclear Enterprises NE461) and logged, with the aid of a special application interface (AIMS 8000, Mumed Ltd), by a microcomputer (Dell System 200).

Following i.v. administration of radiolabelled platelets, counts were monitored for 1 h until they had stabilised. Each animal then received a bolus dose of adenosine diphosphate (ADP; 100 μg kg^{-1}, i.c. or 10 μg kg^{-1}, i.v.) in order to determine whether the platelets were functioning normally, and also to eliminate any that might have been damaged during labelling (May *et al.*, 1990).

After a further 30 min stabilisation, responses to treatment infusions and to administration of platelet agonists were recorded as changes in radioactivity from these later stable baseline values. Agonist-induced responses have been plotted as % change from baseline counts and expressed in the text as % change from counts immediately prior to injection of the agonist. The area under the time curve (AUC) of agonist induced % change in counts has been calculated to give a measure of the response duration.

Thrombin-induced pulmonary embolism in mice

The method has been described elsewhere (Gresele *et al.*, 1990a,b; Momi *et al.*, 1992). Mice were caged and fed a regular diet for at least 1 week before use. The drugs to be tested, or their vehicles, were administered either by the intraperitoneal (i.p.) route in a volume of 20 ml kg^{-1} or i.v. in a fixed volume of 100 μl in a tail vein, prior to the thrombotic challenge. In the majority of these experiments, the thrombotic challenge was induced by 100 μl (containing 1250 u kg^{-1}) of thrombin from human plasma. In some experiments, animals were challenged with collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}) i.v. Mice were accustomed to handling by the investigators and the i.v. injections were carried out by skilled investigators with minimal disturbance to the animals. The total duration of each experiment was 15 min and all surviving animals were immediately killed by exposure to ether vapours. The majority of animals injected with thrombin died within 5 min. Any animals which did not die within this time or which were obviously distressed were killed after 15 min and were recorded as survivors. No anaesthesia was used during the experiments because of the short duration and because it has been shown previously that anaesthesia interferes with the effects of thrombin on pulmonary embolism and mortality (Di Minno & Silver, 1983; Gresele *et al.*, unpublished observations). In each experimental session, at least five animals per treatment group were tested; control groups were run at the beginning and at the end of every session. There was no significant difference in thrombin-induced mortality between any of the control groups and hence these have been pooled. The data have been presented as number of animals dead/total number of animals tested or as % mortality.

Tissue histology in mice

Two minutes after i.v. injection of thrombin or saline, mice were rapidly killed with ether and the lungs, kidneys, liver, spleen and brain removed, rinsed with cold saline and immediately fixed in 10% formalin. The wet tissues were blocked in a uniform manner and paraffin sections, cut at 5–6 μm, were stained with haematoxylin and eosin. The specimens were examined under light microscopy by a pathologist unaware of the treatment administered to the animals. At least four fields, at a magnification of 400 ×, were observed for every specimen using a micrometer eye piece in order to assess vessel wall diameters.

Materials

Drugs and reagents were obtained as follows: adenosine diphosphate (ADP, Sigma, Poole), adrenaline bitartrate (Mascia Brunelli, Milan), aspirin, lysine salt (Italseber, Milan), bovine serum albumin (BSA, Fraction V), bovine thrombin (Sigma, Poole), soluble collagen from bovine ten-

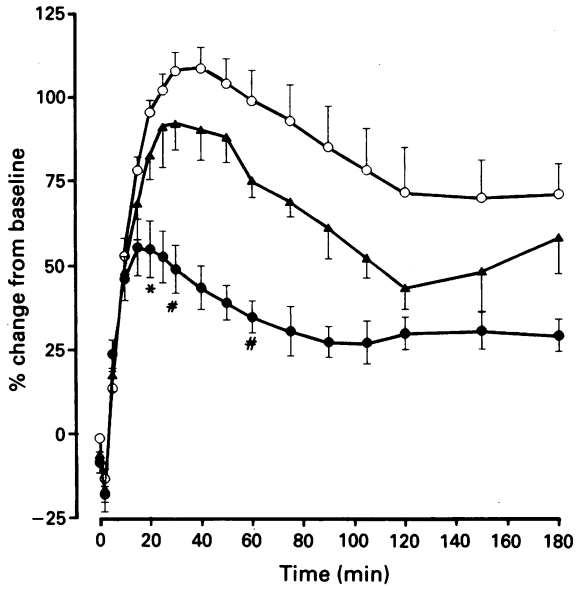


Figure 1 ^{111}In-labelled platelet accumulation in the cerebral circulation of rabbits following intracarotid injection of thrombin (100 u kg^{-1}) at time 0. Animals were pretreated, via a carotid artery, with a bolus injection, followed by 1 h continuous infusion, terminating at time 0, of saline (0; $n = 8$), defibrotide 32 mg kg^{-1} plus 32 mg kg^{-1} h^{-1} (▲; $n = 3$) or defibrotide 64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1} (●; $n = 6$). Results are expressed as mean ± s.e.mean percentage changes from baseline values recorded prior to commencement of the infusions. $*P<0.05$; $\#P<0.01$ compared with saline pretreated controls.

don (Hormon Chemie, Munich), defibrotide (batch no. 82, Crinos SpA, Villa Guardia), diazepam (Valium, Roche), unfractionated heparin (Novo Nordisk, Denmark), Hypnorm (Janssen), L-NAME (Sigma, Poole), PAF (C16 PAF; Novabiochem, Nottingham), PGE_1 (Sigma, Poole), rt-PA (Boehringer Ingelheim, Ingelheim), thrombin from human plasma (Sigma, St. Louis) and tranexamic acid (Malesci, Italy). All materials were dissolved in saline except for human plasma thrombin (distilled water), PAF (saline containing 0.25% BSA) and collagen (isotonic glucose buffer, pH 2.7).

Statistics

Platelet accumulation data are presented as mean values ± standard errors of the means (s.e.mean). Following an analysis of variance, the significance of differences between means was assessed by Student's *t* test (unpaired), Mann Whitney U-test or Wilcoxon test as appropriate. The data for mortality in mice were tested by chi square analysis.

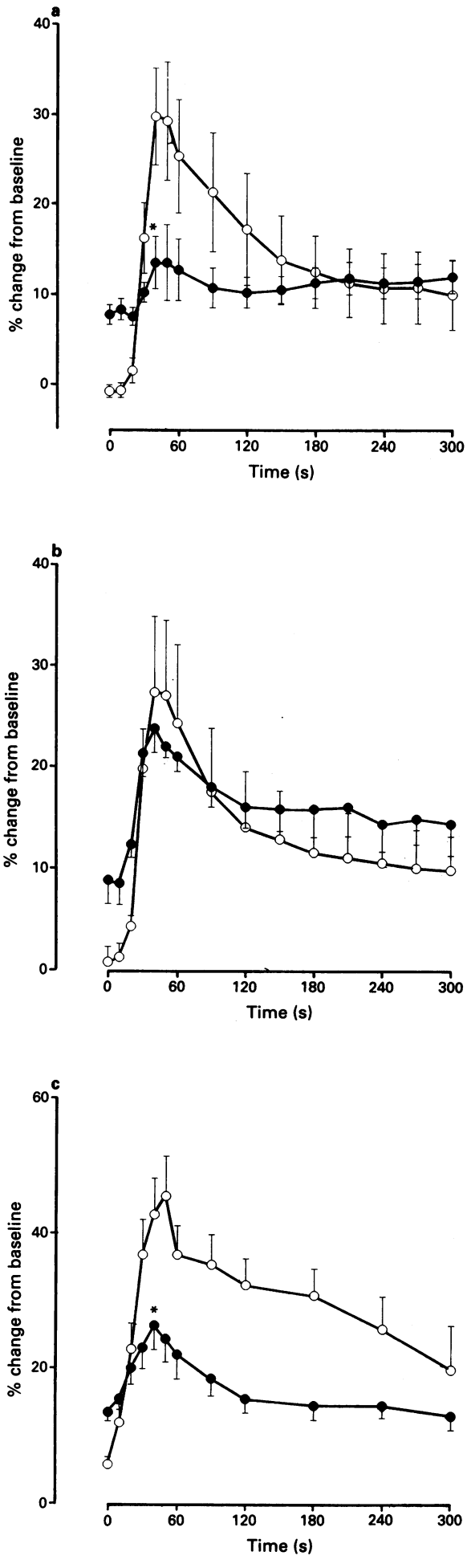


Figure 2 ^{111}In-labelled platelet accumulation in the pulmonary circulation of rabbits following i.v. injection, at time 0, of (a) and (b) thrombin (20 u kg^{-1}) or (c) thrombin (10 u kg^{-1}) 5 min after N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}). Animals were pretreated, via a marginal ear vein, with a bolus injection, followed by 1 h continuous infusion, of saline (○; controls) or defibrotide (●; 64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1}); infusions terminated at time 0 [(a) and (c)] or 1 h prior to thrombin challenge (b). In (a), $n = 6$ per group and in (b) and (c), $n = 4$ per group. Results are expressed as mean (± s.e.mean) percentage changes from baseline values recorded prior to commencement of the infusion. $*P<0.05$ compared with controls.

Results

Effects of defibrotide in rabbit cerebral vasculature

A bolus injection of defibrotide (64 mg kg^{-1}) followed by an infusion (64 mg kg^{-1} h^{-1}) for 1 h via the carotid artery had no noteworthy effect on levels of radiolabelled platelets in the cerebral vasculature. However, the peak platelet accumulation induced by i.c. administration of bovine thrombin (100 u kg^{-1}), injected immediately after the infusion of defibrotide ($n = 6$), was significantly ($P < 0.01$) reduced (57.3 ± 7.6% increase above pre-thrombin baseline) compared with animals receiving an infusion of vehicle ($n = 8$; 109.1 ± 5.5% increase; Figure 1) when measured 30 min post thrombin. Platelet accumulation between 20 and 60 min post thrombin was significantly reduced ($P < 0.05$ to $P < 0.01$) in the defibrotide pretreated animals (see Figure 1). The AUC (0–60 min) was also significantly reduced ($P < 0.01$) from 5243.1 ± 257.1 in controls to 3016.8 ± 376.4 in defibrotide-treated animals. When the dose of defibrotide was reduced to 32 mg kg^{-1} plus 32 mg kg^{-1} h^{-1} ($n = 3$), the effect was no longer significant (98.3 ± 6.3% increase at 30 min and AUC (0–60 min) 4751.3 ± 316.8; Figure 1).

Effects of defibrotide in rabbit pulmonary vasculature

Intravenous treatment with defibrotide (64 mg kg^{-1} bolus plus 64 mg kg^{-1} h^{-1} infusion for 1 h) led to an increase in platelet-associated radioactivity in the lungs. At the end of the infusion, radioactivity was 7.7 ± 1.1% above baseline ($n = 6$). Thrombin (20 u kg^{-1}, i.v.) administered at the termination of the defibrotide infusion produced a markedly depressed ($P < 0.05$) platelet response ($n = 6$; 7.3 ± 3.7% peak increase and AUC (0–5 min) 17.8 ± 6.7) compared with control animals ($n = 6$; 32.5 ± 5.8% peak increase and AUC (0–5 min) 77.4 ± 21.4; Figure 2a). When thrombin injection was delayed for 1 h after termination of the defibrotide infusion, responses to the thrombin were still reduced (17.5 ± 3.0% peak increase and AUC 37.8 ± 2.8), but were no longer significantly different from control animals (27.5 ± 7.3% peak increase and AUC 64.5 ± 22.9) ($n = 4$; Figure 2b). Responses to low dose thrombin (10 u kg^{-1}, i.v.) following L-NAME (10 mg kg^{-1}, i.v.) were significantly ($P < 0.05$) potentiated (39.8 ± 4.7% peak increase and AUC (0–5 min) 120 ± 17.0; $n = 4$) in comparison with the response to thrombin following saline (18.5 ± 3.1% peak increase and AUC 21.4 ± 3.4; $n = 4$). This potentiated response was significantly ($P < 0.05$) reduced following pretreatment with defibrotide (64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1} for 1 h) (12.8 ± 2.3% peak increase and AUC 16.7 ± 4.3; $n = 4$) (Figure 2c). However, the same dose of defibrotide had no significant effect on responses to PAF (50 ng kg^{-1}) or ADP (20 μg kg^{-1}) measured as peak increase (Table 1) or as AUC (data not shown).

Table 1 Intravenous defibrotide on pulmonary platelet accumulation induced by i.v. ADP or PAF in rabbits

Treatment	*Stimulus*	*Peak change* (%)	n
Control (saline)	ADP	31.6 ± 6.1	3
Defibrotide	ADP	27.4 ± 5.2	3
Control (saline)	PAF	26.5 ± 2.5	3
Defibrotide	PAF	24.0 ± 4.7	3

^{111}In-labelled platelet accumulation in the pulmonary circulation was induced by i.v. injection of ADP (20 μg kg^{-1}) or PAF (50 ng kg^{-1}) immediately after a bolus injection, followed by 1 h continuous infusion, of saline (controls) or defibrotide (64 mg kg^{-1} plus 64 mg kg^{-1} h^{-1}) i.v. Values shown represent mean ± s.e.mean peak % changes from levels recorded at the end of the infusions. Responses following defibrotide pretreatment were not significantly different from controls.

Effects of defibrotide on thrombin-induced pulmonary embolism in mice

Defibrotide at 175 mg kg^{-1}, i.v., 2 min before thrombin (1250 u kg^{-1}, i.v.) protected mice from death (7/52 dead/tested vs 171/210 controls, $P < 0.00001$); the effect was smaller at 150 mg kg^{-1} ($P < 0.05$) and insignificant at 100 mg kg^{-1} (23/51 and 30/38, dead/tested, respectively) (Figure 3). Although the protection decreased when the interval between defibrotide (175 mg kg^{-1}, i.v.) injection and thrombin administration was increased, a significant ($P < 0.01$) protec-

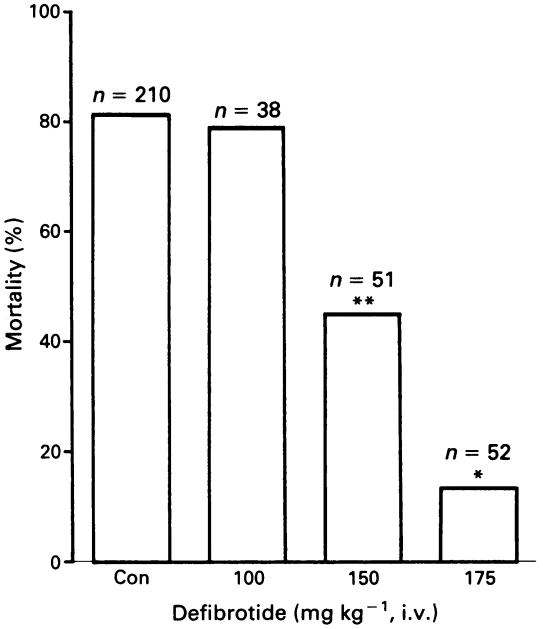


Figure 3 Effect of intravenous injection of defibrotide (100–175 mg kg^{-1}) on mortality in mice injected i.v. 2 min later with thrombin (1250 u kg^{-1}). The number of animals tested per group (n) is shown. *$P < 0.05$; **$P < 0.00001$ compared with controls (Con; saline pretreated).

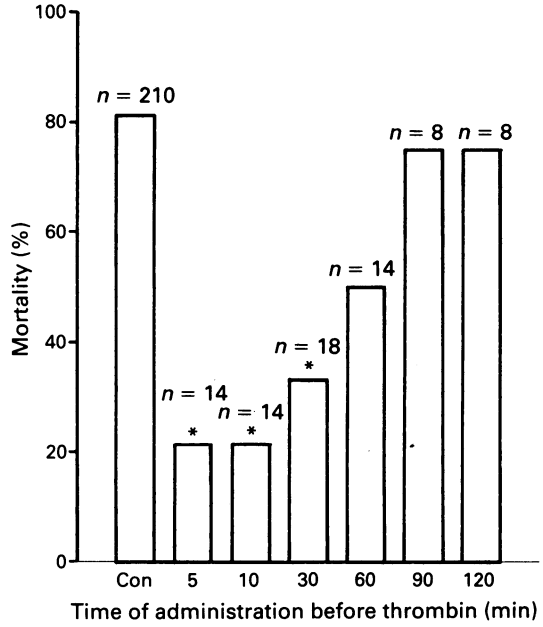


Figure 4 Effect of intravenous injection of defibrotide (175 mg kg^{-1}) at different times (5–120 min) prior to i.v. injection of thrombin (1250 u kg^{-1}) on mortality in mice. The number of animals tested per group (n) is shown. *$P < 0.05$ compared with controls (Con; saline pretreated).

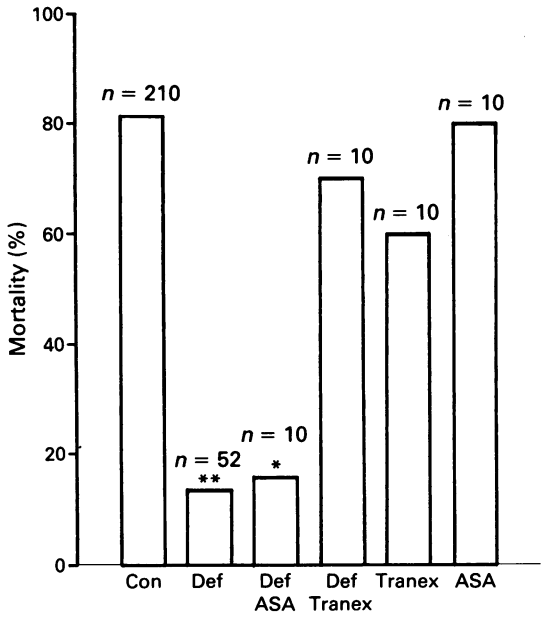


Figure 5 Effect of aspirin (ASA; 300 mg kg^{-1}, i.p.; 1 h before) or tranexamic acid (Tranex; 100 mg kg^{-1}, i.v., 4 min before) pretreatment on the effect of defibrotide (Def; 175 mg kg^{-1}, i.v., 2 min before) on mortality in mice injected i.v. with thrombin (1250 u kg^{-1}). The number of animals tested per group (n) is shown. *$P<0.01$; **$P<0.00001$ compared with controls (Con; saline pretreated).

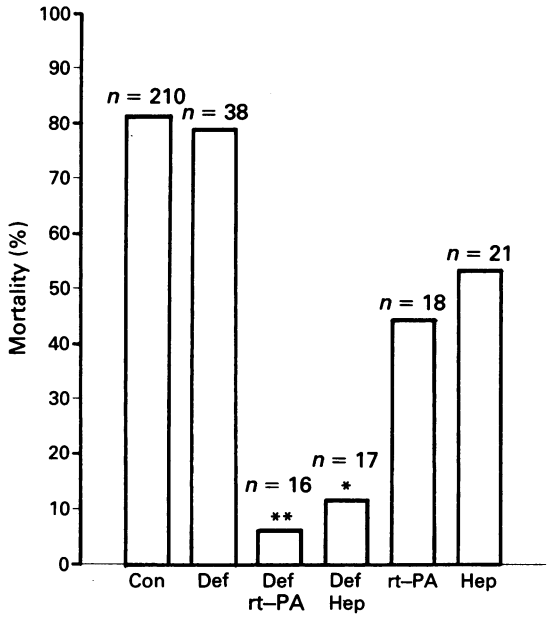


Figure 6 Effect of recombinant tissue-plasminogen activator (rt-PA; 1 mg kg^{-1}, i.v., 4 min before) or unfractionated heparin (Hep; 10 u kg^{-1}, i.v., 4 min before) pretreatment on the effect of defibrotide (Def; 100 mg kg^{-1}, i.v., 2 min before) on mortality in mice injected i.v. with thrombin (1250 u kg^{-1}). The number of animals tested per group (n) is shown. *$P<0.01$; **$P<0.005$ compared with controls (Con; saline pretreated).

Table 2 Intraperitoneal administration of defibrotide on i.v. thrombin-induced mortality in mice

	Dead/Tested	*% mortality*	P
Control	171/210	81.4	–
30 min	14/20	70.0	0.801
60 min	9/20	45.0	0.204
90 min	12/20	60.0	0.524
120 min	15/20	75.0	0.946

Animals were injected i.p. with saline (controls) or defibrotide (350 mg kg^{-1}) at different times (30–120 min) prior to i.v. injection of thrombin (1250 u kg^{-1}). The number of dead animals/total number tested and % mortality are shown for each group.

Table 3 Intravenous defibrotide on i.v. collagen plus adrenaline-induced mortality in mice

	Dead/Tested	*% mortality*	P
Control	64/88	72.7	–
Defibrotide (150 mg kg^{-1})	18/22	81.8	0.881
Defibrotide (175 mg kg^{-1})	17/30	56.6	0.579

Animals were injected i.v. with saline (controls) or defibrotide (150 or 175 mg kg^{-1}) 2 min prior to i.v. injection of collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}). The number of dead animals/total number tested and % mortality are shown for each group.

tion was still present up to 30 min (Figure 4). Defibrotide had some effect when injected intraperitoneally, at a higher dose (350 mg kg^{-1}), though this did not reach statistical significance (Table 2). Aspirin, at a dose known to suppress endogenous PGI_2 synthesis, (300 mg kg^{-1}, i.p., 1 h before thrombin) did not affect the ability of defibrotide (175 mg kg^{-1}, i.v.) to confer protection (3/19 dead/tested), while tranexamic acid (100 mg kg^{-1}), an inhibitor of fibrinolysis, reduced the protective effect (7/10, dead/tested). These doses of aspirin and tranexamic acid did not, by themselves, significantly affect survival (Figure 5). In addition, rt-PA (1 mg kg^{-1}, i.v.) or unfractionated heparin (10 u kg^{-1}, i.v.), at doses which by themselves had no significant effect on thrombin-induced mortality, significantly potentiated the protective activity of defibrotide (100 mg kg^{-1}, i.v.) (Figure 6). Defibrotide (150 or 175 mg kg^{-1}, i.v.) injected 2 min before collagen (1.25 mg kg^{-1}) plus adrenaline (75 μg kg^{-1}) had no significant protective effect (Table 3).

Tissue histology in mice

Microscopic observation of kidney, liver, spleen and brain slices prepared from control (saline pretreated) animals injected i.v. with 1250 u kg^{-1} of human thrombin did not show any significant alterations in vessels in terms of patency or diameter. In contrast, the lung slices showed that 61.5% ($n = 182$) of lung vessels were totally occluded by thrombi. In control animals injected with saline instead of thrombin, only 4.4% ($n = 82$) of vessels were occluded in the lung slices. Vessels filled with thrombotic material had a diameter less than 70 μm, although platelet clumps were observed occasionally in larger vessels. In defibrotide pretreated animals injected with thrombin, only 30% ($n = 20$) of lung vessels were occluded by thrombi.

Discussion

Our data demonstrate significant anti-thrombotic activity of defibrotide following i.v. or i.c. administration of the drug with a lesser, non-significant, effect following i.p. treatment. The ability to inhibit the thromboembolism induced by i.v. thrombin within the pulmonary vasculature or by i.c. thrombin in the cerebral vasculature of the rabbit, but the inability to attenuate significantly the platelet aggregation induced by ADP, PAF or collagen plus adrenaline suggests that the anti-thrombotic effect of defibrotide is not a direct effect of

the drug on platelets. This explanation is supported by the observation that the steady state plasma concentrations (100–200 $\mu g\,ml^{-1}$) measured *in vivo* following pretreatment of rabbits with defibrotide were in the same range as the concentrations of defibrotide (108–161 $\mu g\,ml^{-1}$) which inhibit thrombin- but not collagen-induced platelet aggregation in rabbit PRP (Porta & Bianchi, unpublished observations). The small accumulation of labelled platelets in the pulmonary vascular bed following defibrotide infusion cannot account for the decreased response to thrombin since this increase was also observed in experiments with ADP and PAF in the absence of a significant reduction in responses to these agents.

The experiments performed in mice clearly demonstrate that the protective effect of defibrotide against thrombin-induced death from pulmonary embolism in this species is abrogated by prior treatment with a purported inhibitor of fibrinolysis, tranexamic acid. These results would support the concept that part of the anti-thrombotic action of defibrotide may be secondary to the ability of this drug to promote fibrinolysis. Previous experiments using the rabbit heart and kidney (Thiemermann *et al.*, 1989; Berti *et al.*, 1991) have suggested that the action of defibrotide may be secondary to the ability of this drug to release PGI_2 from the vasculature. However, the protective effect of defibrotide in mice against thrombin-induced death from pulmonary embolism was not influenced by aspirin at doses previously reported to inhibit the synthesis of PGI_2 (Gresele *et al.*, 1990a). The observation that defibrotide was able to potentiate the protective effects of low doses of rt-PA or heparin on thrombin-induced thromboembolism in mice might imply that this drug may render blood clots more susceptible to plasmin digestion. Indeed, recently this has been reported to occur with heparin which may modify the structure of thrombi, making them more readily lysable (Nenci *et al.*, 1992). Certainly, the ability of defibrotide to facilitate fibrinolysis is consistent with the effect of this drug against thrombin-induced embolisation of platelets as thrombin is the only one of the agents used in our experiments which also initiates the coagulation cascade with deposition of fibrin and the subsequent activation of the fibrinolytic system. It is likely that, at the dose of thrombin employed, the deposition of fibrin is responsible for the irreversible nature of the embolisation in both the cerebral and pulmonary vasculature, a situation not observed when lower doses of thrombin, ADP or PAF are used to stimulate platelet aggregation (Barrett *et al.*, 1984). We have previously reported that inhibition of NO synthesis with L-NAME can potentiate the platelet aggregation induced by thrombin, converting a fully reversible platelet aggregation within the pulmonary vascular bed into an irreversible aggregation associated with fibrin deposition and often death (May *et al.*, 1992). Therefore, it is particularly noteworthy that defibrotide was effective in protecting animals against this L-NAME-induced potentiation of thrombin-induced embolisation.

In conclusion, our data suggest that the beneficial effects of defibrotide on thrombin-induced thromboembolism could be due to facilitation of fibrinolysis. However, this may not be the only mechanism involved, considering that defibrotide also inhibits the activation of leucocytes in human blood *in vitro* (Di Perri & Laghi Pasini, 1988), as well as in rat (Bianchi *et al.*, 1992) and rabbit blood (Fumagalli *et al.*, 1991) *in vitro* and *ex vivo*. Therefore, it is not surprising that defibrotide could reduce the fibrin network and the number of leucocytes trapped in clots adhering to a collagen-coated prosthesis inserted into a femoral vein in rabbits, both in fresh and in up to 7 day old thrombi (Fumagalli *et al.*, 1987; 1989). These properties may well also contribute to the clinical effects of this drug in peripheral thrombotic diseases (Strano *et al.*, 1991). The exact mechanism through which defibrotide facilitates fibrinolysis in these animal models of thromboembolism remains to be established.

The authors thank Ms P. Multari for preparation of the manuscript and Ms P. Multari and Dr G.J. Douglas for layout of the Figures. We thank Dr Paolo Alberti (Institute of Pathology, University of Perugia) for the histological screening.

References

BARRETT, P.A., BUTLER, K.D., MORLEY, J., PAGE, C.P., PAUL, W. & WHITE, A.M. (1984). Inhibition by heparin of platelet accumulation in vivo. *Thromb. Haemostas.*, **51**, 366–370.

BERTI, F., ROSSONI, G., BIANCHI, G, ALBERICO, P., TETTAMANTI, R., CALVANI, A.B., MANTOVANI, M. & PRINO, G. (1991). Effects of defibrotide on prostacyclin release from isolated rabbit kidneys and protection from post-ischaemic acute renal failure in vivo. *Eicosanoids*, **4**, 209–215.

BIANCHI, G., ALBERICO, P., TETTAMANTI, R., PORTA, R., MANTOVANI, M. & PRINO, G. (1992). Defibrotide reduces leukocyte adherence in rats by stimulating adenosine receptors. *Pharmacol. Res.*, **26**(S1), 261.

BIANCHI, G. & BERTI, F. (1992). The cardioprotective properties of defibrotide in ischemic perfused rabbit hearts reviewed: highlights on endogenous prostacyclin as a trigger of myocardial salvage. *Current Topics Pharmacol.*, **1**, 1–8.

COCCHERI, S., BIAGI, G., BIANCHINI, B. & GRAUSO, F. (1988). Acute effects of defibrotide, an experimental antithrombotic agent, on fibrinolysis and blood prostanoids in man. *Eur. J. Clin. Pharmacol.*, **25**, 151–156.

DI MINNO, G. & SILVER, J.M. (1983). Mouse antithrombotic assay: a simple method for the evaluation of antithrombotic agents in vivo. Potentiation of antithrombotic activity by ethanol alcohol. *J. Pharmacol. Exp. Ther.*, **225**, 57–60.

DI PERRI, T. & LAGHI PASINI, F. (1988). Effect of defibrotide on polymorphonuclear leukocytes: modulation of calcium entry and availability. *Sem. Thromb. Haemostas.*, **14**, 23–26.

FUMAGALLI, G., ANGELACCIO, E., LOMBARDO, N. & CLEMENTI, F. (1987). Morphometric and ultrastructural study of experimental venous thrombosis. Effects of defibrotide, an antithrombotic agent. *Haemostasis*, **17**, 361–370.

FUMAGALLI, G., ANGELACCIO, E., PORTA, R., PESCADOR, R., MANTOVANI, M. & PRINO, G. (1991). Defibrotide inhibits adhesion of polymorphonuclear leukocytes. *Thromb. Haemostas.*, **65**, 1240.

FUMAGALLI, G., SALA, C., ANGELACCIO, E., LOMBARDO, N. & CLEMENTI, F. (1989). Thrombolytic activity of defibrotide: a morphometric evaluation in experimental venous thrombosis. *Pharmacol. Res.*, **21**, 293–298.

GRESELE, P., CORONA, C., ALBERTI, P. & NENCI, G.G. (1990a). Picotamide protects mice from death in a pulmonary embolism model by a mechanism independent from thromboxane suppression. *Thromb. Haemostas.*, **64**, 80–86.

GRESELE, P., CORONA, C., PERSIA, C., BERRETTINI, M. & NENCI, G.G. (1990b). Platelet lung thromboembolism after intravenous collagen plus epinephrine in mice: characterisation of the model. *Fibrinolysis*, **4** (Suppl. 1), 124.

MAY, G.R., CROOK, P., MOORE, P.K. & PAGE, C.P. (1991). The role of nitric oxide as an endogenous regulator of platelet and neutrophil activation within the pulmonary circulation of the rabbit. *Br. J. Pharmacol.*, **102**, 759–763.

MAY, G.R., HERD, C.M., BUTLER, K.D. & PAGE, C.P. (1990). Radioisotopic model for investigating thromboembolism in the rabbit. *J. Pharmacol. Methods*, **24**, 19–35.

MAY, G.R., PAUL, W., CROOK, P., BUTLER, K.D. & PAGE, C.P. (1992). The pharmacological modulation of thrombin-induced cerebral thromboembolism in the rabbit. *Br. J. Pharmacol.*, **106**, 133–138.

MOMI, S., NENCI, G.G. & GRESELE, P. (1992). Thrombin-induced thromboembolism in mice: a model for testing antithrombotic agents acting on coagulation/fibrinolysis. *Thromb. Res.*, **65**, Suppl. 1, S162.

NENCI, G.G., PARISE, P., MORINI, M., ROSSINI, A. & AGNELLI, G. (1992). Fibrin clots obtained from plasma containing heparin show a higher sensitivity to t-PA-induced lysis. *Blood Coag. Fibrinol.*, **3**, 279–285.

NIADA, R., PESCADOR, R., PORTA, R., MANTOVANI, M. & PRINO, G. (1986). Defibrotide is antithrombotic and thrombolytic against rabbit venous thrombosis. *Haemostasis*, **16** (S1), 3–8.

NUNZIATA, A., MARIANI, M.F., CASCONE, M., CALVANI, A.B. & PESCADOR, R. (1991). Increased plasma t-PA activity and urinary PGE2 after repeated oral administration of defibrotide to the rat and the mouse. *Thromb. Res.*, **64**, 279–284.

PESCADOR, R., MANTOVANI, M., PRINO, G. & MADONNA, M. (1983). Pharmacokinetics of defibrotide and of its profibrinolytic activity in the rabbit. *Thromb. Res.*, **30**, 1–11.

STRANO, A., FAREED, J., SABBA, C. *et al.* (1991). A double blind, multicenter placebo controlled dose comparison study of orally administered defibrotide: preliminary results in patients with peripheral obliterative disease. *Sem. Thromb. Haemostas.*, **17** (S1), 228–234.

THIEMERMANN, C., THOMAS, G.R. & VANE, J.R. (1989). Defibrotide reduces infarct size in a rabbit model of experimental myocardial ischaemia and reperfusion. *Br. J. Pharmacol.*, **97**, 401–408.

(Received June 30, 1993
Revised August 4, 1993
Accepted August 17, 1993)

Br. J. Pharmacol. (1993), **110**, 1572–1578 © Macmillan Press Ltd, 1993

5-HT_4 receptor mediated facilitation of the emptying phase of the peristaltic reflex in the guinea-pig isolated ileum

B. Costall, R.J. Naylor & [1]B.R. Tuladhar

Postgraduate Studies in Pharmacology, The School of Pharmacy, University of Bradford, Bradford BD7 1DP

1 The influence of 5-hydroxytryptamine (5-HT) receptor agonists and antagonists on the emptying phase (circular muscle contraction) of the peristaltic reflex was investigated in the guinea-pig isolated ileum.

2 The effect of drug application to the serosal surface was measured as the changes in threshold pressure required to trigger the peristaltic reflex and the interval between the peristaltic strokes. A facilitation or inhibition of peristalsis was defined as a reduction or increase in threshold pressure respectively.

3 Peristalsis was not modified by the inclusion of methysergide (1 μM) and/or ondansetron (2 μM) in the bathing medium. 5-HT (0.1–1.0 μM) caused a facilitation of the perstaltic reflex; the response curve to 5-HT was not altered by the presence of methysergide (1 μM) and ondansetron (2 μM).

4 In the presence of methysergide (1 μM) plus ondansetron (2 μM), 5-HT (7.36 ± 0.06), 5-methoxytryptamine (7.01 ± 0.17), 5-carboxamidotryptamine (5.43 ± 0.06), renzapride (6.09 ± 0.17), (S)-zacopride (5.99 ± 0.11), (R)-zacopride (5.61 ± 0.13) and metoclopramide (4.8 ± 0.65) caused a concentration-related facilitation of the peristaltic reflex, the pEC_{50} values (mean ± s.e.mean) being shown in parentheses. 2-Methyl-5-HT was ineffective up to 10 μM.

5 The administration of SDZ 205-557 (1 μM) alone failed to modify the peristaltic reflex, but caused a parallel dextral shift in the concentration-effect curve to 5-HT (apparent pK_B 7.38 ± 0.30). It failed to modify the effect of acetylcholine to enhance the peristaltic reflex.

6 It is concluded that the rank order of potency of the 5-HT agonists from the indole and substituted benzamide series to facilitate the emptying phase of the peristaltic reflex in the guinea-pig ileum closely correlates with their published actions as 5-HT_4 agonists in other systems. An agonist action on the 5-HT_4 receptor is also supported by the potency of the 5-HT_3/5-HT_4 antagonist SDZ 205-557 (but not the 5-HT_3 antagonist ondansetron) to inhibit the effects of 5-HT.

Keywords: 5-Hydroxytryptamine; 5-HT_4 receptor; guinea-pig ileum; peristaltic reflex; SDZ 205-557; substituted benzamides

Introduction

The peristaltic reflex in the guinea-pig isolated ileum consists of regular and coordinated contractions of both longitudinal and circular muscles. With the distention of the ileum by the increase of intraluminal pressure, the preparation responds with an increasing longitudinal muscle contraction which is followed, as the pressure reaches a threshold point, by an aborally directed circular muscle contraction which propels the contents in the anal direction. The phase of longitudinal muscle contraction has been termed a preparatory phase and the circular muscle contraction, an emptying phase (Trendelenberg, 1917).

5-Hydroxytryptamine (5-HT) is found in the intestinal mucosa in the entero-chromaffin cells (Erspamer & Asero, 1952) and in the enteric neurones (Gershon *et al.*, 1965) and is considered to play a role in gastrointestinal motility. A stimulant action of exogenous 5-HT has been documented in the dog and the guinea-pig (Bülbring & Crema, 1959; Ormsbee *et al.*, 1981). A stimulatory action of 5-HT followed by an inhibitory effect has also been demonstrated in man (Schmid & Kinzlmeir, 1959). With the guinea-pig isolated ileum as an *in vitro* preparation, both a facilitation and an inhibition of the peristaltic reflex were observed using 5-HT (Bülbring & Crema, 1958). Thus, 5-HT facilitated the peristaltic response when applied to the mucosal surface and abolished it when applied to the serosal surface. However, facilitatory effects of lower concentrations of 5-HT were also observed before the inhibitory effect when 5-HT was applied to the serosal side. An understanding of the 5-HT receptors mediating these effects has awaited a characterization and delineation of 5-HT receptors and it is presently considered that there are four major subtypes 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 (Bradley *et al.*, 1986; Bockaert *et al.*, 1992).

The contractile effects of 5-HT in the longitudinal muscle of the guinea-pig isolated ileum are known to be mediated by 5-HT_2 receptors located on smooth muscle (formerly known as the D receptor) and neuronally located 5-HT_3 receptors (formerly known as the M receptor) (Gaddum & Picarelli, 1957; Butler *et al.*, 1988). However, the role of 5-HT_3 receptors in peristalsis is questionable since 5-HT_3 receptor antagonists fail to block the stimulatory effects of 5-HT on the peristaltic reflex (Craig & Clarke, 1991; Buchheit & Buhl, 1993). The discovery of the prokinetic action of metoclopramide and other substituted benzamides and their agonist action at the 5-HT_4 receptor (Dumuis *et al.*, 1989) indicated a further 5-HT receptor subtype that might be influenced to modulate the peristaltic reflex.

Supportive evidence for a facilitatory role of the 5-HT_4 receptor in the peristaltic reflex was obtained by Craig and Clarke (1991) who found that both 5-HT and the 5-HT_4 agonist (and 5-HT_3 antagonist) renzapride induced intermittent peristalsis in the guinea-pig isolated ileum preparation maintained at a subthreshold intraluminal pressure. These effects were resistant to ondansetron but blocked by the 5-HT_3 receptor antagonist tropisetron, but only at micromolar concentrations considered to antagonize at the 5-HT_4 receptors (Dumuis *et al.*, 1989). However, evidence for a 5-HT_4 receptor involvement remained equivocal since single concentrations of the agonists were used and tropisetron when used at high concentrations has a known variable effect to reduce contractions in both circular and longitudinal muscle (Buchheit & Buhl, 1991). Buchheit and Buhl (1991, 1993)

[1] Author for correspondence.

also studied a 5-HT$_4$ receptor involvement in the peristaltic reflex by using a range of indole and substituted benzamide 5-HT$_4$ receptor agonists and the 5-HT$_3$/5-HT$_4$ receptor antagonist SDZ 205-557. However, the major response examined was the longitudinal muscle activity measured as the area under the contraction time curve. The circular muscle contractions, which are the main component of peristalsis required for the propulsion of the intestinal content, were reported to be nonspecifically decreased due to an increased longitudinal muscle activity. Since the longitudinal muscle contraction contributes little to the propulsion of the intestinal contents (Kosterlitz *et al.*, 1956), and the effect of 5-HT during the preparatory phase to modify longitudinal muscle contraction is different from that known to modify the circular muscle contraction during the emptying phase (Kosterlitz & Robinson, 1957), the study only partially explains the prokinetic role of the 5-HT$_4$ receptor in the peristaltic reflex.

The present study was designed to investigate further the nature of the 5-HT receptors influencing the emptying phase of an ongoing peristaltic reflex using both indole and substituted benzamide agonists and 5-HT receptor antagonists acting on the 5-HT receptor subtypes. Some of these results were presented at the Spring Meeting of the British Pharmacological Society, Aberdeen, 14–16th April 1993.

Methods

Animals

Guinea-pigs (Dunkin Hartley Bradford strain) of either sex weighing 500–1200 g were housed in single sex groups of three in a cage and allowed food (FD1 and ascorbic acid; Special Diet Services) and water *ad libitum*. Hay was provided as a combined food and bedding. The animals were maintained in a constant environment of 18–19°C on a 14 h/10 h light/dark cycle.

Peristalsis

Animals were killed by cervical dislocation and a 30 cm segment of ileum from a distance of some 5 cm from the ileo-caecal junction was removed and kept in Krebs-Henseleit solution (NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 10.0 mM) oxygenated with 95% O_2 and 5% CO_2. The mesentry was removed and the lumen flushed of its contents taking care to reduce distension to the minimum before mounting the ileum in the bath. Peristalsis was induced according to a modification of the method of Bülbring and Lin (1958), the apparatus used being illustrated in Figure 1.

A segment of ileum, about 5 cm in length was cannulated in the oral and aboral ends and secured horizontally in a water-jacketed perspex bath, 7 cm long, 2 cm high and 3 cm wide. The inflow and outflow tubes to which oral and aboral ends of the intestine were cannulated, entered the bath through the opposite walls. The tubes were held in position with the help of rubber gaskets to enable small changes in the horizontal movement of the inflow and outflow tubes to locate precisely the individual length of the ileum, thus avoiding any slackness of the tissue. After the oral and aboral ends had been tied securely to the respective tubes, the ileum was forcibly compressed by use of a watch-maker's forceps at the site just proximal to the aboral output tube. This highly localised destruction prevented spontaneous contraction, the ascending excitatory reflex, observed in some tissues and arising just proximal to the outflow tube caused by the distension of the aboral tube.

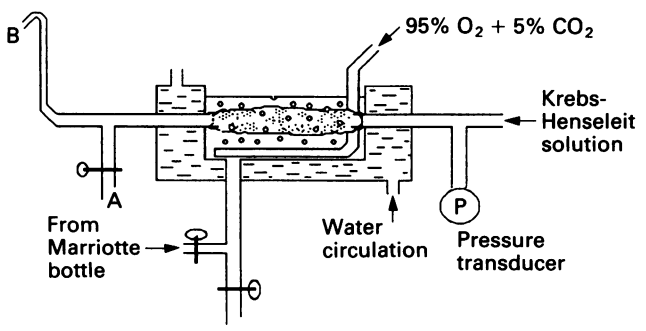


Figure 1 Diagram of the apparatus used to study the emptying phase of the peristaltic reflex in the guinea-pig isolated ileum.

The outflow tube at the aboral end had two outlets, one below the level of the bath (A) and the other about 3 cm above the level of the fluid in the bath (B). The second tube was bent and narrow (1–1.5 mm inside diameter) ensuring that fluid regurgitation into the system at the end of the peristaltic stroke was minimised without producing a high resistance. The Krebs-Henseleit solution in the bath was supplied through a Mariotte bottle and was prewarmed to 37°C by a heating coil. The bath was maintained at 37°C and oxygenated with two gas-distribution tubes running parallel to the ileum. The oxygenation procedure was such as to also reduce the mixing time of any drug addition to less than 10 s. The Krebs-Henseleit solution entering the lumen was also continuously oxygenated with 95% O_2 and 5% CO_2. The intraluminal pressure was measured from the oral side by a pressure transducer (Type 4-422, Bell & Howell Ltd., England) and recorded using a Grass polygraph. The Krebs-Henseleit solution was introduced into the lumen of the ileum by use of a Watson-Marlow peristaltic pump and regular peristalsis was achieved using a flow rate which varied in the different tissues from 1.7 to 4 ml min^{-1}. Pressure was increased slowly resulting in a distension of the ileum and, on reaching the threshold pressure, it triggered the emptying phase of peristalsis, subsequently referred to as the peristaltic stroke, starting as a highly visible contraction of the circular muscle travelling from the oral side to the aboral side. At this point the transducer recorded a sudden sharp increase in the intraluminal pressure. The pressure recorded when the fluid level in the outflow tube was equal to that in the bath was taken as the baseline pressure, the pressure when the sudden increase was recorded was taken as the threshold pressure for peristalsis. During normal peristalsis the pressure returned to the baseline level at the end of every emptying phase. With this technique it was possible to obtain an almost equal rate of peristalsis in all tissues irrespective of any variation in their threshold for at least 15 min.

Experimental design

Four tissues were mounted individually in four baths. The Krebs-Henseleit solution passing through the lumen did not contain any drug. The Krebs-Henseleit solution in the bath routinely contained 1 μM methysergide and 2 μM ondansetron.

The tissues were allowed to equilibrate for at least an hour before peristalsis was induced. During this period, the lumen was continuously flushed with Krebs-Henseleit solution at a rate of 0.5 ml min^{-1} with the drain (A) open. The fluid in the baths was changed at least twice in the first 30 min period of equilibration. The tissues were further equilibrated for another 30–45 min with or without antagonist before the drain (A) in the aboral tube was closed.

After obtaining regular peristalsis of fairly equal intervals in all four tissues, a single but different concentration of 5-HT or agonist was added to each of the four tissues. Any decrease in the threshold and the interval between the peristaltic contractions were observed over a 5 min period. The drain was then opened and the luminal flushing rate decreased to 0.5 ml min^{-1}. The equilibration cycle was then repeated for the second run. SDZ 205-557 was equilibrated

for 45 min before initiation of the peristalsis. The effects of both antagonist and agonists were evaluated using random dose administrations in either the first or second run.

A facilitatory response was defined as any decrease in the threshold (and interval between the peristaltic strokes) below the minimum threshold (and interval between the peristaltic strokes) in the 2 min period before the addition of the agonist drug in any one tissue. It was expressed as the percentage of the minimum threshold (or the interval) in the 2 min period (Figure 2B). Thus any slight differences between the threshold or the interval in the four tissues were normalised. Concentration-effect curves were obtained and the responses are expressed as the percentage reduction in threshold (or interval between the peristaltic strokes).

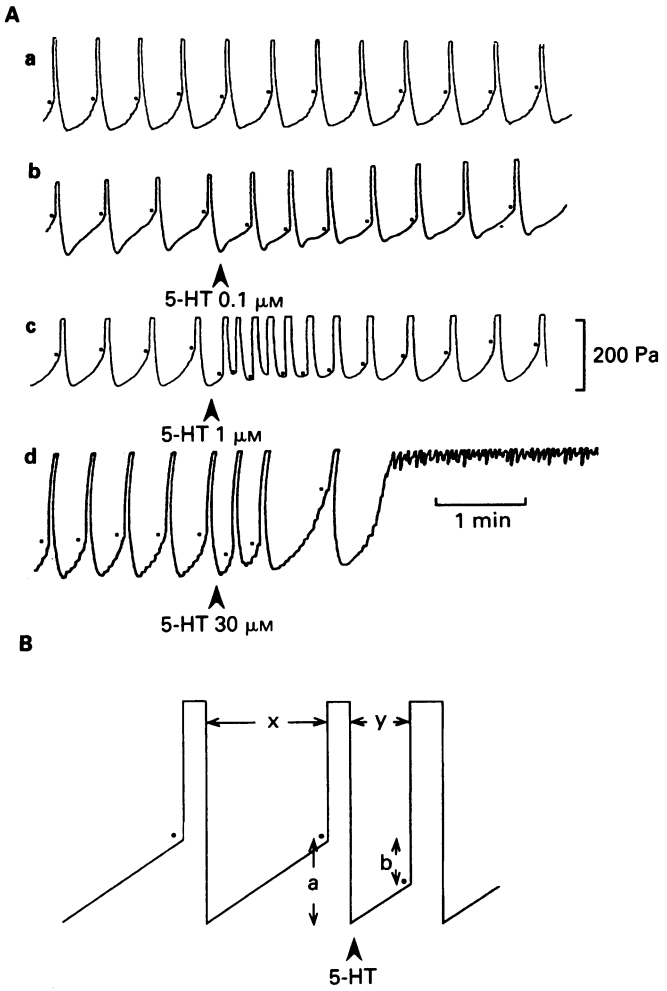


Figure 2 (A) Intraluminal pressure recordings of the peristalsis in the guinea-pig ileum in the presence of methysergide (1 μM) and ondansetron (2 μM). Intraluminal pressure recordings on (a) control tissue and the reduction in peristaltic threshold caused by the addition of (b) 0.1 μM, (c) 1 μM and (d) 30 μM 5-HT. The dots indicate the threshold for peristalsis. (B) Schematic diagram of the tracing showing the variables measured. The % reduction in threshold was calculated as [100b/a] and the % reduction in interval between peristaltic strokes was calculated as [100(x − y)/x].

Analysis of results

The ability of the agonists to facilitate peristalsis was expressed both in absolute terms, as pEC_{50} values (relative to individual maxima) and in terms of their relative potency versus 5-HT. Potency relative to 5-HT was obtained in the experiments in which two concentration-effect curves were obtained from the tissues of the same animal and expressed as the EC_{50} ratio. pEC_{50} values and EC_{50} ratios were determined graphically. All results are expressed as arithmetic mean ± s.e.mean except EC_{50} ratios which are expressed as geometric mean with a 95% confidence limit in parentheses. Apparent pK_B values for SDZ 205-557 were calculated from:

$$\text{apparent } pK_B = \log_{10}(\text{concentration-ratio} - 1) - \log_{10}(\text{antagonist concentration})$$

with a consideration that the slope is unity.

The significance of differences between the values was determined at $P < 0.05$ by use of Student's t test (unpaired two-tail).

Drugs

5-Hydroxytryptamine maleate, 5-methoxytryptamine hydrochloride, metoclopramide monohydrochloride, acetylcholine chloride (Sigma), 2-methyl-5-hydroxytryptamine hydrochloride (Cookson Chemicals Ltd.), ondansetron hydrochloride dihydrate (Glaxo), SDZ 205-557 (2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino)ethyl ester) (Sandoz), (R)- and (S)-zacopride hydrochloride (AH Robins), renzapride hydrochloride (SmithKline Beecham) and 5-carboxamidotryptamine maleate (RBI) were dissolved in distilled water and diluted in Krebs-Henseleit solution. Methysergide hydrogen maleate (Sandoz) was dissolved by sonification. All drugs were added in a volume of 250 μl to a bath volume of 25 ml and to the serosal side. The agonists were added to the bath immediately after the termination of the emptying phase.

Results

General observations

Both the peristaltic threshold and the volume required to reach that threshold varied slightly between the different segments even when taken from the same animal. The mean threshold of 128 segments taken out of 32 animals was 84 ± 2 Pa.

Table 1 Threshold for peristalsis in the guinea-pig isolated ileum in the presence and absence of 5-hydroxytryptamine (5-HT) receptor antagonists

Antagonist(s)	*Threshold for peristalsis* (Pa) *Without antagonist(s)*	*With antagonist(s)*	P *value*	n
Methysergide (1 μM)	75.1 ± 7.0	83.3 ± 7	0.47	3
Ondansetron (2 μM)	66.1 ± 13.3	75.9 ± 14.3	0.63	4
Methysergide (1 μM) + Ondansetron (2 μM)	80.5 ± 5.0	102.0 ± 13.1	0.16	9
Methysergide (1 μM) + ondansetron (2 μM) + SDZ 205-557 (1 μM)	86.1 ± 3.9	93.8 ± 4	0.23	7

P values are for Student's *t* test (unpaired two tail) for the significance of the difference between the thresholds for peristalsis with and without the antagonist(s).

The effect of 5-HT

The addition of 5-HT to the tissues caused concentration-related effects. At concentrations of 5-HT (0.1–1 μM), peristalsis was facilitated (Figure 2). This effect was characterized by a decrease in the threshold for the peristalsis and consequently, the intervals between the peristaltic strokes were reduced. In no case did 5-HT at concentrations below 1 μM exert any inhibitory effect on the peristaltic reflex. At concentrations above 1 μM the responses to 5-HT were complex. An initial facilitatory effect lasting for about 1 min was usually followed in most tissues by inhibition. The inhibitory effect developed slowly over 2–5 min and was characterized by both an increase in the peristaltic threshold and a decrease in the effectiveness of the propulsion of the luminal fluid during the emptying phase. In many tissues and usually only at 10–30 μM the peristalsis was completely inhibited as the threshold pressure increased above the level of the outlet in the tube (B) identified in Figure 1.

Effect of antagonists on the peristaltic reflex

The presence of methysergide (1 μM) and/or ondansetron (2 μM) did not have a significant effect on the threshold for the peristalsis. Moreover, further inclusion of SDZ 205-557 (1 μM) together with methysergide and ondansetron also failed to alter the threshold significantly (Table 1).

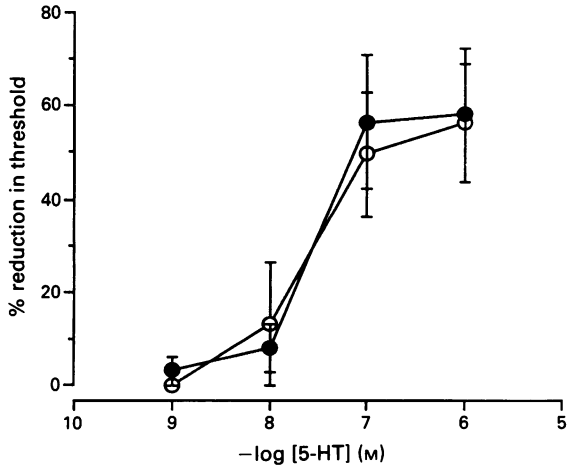


Figure 3 The concentration-effect curves to 5-hydroxytryptamine (5-HT) in the absence (○) and presence (●) of methysergide (1 μM) and ondansetron (2 μM). The reduction in the threshold is expressed as the reduction from the minimum threshold in the 2 min period before the addition of 5-HT. Four points in each concentration-effect curve were obtained from four different tissues from the same animal. Values are the mean from 6 animals with s.e.mean shown by vertical bars.

Table 2 Time taken for the maximum response and the duration of the facilitatory effect of 5-hydroxytryptamine (5-HT) in the presence of methysergide (1 μM) and ondansetron (2 μM)

Concentration of 5-HT	*Time for maximum response* (min)	*Duration of response* (min)	*% of tissues responding**
0.01 μM	1.10 ± 0.23	1.47 ± 0.27	32
0.1 μM	0.53 ± 0.06	1.92 ± 0.13	94
1 μM	0.33 ± 0.05	1.83 ± 0.11	100

*Percentage of the tissues responding are out of 34 tissues tested in each case.

Effect of 5-HT and other 5-HT agonists in the presence of methysergide and ondansetron

5-HT facilitated the peristaltic response in a concentration-dependent manner with maximum effect at 1 μM both in the presence and absence of methysergide (1 μM) and ondansetron (2 μM) (Figure 3). In some tissues at a maximal stimulation (e.g. with 1 μM 5-methoxytryptamine, Figure 5a), the contraction of the circular muscle at the oral side had started before the previous contraction had reached the aboral side. When this occurred both the percentage reduction in the threshold and the percentage reduction in the interval between the peristalsis were taken as 100% for the purpose of the calculation. The time taken for the development of the maximum response and the duration of the facilitatory response varied between the tissues. The average values are shown in Table 2. The sensitivity and hence the maximum effects to 5-HT varied between the tissues from different animals and this variation was most prominent using the concentrations below the one producing the maximum response. This in turn resulted in some variability between different concentration-effect curves (cf. control responses to 5-HT in Figures 4 and 6).

The concentration-effect curves to 5-HT for the reduction of the threshold for peristalsis and reduction in the interval of the peristaltic strokes are shown in Figure 4. The two response curves were indistinguishable. This was shown to occur with all the other agonists. Given that the values so closely followed each other, only the values calculated for the reduction in the threshold are shown in subsequent figures.

Both 5-methoxytryptamine and 5-carboxamidotryptamine caused concentration-related reductions in the threshold for peristalsis with maximum effects not significantly different from that of 5-HT. 2-Methyl-5-HT was completely ineffective up to concentrations of 10 μM. Maximum effects to renzapride, (S)- and (R)-zacopride were also not significantly different from that of 5-HT. However, the maximum effect obtained with 100 μM of metoclopramide was significantly lower (61%) than the maximum effect obtained with 5-HT. Higher doses of metoclopramide could not be tested since such concentrations abolished the peristalsis, the tissues demonstrating numerous uncoordinated contractions. The

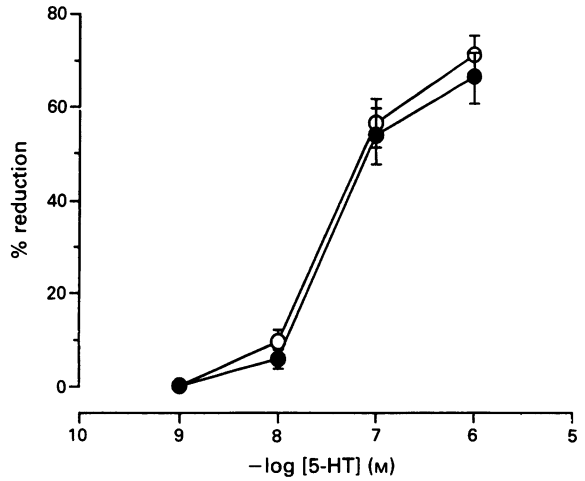


Figure 4 The concentration-effect curves to 5-HT for the reduction of the threshold for peristalsis (○) and the reduction in the interval between the peristaltic strokes (●) in the presence of methysergide (1 μM) and ondansetron (2 μM). Both the reductions in the threshold and the interval between the peristaltic strokes are expressed as the reduction from the minimum threshold and the interval between the peristaltic stroke in the 2 min period before the addition of 5-HT. Four points in each concentration-effect curve were obtained from four different tissues from the same animal. Values are the mean from 34 animals with s.e.mean shown by vertical bars.

Table 3 Agonist potency for the facilitation of the peristaltic reflex in guinea-pig isolated ileum calculated from the decrease in the threshold for peristalsis

Agonist	pEC_{50} (± s.e.mean)	E_{max} (%)	n	EC_{50} ratio (95% CL)
5-HT	7.36 ± 0.06	100	37	1
5-Methoxytryptamine	7.01 ± 0.17	102	6	2.8 (0.6–13)
Renzapride	6.09 ± 0.17	103	5	16.5 (4.5–60)
(S)-zacopride	5.99 ± 0.11	102	5	45 (3–156)
(R)-zacopride	5.61 ± 0.13	82	6	43 (17–68)
5-Carboxamidotryptamine	5.43 ± 0.06	92	4	124 (37–413)
Metoclopramide	4.80 ± 0.15	61	5	135 (43–424)
2-Methyl-5-HT	<5		4	>200

*E_{max} expressed as a percentage of the maximum response to 5-hydroxytryptamine (5-HT).

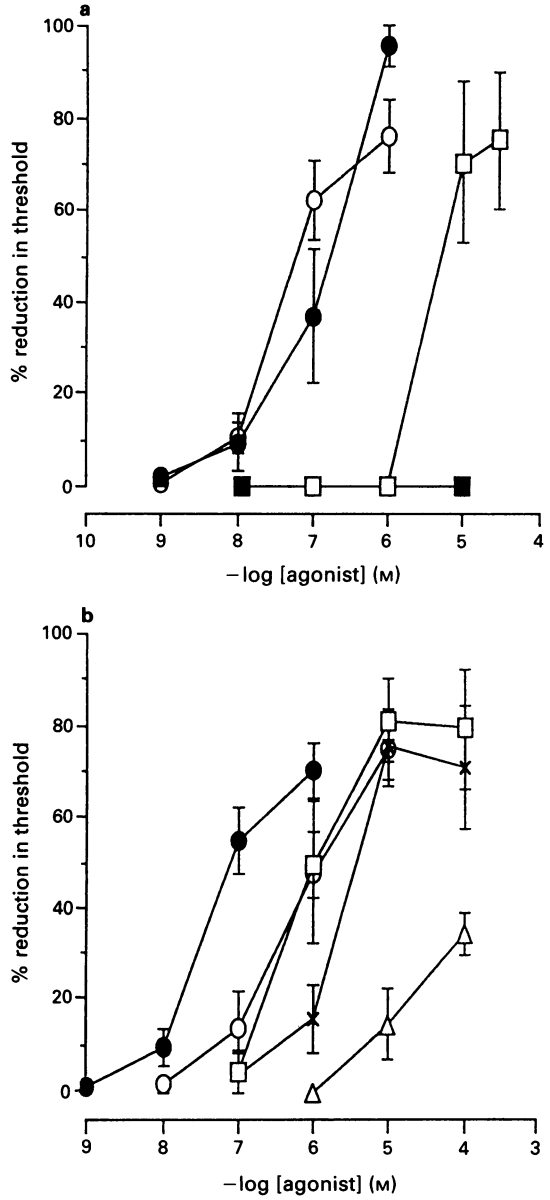


Figure 5 The concentration-effect curves to (a) 5-hydroxytryptamine (5-HT) (○, $n = 14$), 5-methoxytryptamine (●, $n = 6$), 5-carboxamidotryptamine (□, $n = 4$), 2-methyl-5-HT (■, $n = 4$) and (b) 5-HT (●, $n = 20$), renzapride (○, $n = 5$), (S)-zacopride (□, $n = 5$), (R)-zacopride (×, $n = 6$) and metoclopramide (△, $n = 4$) in the presence of methysergide (1 μM) and ondansetron (2 μM). The reduction in the threshold is expressed as the reduction from the minimum threshold in the 2 min period before the addition of the agonists. Four points in each concentration-effect curve were obtained from four different tissues from the same animal. Values are the mean with s.e.mean shown by vertical bars.

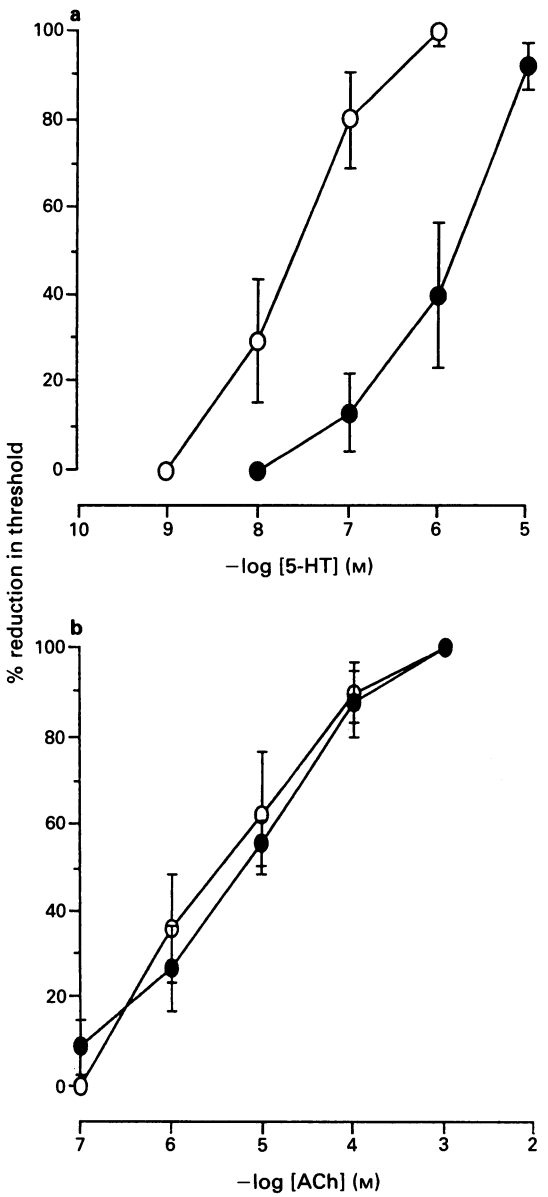


Figure 6 The concentration-effect curves to (a) 5-hydroxytryptamine (5-HT) ($n = 7$) and (b) acetylcholine (ACh) ($n = 4$) in the absence (○) and presence (●) of SDZ-205-557 (1 μM). The reduction in threshold is expressed as the reduction from the minimum threshold in the 2 min period before the addition of 5-HT. Values and the mean with s.e.mean shown by vertical bars.

concentration-effect curves for the indole agonists and substituted benzamides are shown in Figure 5; Table 3 presents the pEC_{50} values, EC_{50} ratios and the maximum effects obtained.

SDZ 205-557 (1 μM) caused a parallel dextral shift of the concentration-effect curves to 5-HT (Figure 6); an apparent pK_B($\pm$ s.e.mean) value of 7.38 $\pm$ 0.30 (n = 7) was calculated. Acetylcholine caused a concentration-related facilitation of peristalsis which was not antagonised by SDZ 205-557 (1 μM). pEC_{50} ($\pm$ s.e.mean) values of acetylcholine with and without SDZ 205-557 were not significantly different (P = 0.76) at 5.32 $\pm$ 0.30 and 5.47 $\pm$ 0.38 respectively (n = 4).

Discussion

A 5-HT$_4$ receptor-mediated increase in cholinergic drive has been demonstrated in the isolated longitudinal muscle preparation of the guinea-pig ileum (Craig & Clarke, 1990; Kilbinger & Wolf, 1992). Buchheit and Buhl (1991) have also shown that the 5-HT induced increase in longitudinal muscle activity during the peristaltic reflex is mediated via 5-HT$_4$ receptor activation. However, although longitudinal muscle contraction during the preparatory phase of peristalsis precedes the circular muscle contraction, the main propulsive drive in the peristaltic reflex comes from the aborally-directed contraction of the circular muscle during the emptying phase (Kosterlitz *et al.*, 1956). It has been demonstrated that a high concentration of acetylcholine and carbachol selectively abolish the longitudinal muscle contraction of the preparatory phase without affecting the wave of circular muscle contraction of the emptying phase of the peristaltic reflex (Kosterlitz *et al.*, 1956; Kosterlitz & Robinson, 1957). Furthermore, when the emptying phase is abolished by the ganglionic blocking agent hexamethonium, the longitudinal muscle contraction in response to the increase in the intraluminal pressure is unaffected (Kosterlitz & Robinson, 1959). These observations suggest that the two phases of the peristaltic reflex are independent of each other (Kosterlitz & Lees, 1964). Differences in the effect of 5-HT in the two phases of the peristaltic reflex have also been demonstrated by Kosterlitz and Robinson (1957), in which it was found that a high dose of 5-HT caused only transient inhibition of the longitudinal muscle contraction whilst the emptying phase was completely inhibited. For these reasons it was the effect of 5-HT on the phase of circular muscle contraction of the peristaltic reflex that was investigated in the present study.

A facilitation of the peristaltic reflex by 5-HT in the present study was obtained in the presence of methysergide and ondansetron. The inclusion of methysergide excludes an involvement of 5-HT$_{1A}$, 5-HT$_{1C}$, 5-HT$_{1D}$ and 5-HT$_2$ receptor as defined by Bradley *et al.* (1986), Peroutka (1990), Zifa and Fillion (1992) and Leysen (1990). The reduced potency of 5-carboxamidotryptamine as compared to 5-HT also indicates the non-involvement of 5-HT$_1$-like receptors (Bradley *et al.*, 1986). The presence of ondansetron at a concentration which is at least 40 times its reported affinity at the 5-HT$_3$ receptor in guinea-pig ileum (Butler *et al.*, 1988) and the inactivity of the 5-HT$_3$ receptor agonist 2-methyl-5-HT (Craig *et al.*, 1990) indicates that it is unlikely that the response is mediated by 5-HT$_3$ receptors.

The most direct evidence that 5-HT mediates its facilitatory effect on the emptying phase of the peristaltic reflex via an agonist action on the 5-HT$_4$ receptor comes from the antagonism of the effects of 5-HT by SDZ 205-557, described as a selective ligand for the 5-HT$_4$ receptor (Buchheit *et al.*, 1991; 1992). This compound has no affinity for 5-HT$_{1A}$, 5-HT$_{1C}$, 5-HT$_{1D}$, 5-HT$_2$, α_1 and α_2-adrenoceptors, opioid μ, muscarinic, nicotinic, or H_1 histamine receptor at the concentration employed in the present study (Buchheit *et al.*, 1991; 1992). The apparent pK_B value of 7.38 for SDZ 205-557 obtained in this study is very much in agreement with the previously obtained value for 5-HT$_4$ receptor antagonism in non-stimulated and electrically stimulated guinea-pig ileum (pA_2 7.4 and 7.3 respectively) (Buchheit *et al.*, 1992); rat oesophageal tunica muscularis mucosae (pA_2 7.3) (Eglen *et al.*, 1993) and guinea-pig hippocampal adenylase cyclase (pA_2 7.5) (Eglen *et al.*, 1993). SDZ 205-557 is also a selective antagonist for 5-HT$_4$ receptor over 5-HT$_3$ in guinea-pig ileum (Buchheit *et al.*, 1992; Eglen *et al.*, 1993). Although such selectivity was not observed in mouse neuroblastoma cells (Eglen *et al.*, 1993) the non selectivity in mouse neuroblastoma cells has been attributed to the species variation in the 5-HT$_3$ receptor (Eglen *et al.*, 1993). Hence the antagonism afforded by SDZ 205-557 in the present study strongly indicates that the effects of 5-HT are mediated via a 5-HT$_4$ receptor. It is unlikely that SDZ 205-557 acts beyond the 5-HT$_4$ receptor at the level of the cholinoceptors since SDZ 205-557 failed to modify the facilitatory effect of acetylcholine itself. Furthermore, the rank order of potency for the four indole agonists to facilitate the peristaltic reflex i.e. 5-HT, 5-methoxytryptamine > 5-carboxamidotryptamine >> 2-methyl-5-HT is in agreement with their reported rank order of potency for the 5-HT$_4$ system in the myenteric plexus of the guinea-pig ileum (Craig & Clarke, 1990), the rat oesophagus tunica muscularis mucosae (Baxter *et al.*, 1991) and the mouse colliculi neurones (Dumuis *et al.*, 1988). It is also relevant that the enhancement of the electrically-induced twitch contractions in the guinea-pig circular muscle preparation of the intestine by 5-HT has been shown to be mediated by a 5-HT$_4$ receptor (Tonini *et al.*, 1992).

Evidence for a 5-HT$_4$ receptor involvement was also obtained from the use of renzapride, metoclopramide and the isomers of zacopride (Sanger, 1987; Sanger & King, 1988; Smith *et al.*, 1988). All such agents are 5-HT$_3$ receptor antagonists and (S)-zacopride has additional 5-HT$_3$ agonist effects (Middlefell *et al.*, 1990; Sancilio *et al.*, 1990). However, such actions are not relevant to the present experiments where even in the presence of ondansetron all the agents mimicked the actions of 5-HT to facilitate circular muscle contraction responses. Again, their potency profile is in agreement with the known 5-HT$_4$ receptor agonist action of these compounds on the myenteric plexus of guinea-pig ileum (Craig & Clark, 1990), the rat oesophagus tunica muscularis mucusoae (Baxter *et al.*, 1991) and mouse colliculi neurones (Dumuis *et al.*, 1989).

The present finding of a facilitation of the circular muscle contractions during peristalsis due to 5-HT$_4$ receptor activation differs from previous studies by Buchheit and Buhl (1992, 1993) in which they found only non-specific inhibition of circular muscle contraction. This discrepancy can probably be explained by the difference in the methodologies employed in the two studies. Thus in the present experiments, the effects of 5-HT$_4$ receptor agonists were studied on on-going peristalsis whereas Buchheit and Buhl (1991, 1993) added the agonists 5 min before the initiation of the peristalsis. The facilitatory effect on the circular muscle contraction in the present study exhibited fade. The response lasted for only about 2 min, after which both threshold and frequency returned to normal. Furthermore, repeated challenge with 0.1 μM 5-HT at 5 min intervals (in the absence of methysergide and ondansetron) produced no response in second and subsequent challenges (Tuladhar, unpublished data). Obviously, such desensitization does not occur in longitudinal muscle contraction as both cumulative and non-cumulative addition of agonists have been shown to produce identical concentration-effect curves for longitudinal muscle activity during peristalsis (Buchheit & Buhl, 1991; 1993). Interestingly, the inhibition of the circular muscle contraction observed by Buchheit and Buhl (1991, 1993) was thought not to be due to 5-HT$_4$ receptor activation but to the indirect consequence of increased longitudinal muscle contraction, since contraction of circular muscle requires elongation in the longitudinal axis (Wood & Perkins, 1970).

In summary, an agonist action by 5-HT, related indoles

and substituted benzamides at the 5-HT_4 receptor enhances the peristaltic response in the guinea-pig isolated ileum. The failure of the administration of SDZ 205-557 alone to modify peristalsis indicates a lack of endogenous 5-HT_4 tone and supports the suggestion made by previous investigators (Craig & Clarke, 1991) that the 5-HT_4 receptor might be involved in a regulatory capacity rather than being directly involved in the neural circuitry of the peristaltic reflex.

We should like to thank the ORS Awards Scheme for providing support to B.R.T. and Sandoz, SmithKline Beecham and Glaxo for gifts of drugs.

References

BAXTER, G.S., CRAIG, D.A. & CLARKE, D.E. (1991). 5-Hydroxytryptamine$_4$ receptors mediate relaxation of the rat oesophageal tunica muscularis mucosae. *Naunyn-Schmiedebergs. Arch. Pharmacol.*, **343**, 439–446.

BOCKAERT, J., FOZARD, J.R., DUMUIS, A. & CLARKE, D.E. (1992). The 5-HT_4 receptor: a place in the sun. *Trends Pharmacol. Sci.*, **13**, 141–145.

BRADLEY, P.B., ENGEL, G., FENIUK, W., FOZARD, J.R., HUMPHREY, P.P.A., MIDDLEMISS, D.N., MY LECHARANE, E.J., RICHARDSON, B.P. & SAXENA, P.R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology*, **25**, 563–576.

BUCHHEIT, K.-H. & BUHL, T. (1991). Prokinetic benzamides stimulate peristaltic activity in the isolated guinea pig ileum by activation of 5-HT_4 receptors. *Eur. J. Pharmacol.*, **205**, 203–208.

BUCHHEIT, K.-H. & BUHL, T. (1993). 5-HT receptor subtypes involved in the stimulatory effect of 5-HT on the peristaltic reflex *in vitro*. *J. Gastrointest. Mot.*, **5**, 49–55.

BUCHHEIT, K.-H., GAMSE, R. & PFANNKUCHE, H.J. (1991). SDZ 205-557, a selective antagonist at 5-HT_4 receptors in the isolated guinea-pig ileum. *Eur. J. Pharmacol.*, **200**, 373–374.

BUCHHEIT, K.-H., GAMSE, R. & PFANNKUCHE, H.-J. (1992). SDZ 205-557, a selective, surmountable antagonist for 5-HT_4 receptors in the isolated guinea pig ileum. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **345**, 387–392.

BÜLBRING, E. & CREMA, A. (1958). Observations concerning the action of 5-hydroxytryptamine on the peristaltic reflex. *Br. J. Pharmacol.*, **13**, 444–457.

BÜLBRING, E. & CREMA, A. (1959). The action of 5-hydroxytryptamine, 5-hydroxytryptophan and reserpine on intestinal peristalsis in anaesthetized guinea-pigs. *J. Physiol.*, **146**, 29–53.

BÜLBRING, E. & LIN, R.C.Y. (1958). The effect of intraluminal application of 5-hydroxytryptamine and 5-hydroxytryptophan on peristalsis; the local production of 5-HT and its release in relation to intraluminal pressure and propulsive activity. *J. Physiol.*, **140**, 381–407.

BUTLER, A., HILL, J.M., IRELAND, S.J., JORDAN, C.C. & TYERS, M.B. (1988). Pharmacologicl properties of GR38032F, a novel antagonist at 5-HT_3 receptors. *Br. J. Pharmacol.*, **94**, 397–412.

CRAIG, D.A. & CLARKE, D.E. (1990). Pharmacological characterization of a neuronal receptor for 5-HT in guinea-pig ileum with properties similar to the 5-hydroxytryptamine$_4$ receptor. *J. Pharmacol. Exp. Ther.*, **252**, 1378–1386.

CRAIG, D.A. & CLARKE, D.E. (1991). Peristalsis evoked by 5-HT and renzapride: Evidence for putative 5-HT_4 receptor activation. *Br. J. Pharmacol.*, **102**, 563–564.

CRAIG, D.A., EGLEN, R.M., WALSH, L.K.M., PERKINS, L.A., WHITING, R.L. & CLARKE, D.E. (1990). 5-Methoxytryptamine and 2-methyl-5-hydroxytryptamine-induced desensitization as a descriminative tool for the 5-HT_3 and putative 5-HT_4 receptors in guinea pig ileum. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **342**, 9–16.

DUMUIS, A., BOUHELAL, R., SEBBEN, M., CORY, R. & BOCKAERT, J. (1988). A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol. Pharmacol.*, **34**, 880–887.

DUMUIS, A., SEBBEN, M. & BOCKAERT, J. (1989). The gastrointestinal prokinetic benzamide derivatives are agonists at the nonclassical 5-HT receptor (5HT_4) positively coupled to adenylate cyclase in neurons. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340**, 403–410.

EGLEN, R.M., ALVAREZ, R., JOHNSON, L.G., LEUNG, E. & WONG, E.H.F. (1993). The action of SDZ 205,557 at 5-hydroxytryptamine (5-HT_3 and 5-HT_4) receptors. *Br. J. Pharmacol.*, **108**, 376–382.

ERSPAMER, V. & ASERO, B. (1952). Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature*, **169**, 800–801.

GADDUM, J.H. & PICARELLI, Z.P. (1957). Two kinds of tryptamine receptor. *Br. J. Pharmacol.*, **12**, 323–328.

GERSHON, M.D., DRAKONTIDES, A.B. & ROSS, L.L. (1965). Serotonin: synthesis and release from myenteric plexus of the mouse intestine. *Science*, **149**, 197–199.

KILBINGER, H. & WOLF, D. (1992). Effects of 5-HT_4 receptor stimulation on basal and electrically evoked release of acetylcholine from guinea-pig myenteric plexus. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **345**, 270–275.

KOSTERLITZ, H.W. & LEES, G.M. (1964). Pharmacological analysis of intrinsic intestinal reflexes. *Pharmacol. Rev.*, **16**, 301–339.

KOSTERLITZ, H.W., PIRIE, V.W. & ROBINSON, J.A. (1956). The mechanism of the peristaltic reflex in the isolated guinea-pig ileum. *J. Physiol.*, **133**, 681–694.

KOSTERLITZ, H.W. & ROBINSON, J.A. (1957). The inhibition of the peristaltic reflex of the isolated guinea-pig ileum. *J. Physiol.*, **136**, 249–262.

KOSTERLITZ, H.W. & ROBINSON, J.A. (1959). Reflex contractions of the longitudinal muscle coat of the isolated guinea-pig ileum. *J. Physiol.*, **146**, 369–379.

LEYSEN, J.E. (1990). Gaps and pecularities in 5-HT_2 receptor studies. *Neuropsychopharmacology*, **3**, 361–369.

MIDDLEFELL, V.C., PRICE, T.L., THOMSON, I.A. & SPOKES, R. (1990). 5-HT_3 agonist effects of zacopride in the ferret. *Br. J. Pharmacol.*, **101**, 560P.

ORMSBEE, H.S. III., SILBER, D.A. & HARDY, F.E.J.R. (1981). Evidence for serotonergic regulation of canine migrating motor complex (MMC). *Gastroenterol.*, **80**, 1244.

PEROUTKA, S.J. (1990). 5-Hydroxytryptamine receptor subtypes. *Pharmacol. Toxicol.*, **67**, 373–383.

SANCILIO, L.F., PINKUS, L.M., JACKSON, C.B. & MUNSON, H.R. (1990). Emetic activity of zacopride in ferrets and its antagonism by pharmacological agents. *Eur. J. Pharmacol.*, **181**, 303–306.

SANGER, G.J. (1987). Increased cholinergic activity and antagonism of 5-hydroxytryptamine M-receptors by BRL 24924. *Br. J. Pharmacol.*, **91**, 77–87.

SANGER, G.J. & KING, F.D. (1988). From metoclopramide to selective gut motility stimulants and 5-HT_3 receptor antagonists. *Drug Design and Delivery*, **3**, 273–295.

SCHMID, E. & KINZLMEIR, H. (1959). Das Verhalten der Magenaciditat und der Motilitat in Verdauungstrakt des Menschen bei Infusion von Serotonin. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **236**, 51–54.

SMITH, W.W., SANCILIO, L.F., OWERA-ATEPO, J.B., NAYLOR, R.J. & LAMBERT, L. (1988). Zacopride, a potent 5-HT_3 antagonist. *J. Pharm. Pharmacol.*, **40**, 301–302.

TONINI, M., CANDURA, S.M., ONORI, L., COCCINI, T., MANZO, L. & RIZZI, C.A. (1992). 5-Hydroxytryptamine$_4$ receptor agonists facilitate cholinergic transmission in the circular muscle of guinea pig ileum: antagonism by tropisetron and DAU 6285. *Life Sci.*, **50**, PL173–178.

TRENDELENBURG, P. (1917). Physiologische und pharmakologische Versuche über die Dünn darmperistaltik. *Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak.*, **81**, 55–129.

WOOD, J.D. & PERKINS, W.E. (1970). Mechanical interaction between longitudinal and circular axes of the small intestine. *Am. J. Physiol.*, **218**, 762–768.

ZIFA, E. & FILLION, G. (1992). 5-Hydroxytryptamine receptors. *Pharmacol. Rev.*, **44**, 401–458.

(Received May 19, 1993
Revised August 16, 1993
Accepted August 20, 1993)

Br. J. Pharmacol. (1993), **110**, 1579–1585 © Macmillan Press Ltd, 1993

Electrophysiological and antiarrhythmic actions of the κ agonist PD 129290, and its **R,R** (+)-enantiomer, PD 129289

M.K. Pugsley, [1]D.A. Saint, M.P. Penz & [2]M.J.A. Walker

Department of Pharmacology & Therapeutics, Faculty of Medicine, The University of British Columbia, 2176 Health Sciences Mall, Vancouver BC, Canada V6T 1Z3

1 The **S,S** (−)-enantiomer PD 129290, a κ agonist, and its corresponding inactive **R,R** (+)-enantiomer (PD 129289) were studied in rat isolated hearts and in intact rats for cardiovascular and antiarrhythmic actions. The electrophysiological actions of PD 129290 were also studied in rat isolated cardiac myocytes using voltage clamp.

2 Ventricular pressure, heart rate and ECG were studied in isolated hearts while blood pressure, heart rate and ECG were studied in pentobarbitone-anaesthetized rats. In the latter, responses to electrical stimulation and coronary occlusion were also investigated.

3 In isolated hearts both enantiomers, over the concentration range 2–16 μM, dose-dependently reduced systolic ventricular pressure and heart rate while prolonging the P–R and QRS intervals of the ECG.

4 At doses of 1–32 μmol kg^{-1} both enantiomers reduced blood pressure and heart rate in anaesthetized rats. In addition, both enantiomers increased the size of the RSh and increased P–R, QRS, and Q–T intervals of the ECG. The thresholds for premature beats and ventricular fibrillation were dose-dependently increased by PD 129289. At lower doses PD 129290 decreased thresholds. These decreases were blocked by naloxone to reveal underlying increases similar to those seen with PD 129289. Both enantiomers increased refractory periods.

5 Naloxone (8 μmol kg^{-1}) did not alter any of the actions of PD 129290, except to abolish the initial decreases in thresholds in intact rats seen with lower doses of PD 129290.

6 Both enantiomers (2 and 8 μmol kg^{-1}) equally reduced arrhythmias in anaesthetized rats subject to occlusion of a coronary artery.

7 In rat isolated cardiac myocytes 20 μM PD 129290, in the presence and absence of naloxone decreased the amplitude of the transient sodium current by about 50% without affecting the voltage-dependence of activation or inactivation of this current.

8 The antiarrhythmic actions of both enantiomers appear to primarily result from their Class I (sodium channel blockade) properties which are independent of κ agonism.

Keywords: Opioid; antiarrhythmic; sodium channel; κ agonist

Introduction

A range of different studies in rats have demonstrated that a wide spectrum of opioid drugs reduce arrhythmias induced by occlusion of a coronary artery (e.g. Sitsapesan & Parratt, 1989; Pugsley *et al.*, 1992b). The degree of antiarrhythmic protection varied with the opioid drug studied and there was no simple correlation between opioid type (agonist or antagonist) and antiarrhythmic activity. After examining a series of agonist and antagonist opioid drugs, Sarne *et al.* (1991) came to the provisional conclusion that sodium channel blockade was responsible for observed antiarrhythmic actions.

We recently studied the antiarrhythmic actions of the κ agonist, U-50,488H in rats and were able to relate its antiarrhythmic actions to sodium channel blockade (Pugsley *et al.*, 1992a,b) and not to κ agonism. PD 129290, an arylbenzacetamide similar in chemical structure to U50,488H, is a newly developed κ agonist (Hunter *et al.*, 1990). PD 129290 is an **S,S** (−)-enantiomer whereas PD 129289 is the corresponding **R,R** (+)-enantiomer which has less than 1/1,000 of the κ receptor binding activity of PD 129290. Its affinity for μ receptors is 0.5 that of the **S,S**-enantiomer (Halfpenny *et al.*, 1990). In view of the large difference in κ agonist activity between the two enantiomers it was apparent that a comparison of the pharmacological and antiarrhythmic profiles of PD 129289 and PD 129290 might help resolve the mechanisms by which such structurally similar, but pharmacologically different, drugs exert an antiarrhythmic action.

We have examined the actions of PD 129289 and PD 129290 in isolated hearts and in intact rats. The latter were subjected either to coronary occlusion, or to electrical stimulation of the left ventricle. The present studies are analogous to those recently completed with U50,488H (Pugsley *et al.*, 1992a,b); similar doses and concentrations were used in both studies. In the U50,488H studies possible opioid receptor-dependent (κ and μ) effects *in vivo* and *in vitro* were abolished with naloxone. In the present study we used naloxone for the same purpose and in addition used the enantiomer PD 129289 which is inactive at κ opioid receptors. We have also examined the inhibitory effects of PD 129290 on sodium currents recorded in isolated cardiac myocytes by use of the whole-cell variant of the patch clamp technique.

Methods

General

Male Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec) weighing 150–200 g were used for all experiments conducted at UBC. The isolated cardiac myocyte experiments conducted at Canberra used the available male Wistar (200–300 g) strain rats. All experiments conducted at U.B.C. were according to guidelines enforced by the U.B.C.

[1]Present address: Faculty of Applied Science, University of Canberra, Belconnen, ACT, Australia

[2]Author for correspondence.

Animal Care Committee while those performed in Australia were according to the guidelines used by the Australian National University.

Intact rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and the trachea cannulated for artificial ventilation at a stroke volume of 10 ml kg^{-1}, 60 × min^{-1}. Body temperature was monitored by a rectal thermometer and maintained between 36–37°C with a heating lamp.

Isolated hearts

Hearts were mounted on a modified Langendorff apparatus (Curtis *et al.*, 1986) and perfused at a pressure of 100 mmHg with Krebs-Henseleit solution (pH 7.4), bubbled with 5% CO_2 and O_2 and maintained at a temperature of 35°C. A compliant saline-filled balloon was inserted into the left ventricle and inflated with approximately 0.5 ml saline to a diastolic pressure of 10 mmHg. The developed pressure in the balloon was recorded together with the maximum rate of intraventricular pressure development ($+dp.dt_{max}^{-1}$) by means of a Grass differentiator, model 7P20C. ECGs were recorded (band width of 0.1–40 Hz) from silver-ball electrodes placed on the epicardial surfaces of the right atrium and left ventricle.

Hearts weighing between 1.2 and 1.6 g were, according to a random design, perfused for 5 min at each concentration with saline or either of PD 129289 or PD 129290 at 1, 2, 4, 8 and 16 μM.

Intact rats

Dose-response curves were constructed for the effects of PD 129289 and PD 129290 (cumulative doses of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 μmol kg^{-1}, i.v.) in pentobarbitone-anaesthetized and artificially ventilated rats ($n = 6$ per group). A carotid artery and jugular vein were cannulated for recording blood pressure and administering drugs, respectively. Drug was given over a 2 min period and recordings made 5 min later, i.e. just before the next dose. In a separate experiment naloxone (8 μmol kg^{-1}) was administered just before dosing with PD 129290. This dose of naloxone was the largest which could be given without influencing ECG, blood pressure or heart rate.

P–R, QRS and Q–T intervals of the ECG were measured. Since the Q–T interval in rats does not change with rate (unpublished observations), it was not corrected for heart rate. A new ECG measure, RSh (mV), was used as a reflection of possible sodium channel blockade (Penz *et al.*, 1992). RSh is the difference in mV between the peak of the R-wave and the trough of the S-wave.

In intact rats, electrical stimulation of the left ventricle was performed via two Teflon-coated silver wire stimulating electrodes inserted through the chest wall and implanted in the left ventricle (Walker & Beatch, 1988); the inter-electrode distance was approximately 1 mm. Square wave stimulation was used to determine the threshold current (i_T–μA) and pulse width (t_T–ms) for induction of extrasystoles, threshold currents for induction of ventricular fibrillation (VF_T–μA), effective refractory period (ERP–ms) and maximum following frequency (MFF–Hz) (Howard & Walker, 1990). Although MFF is related to ERP there are differences in their sensitivity to drugs (Walker & Beatch, 1988) and so both measures were made.

Coronary occlusion studies

The surgical procedures used were those of Au *et al.* (1979) and Paletta *et al.* (1989). In brief, rats were anaesthetized with pentobarbitone and artificially ventilated as described above. A carotid artery cannula was used to record blood pressure and to withdraw blood samples for determination of serum K^+ concentrations (Ionetics Potassium Analyzer). A polyethylene occluder was used to occlude the main left coronary artery in animals whose chest cavity was loosely closed after the occluder had been implanted. Rats were allowed to recover for 30 min after surgical preparation.

All rats were given an initial injection of saline except for one group which received 8 μmol kg^{-1} naloxone. In a random and double-blind manner a second injection of saline, or enantiomer was given 5 min later. Blood pressure and ECG were recorded 5 min after injection and a blood sample (0.25 ml) taken. The occluder was then tightened so as to produce occlusion.

ECG, arrhythmias, blood pressure, heart rate and mortality were monitored for 30 min after occlusion. Arrhythmias, consisting of ventricular premature beats (VPB), ventricular tachycardia (VT) and ventricular fibrillation (VF) were recorded and summarized as an arrhythmia score (AS) according to the system of Curtis & Walker (1988). After 30 min, a second blood sample was taken in surviving animals. Hearts were then removed and perfused via the Langendorff technique with cardiogreen dye (1 mg ml^{-1}) to reveal the underperfused occluded zone (zone-at-risk). The treatment groups were a saline control, PD 129290 (2 or 8 μmol kg^{-1}), PD 129289 (2 or 8 μmol kg^{-1}) and PD 129290 (8 μmol kg^{-1}) after pretreatment with naloxone 8 μmol kg^{-1}. Experimental design and analyses were performed according to the Lambeth Conventions (Walker *et al.*, 1986) with replacement of lost animals to maintain balance between groups. Rats were only included in the study providing that: (1) Mean arterial blood pressure did not fall below 25 mmHg for more than 5 min before and after occlusion. (2) Pre-occlusion ECG had well-defined P, QRS and T waves, was in normal sinus rhythm, and the S–T segment was below the R-wave peak. (3) Arrhythmias before occlusion were less than 15 VPB and no VT or VF occurred. (4) Serum potassium was between 2.9 and 3.9 mM. (5) Occlusion produced an increase in R-wave and elevation of the S–T segment (Johnston *et al.*, 1983). (6) Occluded zone size was 25–50% of total left-ventricular weight.

Statistical analyses

Statistical analyses were performed by use of the NCSS statistical package (Hintze, 1987); values are given as mean ± s.e.mean (n = group size). A general linear model ANOVA was used and multiple comparisons were made by Duncan's test. The statistically significant differences in group incidences of ventricular tachycardia (VT) or ventricular fibrillation (VF) were determined by reference to Mainland's contingency tables (Mainland *et al.*, 1956). The number of VPB was $\log_{10}$ transformed before parametric statistical analyses.

Electrophysiological studies of PD 129290 in isolated myocardial cells

In view of a limited supply of the **R,R**-enantiomer (PD 129289) it was only possible to perform electrophysiological studies on isolated cell using the **S,S**-enantiomer, PD 129290. Single cardiac myocytes were prepared by enzymic dissociation of rat isolated hearts; the method has been described in detail elsewhere (Saint *et al.*, 1992). Isolated cells were allowed to settle onto a glass slide coated with poly-l-lysine in a chamber of 1 ml volume which was perfused at 1 ml min^{-1}.

Evoked currents were recorded by conventional whole-cell patch clamp techniques. The bathing solution (temperature 34°C) had the following composition in mM: NaCl 130, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.0, $CoCl_2$ 5.0, CsCl 5.0, tetraethyl sulphonic acid 10.0, NaOH 5.0, glucose 10.0 with the pH adjusted to pH 7.4. The composition of the electrode solution in mM was: CsF 50.0, NaF 70.0, K-EGTA 20.0, $CaCl_2$ 2.0, tetraethyl sulphonic acid 10.0, ATP-Na_2 5.0, ATP-Mg 5.0, with the pH adjusted to 7.4 with KOH. Microelectrodes were fabricated from borosilicate glass and typically had a resis-

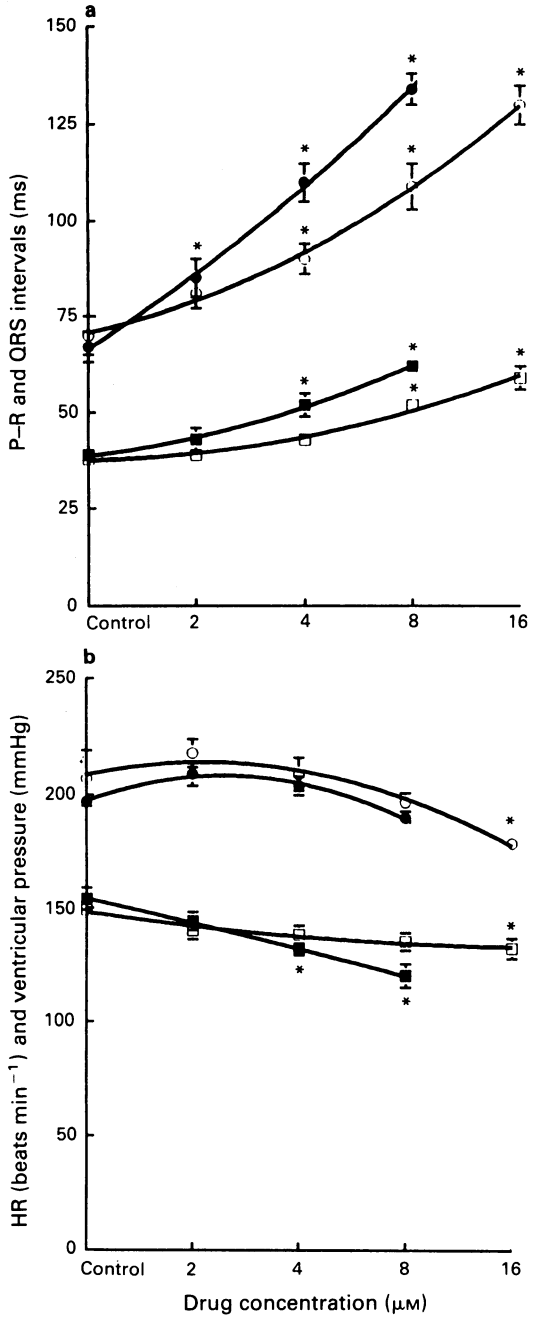


Figure 1 Concentration-response effects of PD 129289 and PD 129290 on ventricular pressure, heart rate and the ECG of rat isolated hearts. Effects of different concentrations of the two enantiomers, PD 129289 (filled symbols) and PD 129290 (open symbols) are shown as changes in ventricular pressure (□), heart rate (○) (b) and the ECG intervals of QRS (□) and P–R (○) (a) from control values after 5 min exposure to different concentrations of drug. Values are mean and vertical lines s.e.mean for $n = 5$. Control values were 154 ± 5 mmHg for ventricular pressure, 197 ± 3 beats min^{-1} for heart rate and 39 ± 2 ms for QRS and 69 ± 4 ms for P–R. *Indicates $P < 0.05$ for statistically significant differences from control.

tance of 10 MΩ. Cells were voltage clamped by use of an Axopatch 1D amplifier. Currents were filtered at 5 KHz, digitized at 10 KHz and stored in a microcomputer which also generated the voltage pulse protocol. Leakage and series resistance compensation was achieved by using the amplifier controls. Voltage pulses were given at 2 s intervals. PD 129290 was applied by adding aliquots of the stock solution of drug to the solution bathing the cells. Naloxone (1 μM) was used to block opioid receptors.

Drugs

PD 129289 (CAM 569; [5**R**-(5α,7α,8β)]-N-methyl-N[7-(1-pyrrolidinyl)-1-oxaspiro [4.5]dec-8-yl]-4-benzofuranacetamide HCl) and PD 129290 (CAM 570 = CI-977), the corresponding **S,S**-enantiomer, were generously supplied by Dr J. Hughes, Parke-Davis Research Institute, Cambridge. Naloxone was from DuPont. All drugs were made up in 0.9% NaCl solution for *in vivo* studies and in appropriate bathing solutions for *in vitro* studies.

Results

Isolated heart studies

Both PD 129289 and 129290 had dose-related effects on the ECG recorded from rat isolated perfused hearts. Figure 1 shows that both enantiomers prolonged P–R and QRS intervals although PD 129289 was somewhat more potent (dose-ratio approximately 2.0) than PD 129290; 8 μM of PD 129289 produced an approximate doubling of P–R and a lesser effect on QRS. Heart rate was reduced by PD 129290 but the change was only statistically significant for the highest concentration (Figure 1b). Dose-related bradycardia was not seen with PD 129289. Both enantiomers tended to reduce ventricular pressure (Figure 1b) and $+dp.dt^{-1}_{max}$ (data not shown). For example with PD 129289 the control peak ventricular systolic pressure of 154 ± 5 mmHg was reduced to 120 ± 5 after 8 μM ($P < 0.05$); similar changes occurred with PD 129290.

The experiments in isolated hearts demonstrated that both enantiomers had direct actions on the ECG. ECG indices were more sensitive to the drugs than either systolic ventricular pressure or heart rate.

Effects in intact rats

Blood presure, heart rate and ECG In saline treated intact rats, blood pressure and heart rate were stable over the measurement period whereas both enantiomers produced falls in blood pressure and heart rate. The fall in blood pressure was dose related for PD 129289. With the initial dose of 0.5 μmol kg^{-1} the change in pressure was − 5 ± 10 mmHg from the pre-drug value of 115 ± 8 mmHg. The fall in pressure increased with dose and was − 35 ± 7 mmHg after 32 μmol kg^{-1}. With PD 129290 the fall after 0.5 μmol kg^{-1} was − 25 ± 6 from a control of 121 ± 5 and − 27 ± 3 from 110 ± 6 mmHg in the presence of naloxone pretreatment. Falls after 32 μmol kg^{-1} were − 34 ± 6 and − 33 ± 7 mmHg in the presence and absence of naloxone, respectively. Between the 0.5 and 32 μmol kg^{-1} doses, falls in blood pressure were not obviously dose-related for PD 129290.

Heart rate in intact rats showed dose-related bradycardia with increasing doses of either enantiomer in the presence or absence of naloxone. A fall in heart rate was seen with PD 129289 after a cumulative dose of 2 μmol kg^{-1} and had fallen 98 ± 17 beats min^{-1} (predrug value 355 ± 27) after 32 μmol kg^{-1}. For PD 129290 in the absence of naloxone the same pattern was seen. The heart rate fell 83 ± 13 from a control of 406 ± 20 beats min^{-1} after 32 μmol kg^{-1}. In the presence of naloxone pretreatment the fall was 32 ± 12 from a control of 391 ± 18 beats min^{-1}.

ECG measures were influenced in a dose-related manner by both compounds (Figure 2). Saline was without effect in that the ECG did not change significantly with time over the experimental period. To an approximately equal extent PD 129289 and PD 129290 (in the presence and absence of naloxone) produced dose-related increases in RSh. For both enantiomers a dose between 1 and 2 μmol kg^{-1} produced a 10% increase in RSh with PD 129289 perhaps being more potent (potency ratio < 2.0).

In terms of effects on QRS duration, PD 129289 (the **R,R**

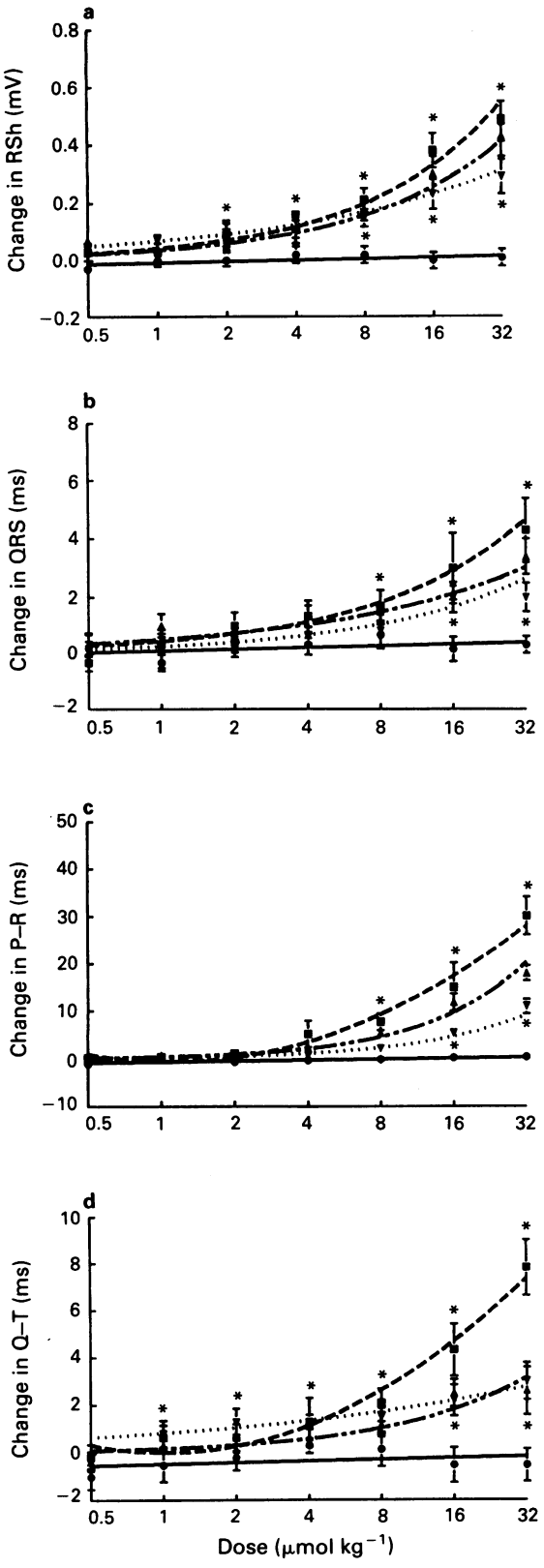


Figure 2 Dose-response curves for the effects of PD 129289 and PD129290 on the ECG of anaesthetized rats. The effects of the two enantiomers in the absence of naloxone, and PD 129290 in the presence of naloxone, are shown as changes in (a) RSh, (b) QRS, (c) P–R and (d) Q–T from control values induced 5 min after administration of a bolus of drug at 1, 2, 4, 8, 16, or 32 μmol kg^{-1}. Values are mean and vertical lines s.e.mean ($n = 8$) for (●) saline, (■) PD 129289, (▲) PD 129290 and (▼) PD 129290 in the presence of naloxone 8 μmol kg^{-1}. Control values were 0.49 ± 0.08 mV for RSh, 28 ± 1 ms for QRS, 53 ± 1 ms for P–R and 38 ± 1 for Q–T. *Indicates $P < 0.05$ for statistically significant differences from control.

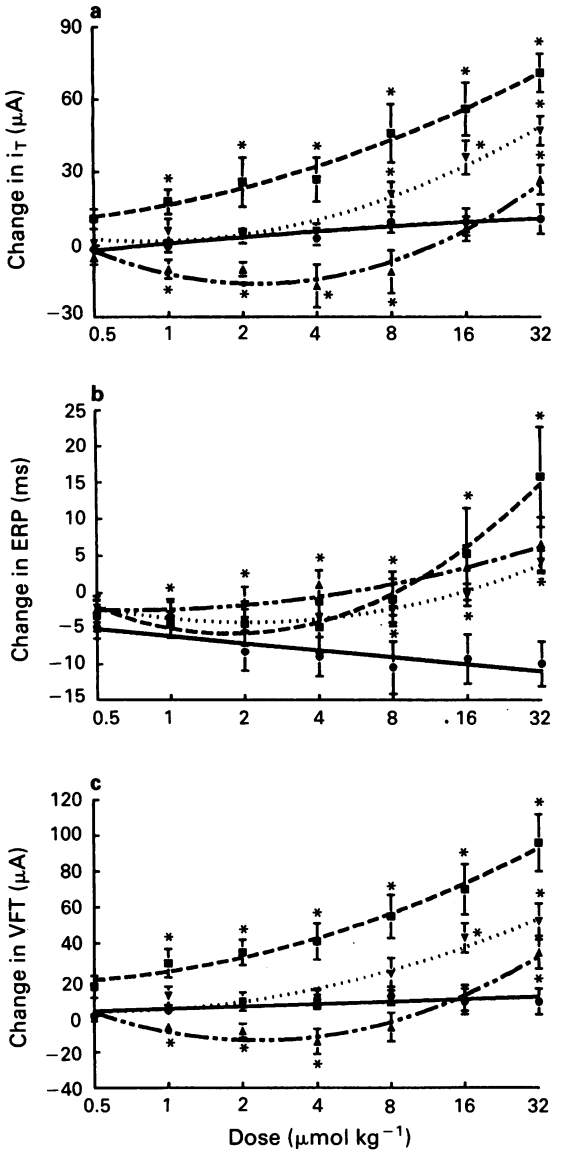


Figure 3 Dose-response curves for the effects of PD 129289 and PD129290 on sensitivity to ventricular electrical stimulation in anaesthetized rats. The effects of the two enantiomers in the absence of naloxone, and PD 129290 in the presence of naloxone, are shown as changes in (a) threshold current (i_T), (b) effective refractory period (ERP) and (c) threshold current for ventricular fibrillation (VF_T) occurring 3 min after administration of either compound at cumulative doses of 1, 2, 4, 8, 16, or 32 μmol kg^{-1}. Values are mean and vertical lines s.e.mean ($n = 6$) for (●) saline, (■) PD 129289, (▲) PD 129290 and (▼) PD 129290 in the presence of naloxone 8 μmol kg^{-1}. Control values were 51 ± 3 μA for i_T, 89 ± 6 μA for VF_T and 54 ± 2 ms for ERP. *Indicates $P < 0.05$ for statistically significant differences from control.

(+)-enantiomer) was slightly more potent than PD 129290 in the presence or absence of naloxone. The dose producing a 10% increase in QRS lay between 16 and 32 μmol kg^{-1}. A similar pattern was seen with P–R interval in that PD 129289 was again more potent (potency ratio < 2.0) than PD 129290 in the presence or absence of naloxone. ED_{10} values for both enantiomers were between 8 and 16 μmol kg^{-1}.

The ECG measurement least influenced by either drug was the Q–T interval. This interval was statistically significantly increased only after the higher doses, such that the approximate ED_{10} was 16 μmol kg^{-1} for PD 129289 and > 32 μmol kg^{-1} for PD 129290, with or without naloxone.

Table 1 Cardiovascular and ECG effects of 2 and 8 μmol kg^{-1} doses of PD 129290 and PD 129289 – antiarrhythmic study

Dose (μmol kg^{-1})	BP	HR	ECG P–R	ECG QRS	ECG Q–T
Saline	105 ± 6	377 ± 12	53 ± 2	29 ± 0.5	38 ± 0.8
PD 129290 2	88 ± 4*	356 ± 16	53 ± 2	31 ± 1.0	37 ± 1.0
PD 129290 8	88 ± 5*	331 ± 11*	62 ± 2*	31 ± 0.3	41 ± 0.4
PD 129290 8 (+N)	76 ± 5*	298 ± 9*	57 ± 2*	33 ± 0.8*	41 ± 1.0
PD 129289 2	71 ± 2*	318 ± 14*	55 ± 1	32 ± 1.0	41 ± 0.6
PD 129289 8	77 ± 6*	338 ± 17*	65 ± 2*	31 ± 0.6	41 ± 0.9

The effects of PD 129290 alone, or in the presence of naloxone (8 μmol kg^{-1}, + N), and PD 129289 are expressed as mean ± s.e.mean ($n = 9$) for the variable indicated. BP = mean blood pressure in mmHg; HR = heart rate in beat min^{-1}; P–R, QRS and Q–T are ECG intervals in ms.
*$P < 0.05$ for comparison with saline.

Table 2 Antiarrhythmic effects of 2 and 8 μmol kg^{-1} doses of PD 129290 and PD 129289 against coronary artery occlusion-induced arrhythmias

Drug and dose (μmol kg^{-1})	Group Incidence VT	VF	VT and/or VF	Arrhythmia score
Saline	8/8	7/8	8/8	5.0 ± 0.6
PD 129290 2	3/8	3/8	3/8	1.1 ± 0.6*
PD 129290 8	4/8	0/8*	4/8	1.5 ± 0.6*
PD 129290 8 (+N)	2/8*	0/8*	2/8*	0.8 ± 0.4*
PD 129289 2	6/8	3/8	6/8	3.0 ± 0.7
PD 129289 8	4/8	0/8*	0/8*	1.3 ± 0.6*
Naloxone 8	6/8	3/8	6/8	3.4 ± 0.2

The antiarrhythmic actions of PD 129290, alone or in the presence of naloxone (8 μmol kg^{-1}), as well as PD 129289, are expressed in terms of the group incidence of one or more episodes of the major arrhythmias of ventricular tachycardia (VT) or ventricular fibrillation (VF), $\log_{10}$ of ventricular premature beats (VPB) and arrhythmia score expressed as mean ± s.e.mean ($n = 9$).
*$P < 0.05$ for comparison with saline.

Effects of treatment on electrical stimulation Figure 3 illustrates the effects of the compounds on sensitivity to electrical stimulation. The control values for thresholds for capture (i_T) and VF (VF_T) were constant over the treatment period whereas those for ERP tended to fall throughout the treatment period. In a clearly dose-related manner PD 129289 increased thresholds and effective refractory periods. PD 129290 in the absence of naloxone initially tended to decrease thresholds (i_T and VF_T) with minima occurring between 2 and 4 μmol kg^{-1}. Thereafter, thresholds increased with increasing doses until finally they rose above control values in the range of 16–32 μmol kg^{-1}. The minimum values with a cumulative dose of 4 μmol kg^{-1} PD 129290 was − 21% from control for i_T and − 14% for VF_T. On the other hand, in the presence of naloxone pretreatment, the i_T and VF_T dose-response curves for PD 129290 were similar in shape to those for PD 129289 and showed no tendency to decrease below control levels. Interestingly in the presence of naloxone the potency of PD 129290 was less than PD 129289 (potency ratio approximately 4.0). Changes in t_T (data not shown) were less consistent.

ERP, and its related variable MFF (not shown), were not markedly influenced by low doses of either drug. The saline control showed a reduction with time for ERP and this was accompanied by the expected increase in MFF. Lower doses of both enantiomers did not have any significant effects on MFF and ERP when compared with saline. At higher doses there were dose-related increases in ERP and corresponding falls in MFF. Notably, responses to PD 129290, in the presence and absence of naloxone, were not markedly different from those for PD 129289.

Antiarrhthmic study In the coronary occlusion-arrhythmia study both 2 and 8 μmol kg^{-1} doses had the ECG and cardiovascular effects (Table 1) expected from the foregoing study. The lower dose (2 μmol kg^{-1}) minimally changed QRS and Q–T intervals. The most noticeable effect of the low dose was on the P–R interval. The large dose (8 μmol kg^{-1}) produced its expected effects on blood pressure, heart rate and ECG. None of the changes induced by the (−)-enantiomer (PD 129290) were reversed by naloxone pretreatment.

Both doses of PD 129289 and PD 129290 statistically significantly reduced arrhythmias induced by coronary occlusion (Table 2). The high dose (8 μmol kg^{-1}) was more antiarrhythmic than the low dose (2 μmol kg^{-1}). Naloxone had no effect on the antiarrhythmic effects of 8 μmol kg^{-1} PD 129290 although, on its own, naloxone appeared to reduce arrhythmias but not to a statistically significant extent.

The antiarrhythmic activity of either enantiomer could not be ascribed to changes in either occluded zone size or serum potassium concentrations. Group mean occluded zone size varied from a group minimum of 30.0 ± 1.2 (% total ventricular weight) to a maximum of 32.5 ± 1.6% (not statistically significant). Similarly, serum potassium concentrations were not influenced by the treatments. Group mean serum potassium concentrations varied from a low of 3.2 ± 0.2 mM to a high of 3.9 ± 0.4 mM and were not related to treatment.

Whole cell voltage clamp studies

Concentrations of PD 129290 (10 and 20 μM) which produced marked effects on isolated hearts (Figure 1) also

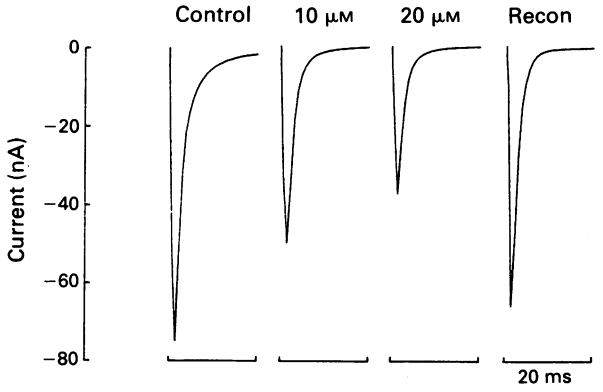


Figure 4 Sodium currents and the effect of PD 129290. Sodium currents were evoked in a single cardiac myocyte by a depolarizing step to − 40 mV from a holding potential of − 140 mV. The current under control conditions had a peak amplitude of 75 nA. This was reduced to 48 nA by 10 μM and to 38 nA by 20 μM PD 129290. These effects could be reversed by wash-out as shown by the re-control (Recon) current obtained 4 min after returning to the control solution. Some decrease in the control sodium current is seen during the course of the experiment, a finding typical of whole-cell records.

reduced sodium currents recorded in whole cell voltage clamp studies (Figure 4). These concentrations are 100–1,000 times greater than those producing κ agonism. The higher concentration (20 μM) of PD 129290 reduced current amplitude by about 50%, without perceptible changes in kinetics. These effects were readily reversible and could not be blocked by naloxone (data not shown).

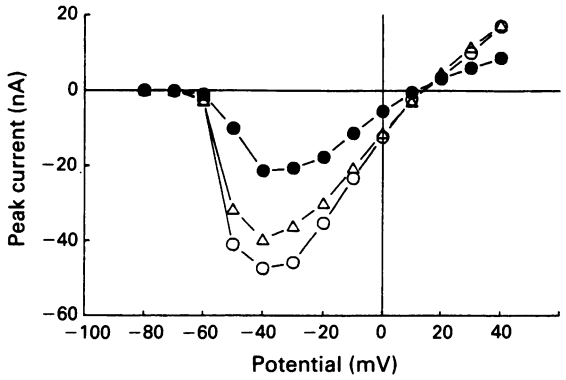


Figure 5 Current-voltage relation for the sodium current and the effect of PD 129290. Peak sodium current evoked by depolarizing steps to various voltages from a holding potential of − 140 mV is shown plotted as a current-voltage relationship. Maximal sodium current of 50 nA was evoked by a step to − 40 mV in control solution (○). The maximal current in the presence of 20 μM PD 129290 was also evoked at − 40 mV, but had a magnitude of only 22 nA (●). The voltage at which the current reversed was not appreciably changed in the presence of PD 129290. It was 14 mV for both curves. E_{NA} was calculated as 14 mV for the solutions used. Recontrol data, after about 4 min of recovery in control solution are shown by the open triangles.

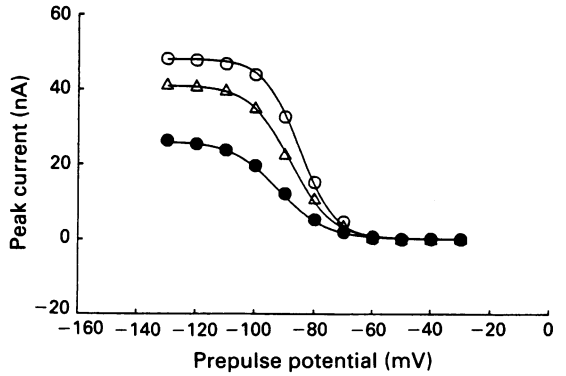


Figure 6 Inactivation curves for the sodium current and the effect of PD 129290. Sodium currents were evoked by a depolarizing step to − 40 mV from prepulses of various potentials. The magnitude of the sodium current is shown plotted against prepulse potential. The inactivation curve so produced was fitted by the Boltzman equation, shown by the line and of the form $y = I_{max}/1 + (e^{((v-v')/k)})$ where v is the prepulse potential, v′ is the voltage at which 50% inactivation occurs and k is a slope factor. The best fits to the data were obtained with values of max = 48 nA, v′ = − 85 mV and k = 6 for the control curve (○), and max = 26 nA, v′ = − 92 mV and k = 8 for the curve obtained in the presence of 20 μM PD 129290 (●). Recontrol data obtained after 4 min of recovery are shown by the triangles.

Figure 5 shows the effect of 20 μM PD 129290 on the current-voltage relationship for the sodium current. Sodium current amplitude was maximum at − 40 mV in control solution and the current reversed at 14 mV. Neither of these values was appreciably altered by the application of 20 μM PD 129290, although maximum sodium current was reduced by about 60%. PD 129290 (20 μM) did not affect the voltage-dependence of inactivation of the sodium current, as shown in Figure 6. Thus, although 20 μM PD 129290 caused large decrease in maximum current amplitude, this was not due to a shift in the voltage-dependence of either the current-voltage relation or inactivation. Thus, in rat isolated heart cells PD 129290 added to the bathing solution was capable of blocking sodium currents.

Discussion

Overall, the actions of the S,S (−)-enantiomer (PD 129290) in these studies were remarkably similar to those of the arylbenzacetamide κ agonist, U50,488H, which we have tested previously under similar conditions (Pugsley *et al.*, 1992a,b). Thus, both U50,488H and PD 129290 (κ agonists) prolonged the P–R and QRS intervals of the ECG in isolated and P–R, QRS, RSh and Q–T in intact hearts. In addition they lowered blood pressure and heart rate and increased threshold currents in the presence of naloxone. Both drugs had lesser effects on the other stimulation variables measured and both were antiarrhythmic against occlusion-induced arrhythmias.

Notably, as was the case with U50,488H, the only unequivocal effect of naloxone pretreatment on the actions of PD 129290 was to antagonize the decrease in threshold currents seen at low doses. Such evidence suggests that most of the antiarrhythmic and other actions of U50,488H and PD 129290 can be ascribed to effects unrelated to κ agonism.

The evidence for the above events being unrelated to the κ receptor is strengthened by findings with PD 129289, the **R,R** (+)-enantiomer of PD 129290, which has very low affinity for κ receptors. Measurement of the binding constants for PD 129289 and PD 129290 on κ receptors indicate at least a 1,000 fold selectivity for PD 129290 (the **S,S** (−)-enantiomer). Thus, it is reasonable to assume that the effects of PD 129290 on RSh, P–R, QRS and Q–T intervals, which were not blocked by naloxone and are similar to those of PD 129289, are probably independent of κ receptors. Similarly, increases in thresholds seen with all doses of PD 129289 and with PD 129290 at higher doses (or at lower doses in the presence of naloxone) may also be independent of κ receptors. Furthermore, PD 129289 was as equally effective as PD 129290 in its antiarrhythmic actions.

Although PD 129289 has very little activity at the κ receptor it has appreciable actions on μ receptors. Thus PD 129289 has almost the same μ receptor binding as PD 129290, and PD 129289 is effective in the hot plate test for analgesia, a test believed to depend upon μ recptors. The ED_{50} for PD 129290 in this test was 2.5 mg kg^{-1} (approx 6 μmol kg^{-1}), i.e. within the dose range tested in this study.

Table 3 Potencies of PD 129290 and PD 129289 with respect to blood pressure (BP), heart rate (HR), and ECG intervals, sensitivity to electrical stimulation (ES) and antiarrhythmic actions

				ECG			*ES*		*Antiarrhythmic*		
Drug	*BP*	*HR*	*P–R*	*QRS*	*Q–T*	i_T	VF_T	*ERP*	*VT*	*VF*	*AS*
PD 129289	16	8	8	>32	>32	8	0.5	3	<2	<2	<2
PD 129290	0.5	0.5	16	>32	>32	32	4	6	2	2	2
PD 129290 (N)	16	32	32	>32	>32	8	4	8	8	<8	<8

In the above table estimates for ED_{25} values (μmol kg^{-1}) have been extrapolated from the data given in the Tables and Figures. PD 129290 (N) refers to PD 129290 in the presence of naloxone. For abbreviations used see text in Methods section.

However naloxone is even more effective in blocking μ receptors than κ receptors (Rees & Hunter, 1990) thus μ effects cannot account for the action of PD 129290 after naloxone treatment.

With respect to the mechanisms underlying the antiarrhythmic actions of the two enantiomers, Table 3 lists the doses of PD 129289 and PD 129290 (in the presence or absence of naloxone) required to produce 25% changes in blood pressure, ECG variables, electrical stimulation variables and antiarrhythmic effects. From this Table it is apparent that marked antiarrhythmic effects occurred at doses which produced limited but consistent changes in the other variables.

In our studies with U50,488H (Pugsley *et al.*, 1992a,b) we suggested that the antiarrhythmic, ECG and most of the electrical stimulation effects of U50,488H were due to sodium channel blockade. In view of the similar actions of PD 129289 and PD 129290 (not blocked by naloxone) it would be reasonable to suggest that sodium channel blockade occurs with all three drugs and that such an action accounts for their antiarrhythmic actions. For PD 129290 we have direct proof that the compound blocks sodium currents in rat isolated cardiac myocytes and that such an action is not blocked by naloxone. Such blockade occurs at concentrations similar to those which produce ECG effects in rat isolated hearts and are consistent with the doses administered to intact rats to produce similar ECG effects. Sodium channel blockade explains the observed P–R prolongation, QRS widening and the increase in threshold currents, all of which occurred at doses which conferred antiarrhythmic actions. However, it should be noted that changes in the Q–T interval of the ECG seen at higher doses suggest that a limited degree of potassium channel blockade may occur with these compounds. It is possible that this action may also contribute to antiarrhythmic actions.

We wish to thank Dr J. Hughes from Parke-Davis Research Laboratories for kindly supplying samples of PD 129289 and PD 129290. This study was funded by the B.C. & Yukon Heart & Stroke Foundation.

References

AU, T.L.S., COLLINS, G.A., HARVIE, C.J. & WALKER, M.J.A. (1979). The action of prostaglandin I_2 and E_2 on arrhythmias produced by coronary occlusion in the rat and dog. *Prostaglandins*, **18**, 707–720.

CURTIS, M.J., MACLEOD, B.A., TABRIZCHI, R. & WALKER, M.J.A. (1986). An improved perfusion apparatus for small animal hearts. *J. Pharmacol. Methods*, **15**, 87–94.

CURTIS, M.J. & WALKER, M.J.A. (1988). Quantification of arrhythmias using scoring systems: an examination of seven scores in an *in vivo* model of regional myocardial ischaemia. *Cardiovasc. Res.*, **22**, 656–665.

HALFPENNY, P.R., HORWELL, D.C., HUGHES, J., HUNTER, J.C. & REES, D.C. (1990). Highly selective kappa opioid analgesics. 3. Synthesis and structure-activity-relationships of novel N-(2-(1-pyrollidinyl)-4- or -5-substituted cyclohexyl) arylacetamide derivatives. *J. Med. Chem.*, **33**, 286–291.

HINTZE, J.L. (1987). *Number Cruncher Statistical System: Version 5.01*. Kaysville, Utah: J.L. Hintze.

HOWARD, P.G. & WALKER, M.J.A. (1990). Electrical stimulation studies with quinacainol, a putative Ic agent, in the anaesthetised rat. *Proc. West. Pharmacol. Soc.*, **33**, 123–127.

HUNTER, J.C., LEIGHTON, G.E., MEECHAM, K.G., BOYLE, S.J., HORWELL, D.C., REES, D.C. & HUGHES, J. (1990). CI-977 a novel and selective agonist for the k-opioid receptor. *Br. J. Pharmacol.*, **101**, 183–189.

JOHNSTON, K.M., MACLEOD, B.A. & WALKER, M.J.A. (1983). Responses to ligation of a coronary artery in conscious rats and the actions of antiarrhythmics. *Can. J. Physiol. Pharmacol.*, **61**, 1340–1353.

MAINLAND, D., HERRERA, L. & SUTCLIFFE, M.I. (1956). *Statistical Tables For Use With Binomial Samples – Contingency Tests, Confidence Limits and Sample Size Estimates*. New York, U.S.A.: Dept. of Medical Statistics Publishers, New York University College of Medicine.

MARTIN, W.R. (1984). Pharmacology of opioids. *Pharmacol. Rev.*, **35**, 285–323.

PALETTA, M.J., ABRAHAM, S., BEATCH, G.N. & WALKER, M.J.A. (1989). Mechanisms underlying the antiarrhythmic properties of β-adrenoceptor blockade against ischaemia-induced arrhythmias in acutely prepared rats. *Br. J. Pharmacol.*, **98**, 87–94.

PENZ, W.P., PUGSLEY, M.K., HSIEH, M.Z. & WALKER, M.J.A. (1992). A new ECG measure (RSh) for detecting possible sodium channel blockade *in vivo* in rats. *J. Pharmacol. Methods*, **27**, 51–58.

PUGSLEY, M.K., PENZ, W.P., WALKER, M.J.A. & WONG, T.M. (1992a). Cardiovascular actions of the kappa agonist, U-50,488H, in the absence and presence of opioid receptor blockade. *Br. J. Pharmacol.*, **105**, 521–526.

PUGSLEY, M.K., PENZ, W.P., WALKER, M.J.A. & WONG, T.M. (1992b). Antiarrhythmic effects of U50,488H in rats subjected to coronary artery occlusion. *Eur. J. Pharmacol.*, **212**, 15–19.

REES, D.C. & HUNTER, J.C. (1990). Opioid receptors. In *Comprehensive Medicinal Chemistry*. ed. Hansch, C., Sammes, P.G. & Taylor, J.B. pp. 806–846. New York: Pergamon Press.

SAINT, D.A., JU, Y.-K. & GAGE, P.W. (1992). A persistent sodium current in rat ventricular myocytes. *J. Physiol.*, **453**, 219–231.

SARNE, Y., FLITSTEIN, A. & OPPENHEIMER, E. (1991). Antiarrhythmic activities of opioid agonists and antagonists and their stereoisomers. *Br. J. Pharmacol.*, **102**, 696–698.

SITSAPESAN, R. & PARRATT, J.R. (1989). The effects of drugs interacting with opioid receptors on the early ventricular arrhythmias arising from myocardial ischaemia. *Br. J. Pharmacol.*, **97**, 795–800.

WALKER, M.J.A. & BEATCH, G.N. (1988). A new method for electrical stimulation of the heart in rats. *Proc. West. Pharmacol. Soc.*, **31**, 167–170.

WALKER, M.J.A., CURTIS, M.J., HEARSE, D.J., CAMPBELL, R.W.F., JANSE, M.J., YELLON, D.M., COBBE, S.M., COKER, S.J., HARNESS, J.B., HARRON, D.W.G., HIGGINS, D.G., LAB, M.J., MANNING, A.S., NORTHOVER, B.J., PARRATT, J.R., RIEMERSMA, R.A., RIVA, E., RUSSEL, D.C., SHERIDAN, D.J., WINSLOW, E. & WOODWARD, B. (1986). The Lambeth convention: guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion. *Cardiovasc. Res.*, **22**, 447.

(Received March 22, 1993
Revised June 25, 1993
Accepted August 23, 1993)

Br. J. Pharmacol. (1993), **110**, 1586–1592 © Macmillan Press Ltd, 1993

$GABA_B$ receptors modulate catecholamine secretion in chromaffin cells by a mechanism involving cyclic AMP formation

[1]M.J. Oset-Gasque, M. Parramón & M.P. González

Instituto de Bioquímica, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain

1 The function of γ-aminobutyric $acid_B$ ($GABA_B$) receptors in modulation of catecholamine secretion by chromaffin cells and the possible mechanism involved in this action have been examined.

2 The $GABA_B$ agonists (−)-baclofen and 3-aminopropylphosphinic acid (3-APPA) were found to induce a dose-dependent increase of basal catecholamine secretion. The EC_{50}s were 151 ± 35 μM and 225 ± 58 μM for baclofen and 3-APPA, respectively. This stimulatory effect was specific since it could be blocked by 0.5 mM of the specific $GABA_B$ antagonist CGP-35348.

3 In contrast, preincubation of chromaffin cells with the $GABA_B$ agonists was found to inhibit, in a dose-dependent manner, the catecholamine secretion evoked by 10 μM nicotine and 200 μM muscimol.

4 The effects of $GABA_B$ agonists on both basal and evoked catecholamine secretion were found to be accompanied by parallel changes in intracellular calcium concentration ($[Ca^{2+}]_i$). $GABA_B$ agonists produced a dose-dependent increase in $[Ca^{2+}]_i$ which was partially blocked by CGP 35348, but they produced a strong inhibition of the $[Ca^{2+}]_i$ increase induced by nicotine and muscimol.

5 The $GABA_B$ agonists also produced a dose-dependent increase in intracellular cyclic AMP levels, there being a direct correlation between both increase in catecholamine secretion and in intracellular cyclic AMP levels.

6 The pretreatment of chromaffin cells with pertussis toxin doubled the catecholamine secretion and increased by four times the intracellular cyclic AMP levels evoked by $GABA_B$ agonists.

7 The possible involvement of adenylate cyclase in the mechanism of $GABA_B$ receptor modulation of catecholamine secretion is discussed.

Keywords: $GABA_B$ receptors; catecholamine secretion; chromaffin cells; cyclic AMP; adrenal medulla

Introduction

Different biochemical and physiological evidence concerning the regulation of catecholamine secretion by chromaffin cells show that, in addition to nicotinic cholinoceptors (nAChR) whose role in adrenal medulla function is well established, there are other receptors implicated in the modulation of this response. These include muscarinic cholinoceptors (Cheek & Burgoyne, 1985), opioid peptide (Lemaire *et al.*, 1981), substance P (Livett *et al.*, 1979), bradykinin (Owen *et al.*, 1989), histamine H_1 (Chaliss *et al.*, 1991), dopamine D_1 and D_2 (Stauderman & Pruss, 1990; Huettl *et al.*, 1991), prostaglandin E_2 (Negishi *et al.*, 1989; Ito *et al.*, 1991), adenosine (Chern *et al.*, 1988), angiotensin II (Marley *et al.*, 1989; O'Sullivan & Burgoyne, 1989) and $GABA_A$ receptors (Castro *et al.*, 1989).

In relation to γ-aminobutyric acid (GABA) regulation of chromaffin cell secretion, previous work by our group has demonstrated the presence of all the enzymes implicated in GABA metabolism in the bovine adrenal medulla (Fernández-Ramil *et al.*, 1982; 1983) as well as the existence of an active uptake (Oset-Gasque & Aunis, 1989), and an exocytotic release of this neurotransmitter (Oset-Gasque *et al.*, 1990). Moreover, the presence of the two subtypes of GABA receptors, one sensitive to muscimol ($GABA_A$) and the other insensitive to muscimol but sensitive to baclofen ($GABA_B$) has also been described (Castro *et al.*, 1988).

The function and molecular mechanism of $GABA_A$ receptors in the adrenal medulla is now well established. Our recent studies demonstrated that when GABA binds to $GABA_A$ receptors in the adrenal medulla it modulates catecholamine secretion by a mechanism dependent on the membrane potential (González *et al.*, 1992). Nevertheless the function and mechanism of action of $GABA_B$ receptors in adrenal medulla function has not yet been investigated.

$GABA_B$ receptors have recently assumed additional importance because of evidence that they could mediate both pre- and postsynaptic actions in the brain (see Bowery, 1989 for a review). Their presence has also been demonstrated in peripheral tissues and enteric nerve terminals, where they appear to have a function in the regulation of the evoked release of excitatory neurotransmitters and hormones (Bowery *et al.*, 1980; Huston *et al.*, 1990), in the regulation of muscular contraction and tone (see Bowery, 1989, for a review) and in the facilitation of the induction of long-term potentiation (Mott & Lewis, 1991).

These actions seem to be mediated by molecular mechanisms involving the participation of second messenger systems such as adenylate cyclase, inositolphosphate turnover or regulation of Ca^{2+} or K^+ conductance. However it is not clear whether or not these reactions occur sequentially or independently and which component is directly coupled with $GABA_B$ receptors.

In this paper we examine (1) the role of $GABA_B$ receptors in the regulation of chromaffin catecholamine secretion, (2) the possible interactions between this $GABA_B$ and other receptor systems known to regulate this process and (3) the possible molecular mechanism linked to these actions.

Methods

Cell isolation and culture

Bovine chromaffin cells were isolated from adrenal glands and cultured as described by Bader *et al.* (1981) with minor

[1] Author for correspondence.

modifications. Cell viability was checked with trypan blue and chromaffin cell purity was assessed by the specific incorporation of 3 mg ml^{-1} neutral red, which dyes red specifically only the cells which contain catecholamines, to these cells. Viability and purity were always higher than 90%. Cells were suspended in DMEM containing 10% FCS, antibiotics (100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 40 μg ml^{-1} gentamicin), and cytostatics (10 μM fluoro-deoxyuridine and 10 μM cytosine arabinoside), plated in 24 well Costar cluster dishes, at a density of 0.5×10^6 cells per well, and used 3–7 days after plating.

Measurement of catecholamine secretion

Chromaffin cells were washed twice, at 10 min intervals with 1 ml of a Krebs HEPES solution (Locke medium) containing (mM) NaCl 140, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 11, ascorbic acid 0.5 and HEPES 15, pH = 7.5, at 37°C. This medium was removed and cells were stimulated, during 10 min periods at 37°C, with 0.5 ml of a fresh Locke medium containing the different secretagogues used as indicated in each type of experiment. At the end of incubation the medium was removed and cells lysed with 0.4 M perchloric acid, and then scraped off the plates. Incubation medium and cell lysates were both used for the determination of catecholamine secretion and total catecholamine content, respectively. The catecholamine determination, in both sample types, adequately diluted and neutralized (see below), was performed with an electrochemical detector (Metrohm 641 VA-detector) adjusted to + 580 mV and registered in an LKB recorder model 2210, using for this calibration a standard curve with noradrenaline. Results are presented as a percentage of catecholamine released in the incubation medium in respect of the total catecholamine content (incubation medium + pellet).

Measurement of intracellular levels of cyclic AMP

Chromaffin cells, prepared as indicated above, were stimulated for 10 min with appropriate secretagogues as indicated. Cells were incubated in the presence of 0.5 mM isobutyl methyl xanthine (IBMX) in order to prevent adenosine 3':5'-cyclic monophosphate (cyclic AMP) degradation. The medium was removed, cells lysed with 200 μl of 0.4 M $HClO_4$, and scraped off the plates. These cell lysates were neutralized with 1 M KOH, centrifuged in an Eppendorf centrifuge and supernatants were used for assay of cyclic AMP. Intracellular cyclic AMP measurements were performed by using the cyclic AMP Radioimmunoassay Kit of Amersham Int., as described in the manufacturer's instructions.

Measurement of cytosolic [Ca^{2+}]

Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were monitored by measuring Fura 2AM fluorescence. Cells were washed twice with Locke medium and incubated with 5 μM Fura 2AM for 45 min at 37°C. Excess dye was removed by washing the cells twice with fresh Locke medium and suspended in this medium at 10^6 cells ml^{-1}. Fluorescence (excitation wavelength = 380 nm, emission = 510 nm, slits, 5 nm exc. 10 nm em.) was monitored at 37°C in a well stirred cuvette containing 1 ml of this suspension with a Perkin-Elmer LS-50 spectrofluorimeter. At the end of each experiment 0.5% triton X-100 was added to permeabilize the cells, allowing the dye to gain access to the extracellular [Ca^{2+}] ($[Ca^{2+}]_e$). This $[Ca^{2+}]_e$ saturated the dye and provided a measure of the maximum fluorescence signal (F_{max}). To determine the intensity of the minimum fluorescence signals (F_{min}), 20 mM Tris base was added to raise the pH above 8.2 followed by 10 mM EGTA which reduced the [Ca^{2+}] to less than 1 nM. The $[Ca^{2+}]_i$ was calculated with the equation:

$$[Ca^{2+}]_i = K_D\ (F - F_{min})/(F_{max} - F)$$

the equilibrium dissociation constant (K_D) for [Ca^{2+}]-Fura being 224 nM.

Materials

Dulbecco's Modified Eagle's Medium (DMEM) and foetal calf serum (FCS) were from GIBCO. Collagenase (E.C.3.4.4.19) and 8-(4-chlorphenylthio)-adenosine 3':5' cyclic monophosphate (pClp cyclic AMP), were obtained from Boehringer Mannheim S.A. (Barcelona, Spain). Muscimol, nicotine, forskolin, and 3-isobutyl-l-methyl xanthine (IBMX) were from Sigma Chemical Co (St. Louis, MO, U.S.A.). (−)-Baclofen, CGP 35348 (3-aminopropyl-diethoxymethyl-phosphinic acid) and 3-aminopropylphosphinic acid (3-APPA) were a gift from Ciba-Geigy (Basel, Switzerland). Faclofen was purchased from Tocris Neuramin (Essex, U.K.). The kit for cyclic AMP assay was from Amersham Int. (Bucks., U.K.) and Fura-2 AM was obtained from Molecular Probes (Cambridge, U.K.). All other chemicals were reactive grade products from Merck (Darmstadt, Germany).

Statistics

The data shown are means ± s.e.mean of at least three experiments each one performed in duplicate. Statistical significance was estimated with Student's t test for unpaired observations. A P value of less than 0.05 was considered significant. Linear regressions were made by the method of minimum squares and fittings of concentration-response curves for estimation of EC_{50} or IC_{50} values by a weighed non linear regression of minimum squares, using logistic curves.

Results

Effect of $GABA_B$ agonists on basal catecholamine secretion

Stimulation of $GABA_B$ receptors with their specific agonists baclofen and 3-APPA increase basal catecholamine secretion from chromaffin cells in culture. This effect was dose-dependent in both cases with EC_{50}s of 151 ± 35 μM and 225 ± 58 μM for baclofen and 3-APPA, respectively (Figure 1). Maximal responses were achieved at concentrations between 0.5 and 1 mM of agonists and were 2.73 ± 0.52 and of 2.32 ± 0.44 times the basal catecholamine secretion for stimulation with 3-APPA and baclofen, respectively.

The stimulatory effect of $GABA_B$ agonists on basal catecholamine secretion was Ca^{2+}-dependent and specific since it was inhibited between 75 and 95% in an extracellular medium containing 200 nM $CaCl_2$ and it could be blocked by the specific $GABA_B$ antagonist CGP 35348. At a dose of 0.5 mM this compound was able to inhibit the agonist response between 67 and 100%. In contrast, the antagonist faclofen was ineffective at the same dose (Figure 2). None of the antagonists had any significant effect on basal catecholamine secretion at doses between 10 μM and 1 mM (Figure 2).

To test the possible cell damage produced by $GABA_B$ agonists on chromaffin cells, experiments of trypan blue permeation and LDH release were carried out. These experiments showed no significant differences from controls (cellular viability between 90–95%, and LDH release of 3.98 ± 1.18% over total cellular content).

$GABA_B$ agonists were also able to evoke secretion of the intravesicular component dopamine-β-hydroxylase (DBH) in a similar percentage to that for catecholamines (0.5 mM 3-

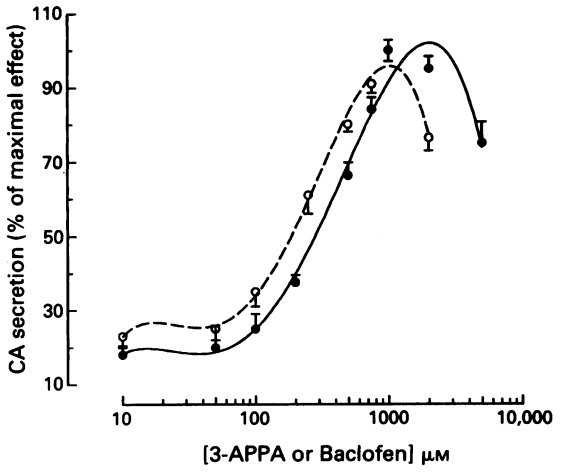


Figure 1 Dose-response curves for the effect of $GABA_B$ agonists on basal catecholamine (CA) release by chromaffin cells. Cells were incubated with the indicated doses of 3-aminopropylphosphinic acid (3-APPA, ●) or baclofen (○) at 37°C. Catecholamine release over a 10 min period is expressed as percentage of maximal responses (6.84 ± 0.59% and 5.8 ± 0.45% of total cellular catecholamine content for 3-APPA and baclofen, respectively) in which basal catecholamine secretion (2.58 ± 0.55% of total cellular catecholamine content) was subtracted. Release data are means ± s.e.mean of three independent determinations each performed in duplicate. EC_{50} for 3-APPA = 225 ± 58 μM and for baclofen = 151 ± 35 μM.

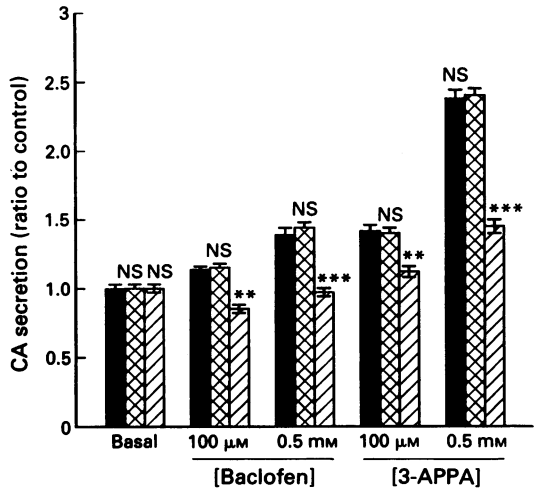


Figure 2 Effect of antagonists of $GABA_B$ receptors on catecholamine (CA) secretion evoked by $GABA_B$ antagonists. Cells were incubated with Locke solution (■) or the antagonists faclofen 0.5 mM (▩) or CGP 35348 0.5 mM (▨) at 37°C for 10 min. The medium was removed and cells restimulated with a mixture of the indicated $GABA_B$ agonists and antagonists. Results are expressed as ratios to their respective basal values, which were taken as the unit. Columns represent means and bars s.e.mean of three duplicate determinations. Significantly different from the corresponding controls without antagonist at $*P<0.05$, $**P<0.01$ and $***P<0.001$. Basal catecholamine secretions were: Locke solution = 2.5 ± 0.1, CGP 35348 0.5 mM = 2.47 ± 0.09 and faclofen 0.5 mM = 2.61 ± 0.16%' over total cellular catecholamine content.

APPA and 0.5 mM baclofen produced an increase in DBH release of 2.03 ± 0.11 and 1.69 ± 0.09 times, respectively, the control values).

Effect of $GABA_B$ agonists on evoked catecholamine secretion

In contrast to the effect of $GABA_B$ agonists on basal catecholamine secretion, preincubation of chromaffin cells

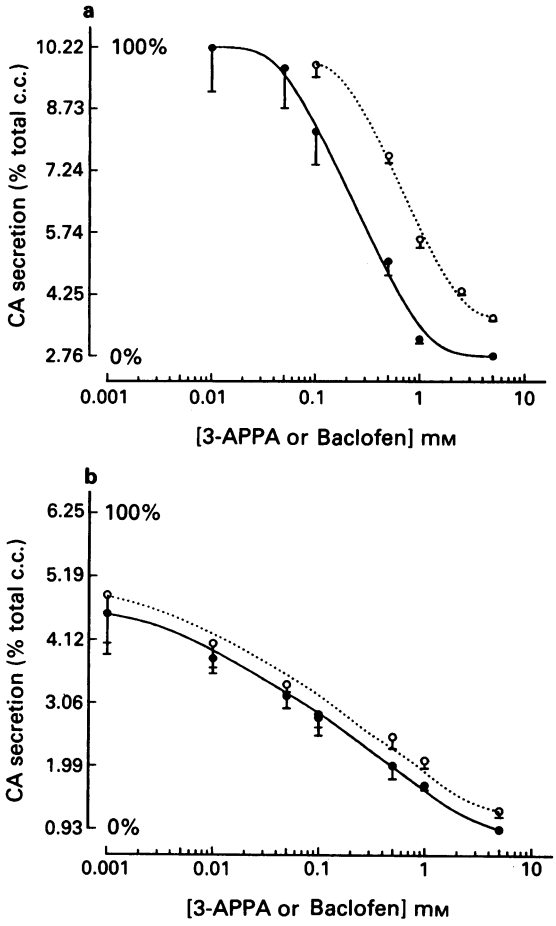


Figure 3 Concentration-dependent inhibition of (a) nicotine and (b) muscimol-evoked catecholamine (CA) release by $GABA_B$ agonists. Chromaffin cells were incubated with the indicated concentrations of the $GABA_B$ agonists (○, baclofen; ●, 3-aminopropylphosphinic acid) at 37°C for 10 min before stimulation with (a) 10 μM nicotine or (b) 200 μM muscimol. Results are expressed as percentages of catecholamine release over total cellular content (c.c.). Values represent means ± s.e.mean of three experiments each one performed in duplicate. (a) IC_{50} for baclofen = 354 ± 98 μM and for 3-APPA = 240 ± 59 μM. (b) IC_{50} for baclofen = 45 ± 9 μM and for 3-APPA = 21 ± 7 μM.

with baclofen or 3-APPA inhibited catecholamine secretion evoked by 10 μM nicotine. This effect was dose-dependent (Figure 3a) with an IC_{50} estimated by log-logit analysis of 240 ± 59 μM for 3-APPA and 354 ± 98 μM for baclofen.

An inhibitory effect was also observed in the case of the catecholamine secretion evoked by 200 μM muscimol. The effect was dose-dependent the IC_{50} being 21 ± 7 μM for 3-APPA and 45 ± 9 μM for baclofen (Figure 3b), doses lower than those required for inhibition of 50% of nicotinic responses.

Effect of $GABA_B$ agonists on intracellular levels of calcium

The effect of $GABA_B$ agonists on basal catecholamine secretion was accompanied by a dose-dependent increase in the intracellular calcium concentration. The EC_{50}s were 25 ± 6 and 60 ± 10 μM for 3-APPA and baclofen, respectively (Figure 4). Maximal effects were obtained with 0.5 mM of the agonists and were 250 ± 40 nM for 3-APPA and 225 ± 30 nM for baclofen. This effect was partially blocked by the specific antagonist CGP 35348 which, at 0.5 mM, inhibited 50% of the effect evoked by 0.5 mM 3-APPA (increase of about 200 nM in $[Ca^{2+}]_i$). This increase in $[Ca^{2+}]_i$ was also depen-

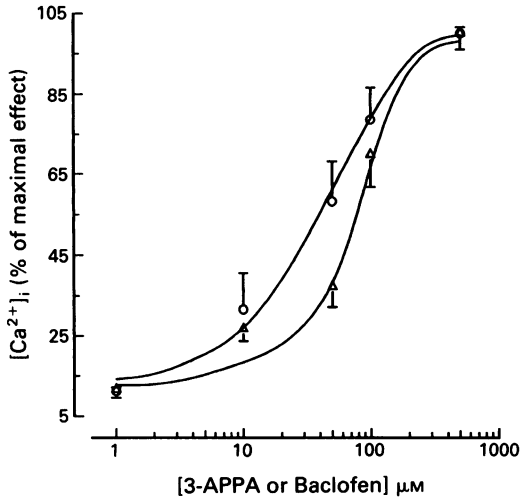


Figure 4 Dose response curves of the effect of $GABA_B$ agonists on intracellular calcium concentrations. Cytosolic calcium was measured, as indicated in Methods, after stimulation of cells with indicated doses of 3-aminopropylphosphinic acid (3-APPA, O) or baclofen (Δ). $[Ca^{2+}]_i$ is expressed as percentage of maximal effects (250 ± 40 nM and 225 ± 30 nM for 3-APPA and baclofen, respectively) in which, basal $[Ca^{2+}]_i$ (175 ± 25 nM) was subtracted. Points are means ± s.e.mean of 4–5 independent determinations with three different cell batches. EC_{50} for 3-APPA = 25 ± 6 μM and for baclofen = 60 ± 10 μM.

dent on Ca^{2+} entry from extracellular medium since in a 200 nM Ca^{2+}-containing medium the increase in $[Ca^{2+}]_i$ was inhibited between 80 and 100%.

In addition, $GABA_B$ agonists decreased the rise in $[Ca^{2+}]_i$ induced by nicotine and muscimol. Figure 5a shows that 0.5 mM 3-APPA or baclofen was able to reduce by 80–90% the increase in $[Ca^{2+}]_i$ evoked by 10 μM nicotine. Similar results were obtained in the case of stimulation of cells with 200 μM muscimol (Figure 5b).

$GABA_B$ agonists increase intracellular levels of cyclic AMP

Stimulation of cells with $GABA_B$ agonists produced a time-dependent increase in the intracellular concentration of cyclic AMP, reaching the maximum effect at 10 min of incubation (data not shown). This effect was dose-dependent (Figure 6a) with EC_{50}s for baclofen of 210 ± 47 μM and 146 ± 20 μM determined in the absence or presence, respectively, of 0.5 mM IBMX, an inhibitor of phosphodiesterase used to prevent cyclic AMP degradation.

Correlation between catecholamine secretion and intracellular cyclic AMP levels evoked by $GABA_B$ agonists

Stimulation of $GABA_B$ receptors produced an increase in basal catecholamine secretion as well as in intracellular levels of cyclic AMP. As shown in Figure 6b both parameters are directly related either in the absence or presence of 0.5 mM IBMX. This compound did not have any effect on catecholamine secretion and did not interfere with cyclic AMP determination.

Effect of forskolin and cyclic AMP analogues on catecholamine secretion and intracellular cyclic AMP levels evoked by $GABA_B$ agonists

When cells were stimulated with 3-APPA or baclofen added together with forskolin, the intracellular levels of cyclic AMP increased significantly, as a result of an additive effect (Table 1). However, stimulation of cells with forskolin or pClp

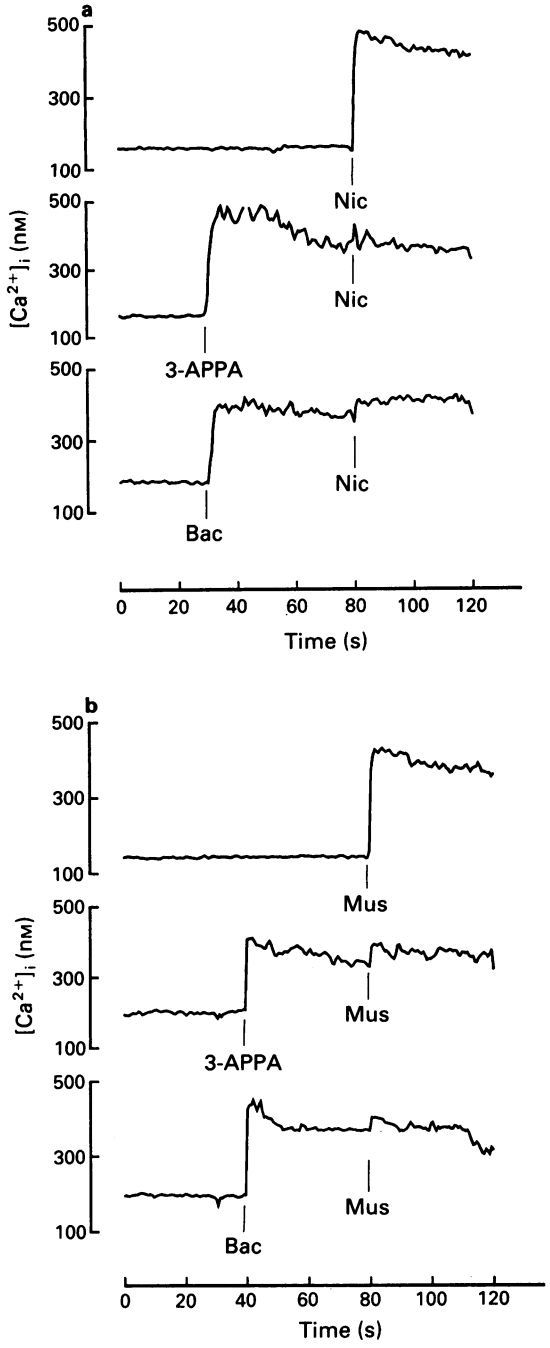


Figure 5 Effect of $GABA_B$ agonists on intracellular calcium concentrations evoked by nicotine and muscimol. Cytosolic Ca^{2+} transients elicited by stimulation of chromaffin cells with (a) 10 μM nicotine and (b) 200 μM muscimol in the absence or presence or 0.5 mM 3-APPA or baclofen. Superimposed records from different cell samples are traced. Drugs were added at times indicated by the arrows. Nic, 10 μM nicotine; Mus, 200 μM muscimol; 3-APPA, 0.5 mM 3-aminopropylphosphinic acid; Bac, 0.5 mM baclofen. Similar records were obtained from different samples in three different cell batches.

cyclic AMP together with $GABA_B$ agonists, baclofen or 3-APPA, did not produce any further increase in catecholamine secretion but instead resulted in a reduction of secretion evoked by each agent independently (Table 1).

Effects of preincubation with bacterial toxins on $GABA_B$ agonist-mediated responses in chromaffin cells

To study the participation of G proteins in the mechanism of action of $GABA_B$ receptors, cells were preincubated with

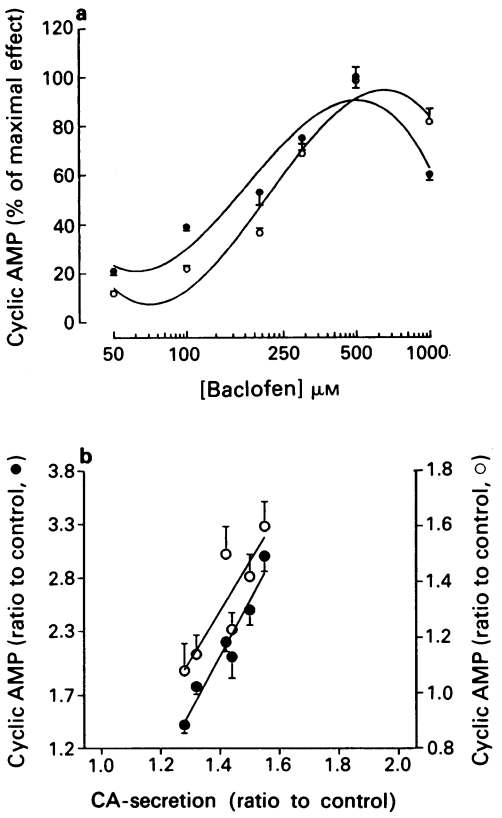


Figure 6 Effect $GABA_B$ agonists on intracellular levels of cyclic AMP in chromaffin cells. Cells were incubated with indicated concentrations of baclofen in the absence (○) or presence (●) of 0.5 mM isobutyl methylxanthine (IBMX) for 10 min and then cyclic AMP levels measured as indicated in Methods. (a) Dose-response curves at indicated doses of baclofen in the presence or absence of IBMX. Results are expressed as percentages of maximal effects (7.2 ± 0.5 and 6.16 ± 0.34 pmol cyclic AMP/10^6 cells for baclofen in the presence or absence, respectively of IBMX) in which basal [cyclic AMP] values (3.85 ± 0.15 pmol cyclic AMP/10^6 cells in the absence and 3.6 ± 0.2 pmol cyclic AMP/10^6 cells in the presence of 0.5 mM IBMX) were subtracted. Values are means ± s.e.mean of three independent determinations each performed in duplicate. EC_{50} = 146 ± 20 μM in the presence of IBMX and 210 ± 47 μM in the absence of IBMX. (b) Correlation between catecholamine (CA) secretion and cyclic AMP level rises induced by $GABA_B$ agonists. Data represent % of variation over the corresponding controls of catecholamine secretion (3.2 ± 0.1% of total cellular content) and of cyclic AMP levels (4.16 ± 0.23 pmol cyclic AMP/10^6 cells). Rects were fitted by minimum squares and were of $y = 5.18x - 5.18$; $^{***}P < 0.001$, $n = 13$ (without IBMX); and $y = 1.77x - 1.18$; $^{**}P < 0.01$, $n = 16$ (with IBMX). In the absence of IBMX $r = 0.87$ and in its presence $r = 0.96$.

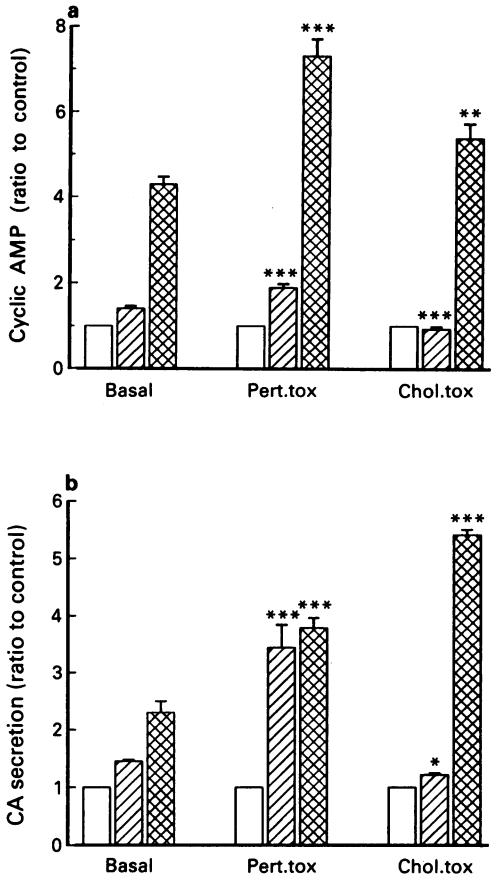


Figure 7 Effect of bacterial toxins on (a) intracellular levels of cyclic AMP and (b) catecholamine (CA) secretion induced by baclofen and forskolin. Cells were incubated with Locke solution (control), 300 ng ml^{-1} choleric toxin or 100 ng ml^{-1} pertussis toxin for 4 h at 37°C. Then the medium was removed and cells incubated with normal Locke (□), 100 μM baclofen (▨) or 1 μM forskolin (▩) for 10 min and cyclic AMP levels (a) or catecholamine secretion (b) measured. Results are means ± s.e.mean of three determinations each one performed in duplicate. Significantly different from the corresponding controls without toxins at $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$.

cholera toxin (CTX) or pertussis toxin (PTX) for 4 h and then the effect of $GABA_B$ agonists on catecholamine secretion and intracellular cyclic AMP levels determined.

Figure 7a shows that the effect of baclofen on cyclic AMP levels was approximately doubled in cells pretreated with PTX, but was inhibited by pretreatment with CTX. Moreover, pretreatment of cells with PTX increase by 4.5 times the catecholamine secretion evoked by baclofen but the effect of baclofen was not inhibited after pretreatment with CTX (Figure 7b).

The same effects on cyclic AMP levels and catecholamine secretion were shown with 1 μM forskolin when cells were preincubated with PTX but was different when cells were pretreated with CTX. In the latter case there was a rise in intracellular levels of cyclic AMP and, as a consequence, an increase in catecholamine secretion (Figure 7a and b).

Table 1 Effect of preincubation with forskolin on catecholamine secretion and cyclic AMP levels evoked by $GABA_B$ agonists

Drug	*Intracellular cyclic AMP* (pmol/10^6 cells)	*Catecholamine secretion* (% of total c.c.)
– Forskolin		
Control	4.43 ± 14	2.79 ± 0.10
Baclofen 100 μM	6.20 ± 0.19***	4.30 ± 0.27***
Baclofen 500 μM	8.95 ± 0.61***	6.28 ± 0.80**
3-APPA 100 μM	8.42 ± 0.42***	4.60 ± 0.52**
3-APPA 500 μM	13.00 ± 0.83***	7.40 ± 1.04**
+ Forskolin 1 μM		
Control	25.36 ± 1.97	6.13 ± 0.47
Baclofen 100 μM	30.25 ± 0.52*	2.34 ± 0.05***
Baclofen 500 μM	33.70 ± 1.97**	3.42 ± 0.05***
3-APPA 100 μM	34.10 ± 0.50**	3.18 ± 0.04***
3-APPA 500 μM	38.09 ± 0.98***	2.62 ± 0.18***

Cells were incubated with Locke solution (control) or $GABA_B$ agonists at the indicated concentrations in the absence or presence of 1 μM forskolin and then catecholamine secretion and $[\text{cyclic AMP}]_i$ measured. Results are means ± s.e.mean of three duplicate determinations. Significantly different from the corresponding controls (in the absence or presence of forskolin) at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. c.c = cellular content.

Discussion

Exocytotic release of catecholamines is a very complex event in the regulation of which numerous proteins are involved. A few years ago, the only receptor clearly linked to the stimulation of the exocytosis of catecholamine in bovine chromaffin cells was nAChR, but at present, a large number of other receptors are known to be able to stimulate catecholamine secretion by themselves or modulate nicotine-induced secretion (for a review see Burgoyne, 1991). At present, one of the most important neutrotransmitters implicated in this regulation is GABA.

GABA has two binding sites in the adrenal medulla, characterized as $GABA_A$ and $GABA_B$ receptors because of their capacity to be blocked with saturating concentrations of muscimol or baclofen, respectively and their sensitivity to Ca^{2+} ions (Castro *et al.*, 1988). The function of $GABA_A$ receptors has been clearly established (Kataoka *et al.*, 1986; Kitayama *et al.*, 1986; Castro *et al.*, 1989). It is known that stimulation of cells by $GABA_A$ agonists depolarizes the chromaffin cell membrane producing an increase in Cl^- efflux through the Cl^- channel associated with the $GABA_A$ receptor and as a consequence, a membrane depolarization which culminates in an increase in basal catecholamine secretion (González *et al.*, 1992).

The function of $GABA_B$ receptors in adrenal chromaffin cells has not been studied before. Results in this paper show for the first time that the $GABA_B$ agonists baclofen and, the most potent agonist available, 3-APPA increase basal catecholamine secretion in a dose-dependent manner. The EC_{50} of this effect was estimated as $225 \pm 58\ \mu M$ for 3-APPA and $151 \pm 35\ \mu M$ for baclofen, respectively. These doses are high compared with the concentrations of other agonists needed to stimulate 50% of catecholamine secretion (i.e. nicotine, muscimol and histamine have an EC_{50} of $5-10\ \mu M$), and even higher than the doses required to stimulate other $GABA_B$ responses in the mammalian brain (i.e. between $10-100\ \mu M$ in $GABA_B$ mediated inhibitory post-synaptic potentials or hyperpolarizations in the pyramidal cells of the hypocampus). However they are of the same order of magnitude as those required to stimulate $GABA_B$ responses in other peripheral tissues such as the inhibition of ileum peristalsis or inhibition of glutamate, acetylcholine and noradrenaline release in the peripheral nervous system ($100-300\ \mu M$), although in all these cases $GABA_B$ responses were inhibitory. These findings together with the fact that $GABA_B$ responses on chromaffin cell catecholamine secretion represent between 40–50% of those obtained with nicotine at the same doses, seem to suggest either a low affinity of $GABA_B$ receptors or the existence of a low number of $GABA_B$ binding sites in adrenal medullary cells (Castro *et al.*, 1988).

Nevertheless, in spite of the high doses required to stimulate these receptors, the $GABA_B$-mediated increase in catecholamine secretion from chromaffin cells seems to be a true exocytotic and specific effect. This is supported by the findings that (1) this effect was accompanied by a dose-dependent increase in $[Ca^{2+}]_i$ measured by Fura-2 methods (Figure 5a) and was almost completely blocked when catecholamine secretion by these agonists was measured in a low Ca^{2+}-containing medium (200 nM), (2) $GABA_B$ agonists were able to release other secretory granular components such as DBH to a similar extent as catecholamines and (3) their effects were able to be blocked by the specific $GABA_B$ antagonist CGP-35348, which, at a dose of 0.5 mM inhibited by 70 to 100% the agonist-evoked responses (Figure 2). Faclofen, another specific $GABA_B$ antagonist, was unable to inhibit $GABA_B$ agonist responses. On the basis of the pharmacological classification of $GABA_B$ receptors of Dutar & Nicholl (1988), this insensitivity to faclofen could mean that $GABA_B$ receptors in the adrenal medulla belong to a presynaptic type, although the finding that the same data were obtained with well characterized postsynaptic $GABA_B$ receptors (Huston *et al.*, 1990) suggests that this rule may not be universally applicable. Faclofen may be a non-specific and potent $GABA_B$ antagonist in some tissues. In fact this compound has been shown to have $GABA_B$ agonist behaviour in some responses (Michler & Erdö, 1989). Therefore, the precise nature, pre- or post-synaptic, of adrenal medullary $GABA_B$ receptors remains to be determined.

In addition to the effect of $GABA_B$ agonists on basal catecholamine secretion, stimulation of chromaffin cells by these compounds also has an effect on the catecholamine secretion evoked by nACh and $GABA_A$ agonists. When cells were preincubated with $GABA_B$ agonists before nicotine was added, these agonists strongly inhibited nicotine-evoked catecholamine secretion in a dose-dependent manner; the IC_{50}s obtained were of the same order of magnitude as those for activation of basal catecholamine secretion (Figure 3a). This inhibitory effect was accompanied by a reduction in the intracellular Ca^{2+} levels induced by nicotine, results which support the hypothesis of a true inhibitory modulation of nACh receptors by $GABA_B$ ones (Figure 5a). Similar results were also obtained for catecholamine secretion evoked by $GABA_A$ agonists (Figure 3b) and for increases in $[Ca^{2+}]_i$ (Figure 5b).

With respect to the molecular mechanism by which $GABA_B$ agonists produce these effects in chromaffin cells it seems to be mediated by the activation of adenylate cyclase (AC). This conclusion is supported by the following experimental data: (1) $GABA_B$ agonists increase intracellular levels of cyclic AMP in a dose-dependent fashion, effects which are well correlated with increases obtained in catecholamine secretion (Figure 6). (2) Several activators of AC as well as forskolin and cyclic AMP analogues increase basal catecholamine secretion in a dose-dependent manner, a direct correlation existing between both parameters, intracellular cyclic AMP levels and catecholamine secretion (Oset-Gasque *et al.*, 1991). (3) Pretreatment of cells with PTX, which by ADP-ribosylation of G_i proteins produces an inhibition of negative control of AC, potentiates cyclic AMP levels and catecholamine secretion induced by $GABA_B$ agonists as well as by forskolin (Figure 7a and b). (4) Pretreatment of cells with CTX, which permanently activates AC, does not produce additional increases in either the cyclic AMP levels or in catecholamine secretion induced by baclofen.

The inhibitory actions of $GABA_B$ agonists on catecholamine secretion evoked by nicotinic and $GABA_A$ receptors could also be explained by this mechanism. This conclusion seems to be supported both by our results which show that forskolin and pClp cyclic AMP have a dose-dependent inhibitory effect on catecholamine secretion evoked by nicotine and muscimol (Oset-Gasque *et al.*, 1991) and by other work showing that cyclic AMP, or agonists which activate AC, may produce an inactivation of nAChR and $GABA_A$ receptors by phosphorylation (Alburquerque *et al.*, 1986; Huganir *et al.*, 1986; Harrisson & Lambert, 1989; Leidenheimer *et al.*, 1991). However, although $GABA_B$ agonists seem to mediate their effects on catecholamine secretion by a mechanism implicating cyclic AMP formation, we do not exclude the possibility of the existence of an additional mechanism for the action of these receptors.

Specific interactions between $GABA_A$ and $GABA_B$ receptors have not been described until very recently (Hahner *et al.*, 1991). Our results seem to support a specific modulation of $GABA_A$ receptors by the $GABA_B$ ones, since the IC_{50} required for inactivation of $GABA_A$ receptors by $GABA_B$ agonists was very much smaller than that required for inactivation of nAChR.

In conclusion, GABA through both its $GABA_A$ and $GABA_B$ receptors, seems to play an important role in the modulation of chromaffin cell function and therefore, GABA could be added to the long list of neurotransmitters and hormones implicated in the modulation of this complex function. The physiological significance of the results presented here is not yet known. It could be possible that ionotropic

GABA receptors, as well as nACh receptors, play a major role in the initiation of catecholamine release *in vivo* while metabotropic receptors, including those for $GABA_B$, have a more important role in the modulation of the secretion evoked by ionotropic receptors.

This work was supported by Research Grants CAM C168/91 from Comunidad Autónoma de Madrid and FISS 92/0241 from Fondo de Investigaciones Sanitarias de la Seguridad Social of Madrid (Ministry of Health, Spain). The authors thank D.A. Gilson for improving the manuscript.

References

ALBURQUERQUE, E.X., DESHPANDE, S.S., ARAVACA, Y., ALKONDON, M. & DALY, J.M. (1986). A possible involvement of cyclic AMP in the expression of desensitization of the nicotinic acetylcholine receptor. *FEBS Lett.*, **199**, 113–120.

BADER, M.F., CIESIELSKI-TRESKA, J., THIERSE, D., HESKETCH, J.E. & AUNIS, D. (1981). Immunocytochemical study of microtubules in chromaffin cells in culture and evidence that tubulin is not an integral protein of the chromaffin granule membrane. *J. Neurochem.*, **37**, 917–933.

BOWERY, N.G. (1989). $GABA_B$ receptors and their significance in mammalian pharmacology. *Trends Pharmacol. Sci.*, **10**, 401–407.

BOWERY, N.G., HILL, D.R., HUDSON, A.L., DOBLE, A.A., MIDDLEMISS, D.N., SHAW, J. & TURNBULL, M.J. (1980). Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. *Nature*, **283**, 92–94.

BURGOYNE, R.D. (1991). Control of exocytosis in adrenal chromaffin cells. *Biochim. Biophys. Acta*, **1071**, 174–202.

CASTRO, E., OSET-GASQUE, M.J., CAÑADAS, S., GIMÉNEZ, A. & GONZÁLEZ, M.P. (1988). $GABA_A$ and $GABA_B$ sites in bovine adrenal medulla membranes. *J. Neurosci. Res.*, **20**, 241–245.

CASTRO, E., OSET-GASQUE, M.J. & GONZÁLEZ, M.P. (1989). $GABA_A$ and $GABA_B$ receptors are functionally active in regulation of catecholamine secretion by bovine chromaffin cells. *J. Neurosci. Res.*, **23**, 290–296.

CHALISS, R.A.J., JONES, J.A., OWEN, P.J. & BOARDER, M.R. (1991). Changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulations in cultured adrenal chromaffin cells in response to bradykinin and histamine. *J. Neurochem.*, **56**, 1083–1086.

CHEEK, T.R. & BURGOYNE, R.D. (1985). Effect of activation of muscarinic receptors on intracellular free calcium and secretion in bovine adrenal chromaffin cells. *Biochim. Biophys. Acta.*, **846**, 167–173.

CHERN, Y.J., KIM, K.T., STARLEY, L.L. & WESTHEAD, E.W. (1988). Adenosine receptors activate adenylate cyclase and enhance secretion from bovine adrenal chromaffin cells in the presence of forskolin. *J. Neurochem.*, **50**, 1484–1493.

DUTAR, P. & NICOLL, R.A. (1988). A physiological role for $GABA_B$ receptors in the central nervous system. *Nature*, **332**, 156–158.

FERNÁNDEZ-RAMIL, J.M., SÁNCHEZ-PRIETO, J. & GONZÁLEZ, M.P. (1982). Presence of glutamate decarboxylase in bovine adrenal medullary cells. *Rev. Esp. Fisiol.*, **38**, 91–96.

FERNÁNDEZ-RAMIL, J.M., SÁNCHEZ-PRIETO, J., CAÑADAS, S. & GONZÁLEZ, M.P. (1983). GABA-T in bovine adrenal medulla cells. Kinetic properties and comparison with GABA-T from other tissues. *Rev. Esp. Fisiol.*, **39**, 299–304.

GONZÁLEZ, M.P., OSET-GASQUE, M.J., CASTRO, E., BUGEDA, J., ARCE, C. & PARRAMÓN, M. (1992). Mechanism through which $GABA_A$ receptor modulates catecholamine secretion from bovine chromaffin cells. *Neuroscience*, **47**, 487–494.

HAHNER, L., MAQUILKIN, S. & HARRIS, R.A. (1991). Cerebellar $GABA_B$ receptors modulate function of $GABA_A$ receptors. *FASEB J.*, **5**, 2466–2472.

HARRISON, N.L. & LAMBERT, N.A. (1989). Modification of $GABA_A$ receptor function by an analog of cyclic AMP. *Neurosci. Lett.*, **105**, 137–142.

HUETTL, P., GERHARDT, G.A., BROWNING, M.D. & MASSERANO, J.M. (1991). Effects of dopamine receptor agonists and antagonists on catecholamine release in bovine chromaffin cells. *J. Pharmacol. Exp. Ther.*, **257**, 567–574.

HUGANIR, R.L., DELCOUR, A.H., GREENGARD, P. & HESS, G.P. (1986). Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. *Nature*, **321**, 774–776.

HUSTON, E., SCOTT, R.H. & DOLPHIN, A.C. (1990). A comparison of the effect of calcium channel ligands and $GABA_B$ agonists and antagonists on transmitter release and somatic calcium channel. *Neuroscience*, **38**, 721–729.

ITO, S., MOCHIZUKI-ODA, N., HORI, K., OZAKI, K., MIYAKAWA, A. & NEGISHI, M. (1991). Characterization of prostaglandin-E2-induced Ca^{++} mobilization in single bovine adrenal chromaffin cells by digital image microscopy. *J. Neurochem.*, **56**, 531–540.

KATAOKA, Y., FUJIMOTO, M., ALHO, H., GUIDOTTI, A., GEFFARD, M., KELLY, G.D. & HANBAUER, I. (1986). Intrinsic gamma aminobutyric acid receptors modulate the release of catecholamine from canine adrenal gland *in situ*. *J. Pharmacol. Exp. Ther.*, **239**, 584–590.

KITAYAMA, S., KOYAMA, Y., MORITA, K., DOHI, T. & TSUJIMOTO, A. (1986). Increase in catecholamine release and Ca^{++} uptake induced by GABA in cultured bovine adrenal chromaffin cells. *Naunyn-Schmied. Arch. Pharmacol.*, **341**, 414–418.

LEIDENHEIMER, N.J., BROWNING, M.D. & HARRIS, R.A. (1991). $GABA_A$ receptor phosphorylation: multiple sites, actions and artifacts. *Trends Pharmacol. Sci.*, **12**, 84–87.

LEMAIRE, S., LIVETT, B.G., TSENG, R., MERCIER, P. & LEMAIRE, I. (1981). Studies on the inhibitory action of opiate compounds in isolated bovine adrenal chromaffin cells: non-involvement of stereospecific opiate binding sites. *J. Neurochem.*, **36**, 886–892.

LIVETT, B.G., KOZOUSCK, V., MIZOBE, F. & DEAN, D.M. (1979). Substance P inhibits nicotine activation of chromaffin cells. *Nature*, **278**, 256–257.

MARLEY, P.D., BUNN, S.J., WAN, D.C.C., ALLEN, A.M. & MENDELSOHN, F.A.O. (1989). Localization of angiotensin II binding sites in the bovine adrenal medulla using a labelled specific antagonist. *Neuroscience*, **28**, 777–787.

MICHLER, A. & ERDÖ, A.L. (1989). Stimulation by phaclofen of inositol 1,4,5-triphosphate production in cultured neurons from chick tectum. *Eur. J. Pharmacol.*, **167**, 423–425.

MOTT, D.D. & LEWIS, D.V. (1991). Facilitation of the induction of long-term potentiation by $GABA_B$ receptors. *Science*, **252**, 1718–1720.

NEGISHI, M., ITO, S. & HAYAISHI, O. (1989). Prostaglandin E receptors in bovine adrenal medulla are coupled to adenylate cyclase via Gi and to phosphoinositide metabolism in a pertussis toxin-insensitive manner. *J. Biol. Chem.*, **264**, 3916–3922.

OSET-GASQUE, M.J. & AUNIS, D. (1989). Gamma-aminobutyric acid uptake in bovine chromaffin cells in culture. *Biochem. Pharmacol.*, **38**, 2227–2232.

OSET-GASQUE, M.J., CASTRO, E. & GONZÁLEZ, M.P. (1990). Mechanism of [^{3}H] gamma-aminobutyric acid release by chromaffin cells in primary culture. *J. Neurosci. Res.*, **26**, 181–187.

OSET-GASQUE, M.J. PARRAMÓN, M. & GONZÁLEZ, M.P. (1991). *XII Reunión del Grupo Español de la Célula Cromafín*, Salamanca, Spain.

O'SULLIVAN, A.J. & BURGOYNE, R.D. (1989). A comparison of bradykinin, angiotensin II and muscarinic stimulation of cultured bovine adrenal chromaffin cells. *Biosci. Rep.*, **9**, 243–252.

OWEN, P.J., PLEVIN, R. & BOARDER, M.J. (1989). Characterization of bradykinin-stimulated release of noradrenaline from cultured bovine adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.*, **248**, 1231–1235.

STAUDERMAN, K.A. & PRUSS, R.M. (1990). Different patterns of agonist-stimulated increase of [^{3}H]-inositol phosphate isomers and cytosolic Ca^{++} in bovine adrenal chromaffin cells: comparison of the effects of histamine and angiotensin II. *J. Neurochem.*, **54**, 946–953.

(Received May 4, 1993
Revised July 27, 1993
Accepted August 23, 1993)

Br. J. Pharmacol. (1993), **110**, 1593–1599 © Macmillan Press Ltd, 1993

The guinea-pig distal colon – a sensitive preparation for the investigation of 5-HT_4 receptor-mediated contractions

[1]Kay A. Wardle & Gareth J. Sanger

SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD

1 Experiments were designed to characterize pharmacologically the contractile responses to 5-hydroxytryptamine (5-HT) in the guinea-pig isolated distal colon longitudinal muscle-myenteric plexus preparation (LMMP).

2 In the presence of methiothepin (100 nM) and granisetron (1 μM), 5-HT (10 pM–10 nM) produced concentration-dependent contractile responses of the guinea-pig distal colon LMMP, with a pEC_{50} of 9.2 ± 0.08.

3 Responses to 5-HT were mimicked by a series of tryptamine analogues, with the following rank order of potency; 5-HT > 5-MeOT > > 5-CT > tryptamine > 2-Me-5-HT. All were found to be full agonists.

4 Responses to 5-HT were also mimicked by a series of substituted benzamide analogues. Their rank order of potency was 5-HT > renzapride > cisapride > (**S**)-zacopride > (**R**)-zacopride > metoclopramide. All were full agonists relative to 5-HT.

5 The benzimidazolone derivatives, BIMU 1 and BIMU 8 were approximately equipotent partial agonists (intrinsic activities of 0.8 ± 0.07 and 0.5 ± 0.08 respectively) in the guinea-pig distal colon.

6 Tropisetron produced a rightward displacement of the 5-HT concentration-effect curve, yielding an apparent pA_2 of 6.4 ± 0.1. The slope of the Schild plot (1.3 ± 0.1) was significantly greater than unity.

7 SDZ 205 557 produced a concentration-dependent shift to the right of the 5-HT concentration-response curve, yielding an estimated pA_2 of 7.8 ± 0.1 and a slope which did not significantly deviate from unity. SDZ 205 557 produced similar pK_B estimates (7.3–7.9) when tested against 5-MeOT, renzapride and 5-CT, indicating a common site of action.

8 The pharmacological profile of the 5-HT-evoked contractions of the guinea-pig distal colon LMMP are consistent with activity at the 5-HT_4 receptor. Furthermore, of the models of this receptor described in the literature, the guinea-pig distal colon appears to be the most sensitive model to date, making it a useful tool in the investigation of 5-HT_4 receptor-mediated events.

Keywords: Guinea-pig colon; 5-HT_4 receptors; 5-HT (5-hydroxytryptamine); tryptamine analogues; substituted benzamide analogues; benzimidazolone derivatives; tropisetron; SDZ 205 557

Introduction

Based on an elegant series of experiments carried out in mouse embryo colliculi neurones and guinea-pig hippocampus (Dumuis *et al.*, 1988a,b; Bockaert *et al.*, 1990) it has been proposed that at least some of the reported 'atypical' responses to 5-hydroxytryptamine (5-HT) can be attributed to stimulation of a novel receptor, termed 5-HT_4. Thus, the 5-HT_4 receptor is characterized by a high sensitivity to both 5-HT and 5-methoxytryptamine (5-MeOT) and is stimulated by certain substituted benzamides such as renzapride, cisapride and metoclopramide. Antagonists at this site include high concentrations of tropisetron (ICS 205 930, Dumuis *et al.*, 1988a), SDZ 205 557 (Buchheit *et al.*, 1991; 1992) and DAU 6285 (Dumuis *et al.*, 1992).

5-HT_4 receptor-mediated functional responses have been detected in a variety of tissues including the guinea-pig ileum (Craig & Clarke, 1990) and ascending colon (Elswood *et al.*, 1991), rat oesophagus (Baxter *et al.*, 1991), frog and human adrenocortical cells (Idres *et al.*, 1991; Lefebvre *et al.*, 1992), sheep pulmonary vein (Cocks & Arnold, 1992) and in both pig (Villalon *et al.*, 1990) and human heart (Kaumann *et al.*, 1990a,b). The sensitivity of each of these preparations to 5-HT is in the sub-micromolar range, with pEC_{50} values ranging from 6.7 in the piglet heart (Lorrain *et al.*, 1992) to 8.6 in the guinea-pig ileum (Craig & Clarke, 1990). In the current investigation we describe a highly sensitive model of the 5-HT_4 receptor, namely the guinea-pig distal colon longitudinal muscle myenteric plexus (LMMP).

Some of these results have been presented to the British Pharmacological Society (Wardle & Sanger, 1992).

Methods

Preparation of tissues

Distal colon (approximately 7–8 cm from the anus) was removed from young male Dunkin Hartley guinea-pigs (200–300 g) and placed in Krebs solution of the following composition (mM): NaCl 121.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 5.6

Sections of colon (2–3 cm in length) were slipped over a glass rod. With a scalpel blade, the longitudinal muscle layer was cut along the mesenteric border, taking care not to cut through all the muscle layers. The longitudinal muscle-myenteric plexus was then stripped off with a piece of moist cotton wool and mounted under a 0.5 g load in a 10 ml tissue bath. Tissues were bathed with Krebs solution at 37°C gassed with 5% CO_2 in O_2 and containing granisetron (1 μM) and methiothepin (100 nM) to inhibit responses mediated by 5-HT_3 receptors and 5-$HT_{1\text{-like}}$ and 5-HT_2 receptors respectively. Responses were recorded isotonically and displayed on a Lectromed MT8P multitrace chart recorder. Tissues were allowed to stabilize for 45 min (washing every 15 min) prior to starting the experiment.

[1] Author for correspondence.

In a few experiments, sections of guinea-pig ileum, 10 cm proximal to the caecum were removed, dissected and set up in the same way as described for the distal colon.

Experimental protocol and concentration-response curves

Tissues were first exposed to a supramaximal concentration of acetylcholine (ACh, 40 μM) to establish the maximum response evoked by each tissue. Initial responses to 5-HT were found to be small, but increased on repeated dosing. Hence it was deemed necessary to sensitize each tissue to 5-HT before constructing the first concentration-response curve. This was done by dosing the preparations every 15 min with a supramaximal concentration of 5-HT (1 μM) four to five times until consistent responses were produced.

Following sensitization, agonist-concentration response curves were constructed non-cumulatively, by adding increasing concentrations of agonist at 15 min time intervals. The agonist was left in contact with the tissue until a maximum response was obtained (usually 30 s). In each tissue, two agonist concentration-response curves were constructed. In all experiments, the first curve was to 5-HT itself. In experiments to investigate 5-HT_4 receptor agonist activity, the second curve was constructed to either 5-HT (in time control experiments) or to a test agonist. In time control experiments, the two concentration-response curves to 5-HT were super-imposable (pEC_{50} and E_{max} values for first and second curves were respectively 9.2 ± 0.1, 100 ± 0 and 9.2 ± 0.2, 98 ± 4, $n = 6$). For antagonist experiments, the second curve was constructed to 5-HT in the presence of the appropriate concentration of antagonist, added immediately after completion of the first curve. In all experiments, a minimum of 45 min was left between successive curves. Responses were expressed as a percentage of the maximum 5-HT-evoked contraction in the first concentration-response curve in each tissue.

All agonist concentration-response curves were fitted using a non-linear iterative fitting programme (Kaleidagraph, Synergy Software, PCS Inc., Reading, Pa) according to the following relationship (Parker & Waud, 1971):

$$E = M[A]^p/([A]^p + [K]^p)$$

This relationship describes a curve with a maximum response M, an EC_{50} equal to K and a slope determined by the power p. [A] represents agonist concentration and E is the response.

Agonist potency

The ability of agonists to evoke contraction of the guinea-pig isolated distal colon was expressed in terms of a pEC_{50} value (relative to their own maxima) and in terms of a maximum response, relative to 5-HT (intrinsic activity, where intrinsic activity of 5-HT = 1.0). The potency relative to 5-HT was calculated from experiments in which two concentration-response curves were constructed in each preparation; the first curve to 5-HT itself, the second curve to the test agonist.

Antagonist potency

pA_2 values for a variety of antagonists versus 5-HT were determined according to the method of Arunlakshana & Schild (1959). Two concentration-response curves were constructed in each tissue, the first in the absence, the second in the presence of the appropriate concentration of antagonist.

Apparent pK_B values for SDZ 205 557 versus all other agonists were determined by the method of Furchgott (1972) using a single concentration of antagonist (100 nM).

Drugs

The following were used: 5-hydroxytryptamine creatinine sulphate (5-HT), tryptamine hydrochloride, cocaine hydrochloride, tetrodotoxin (TTX), atropine sulphate, pargyline hydrochloride, acetylcholine perchlorate, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) (Sigma), methiothepin hydrochloride (Roche), 5-methoxytryptamine hydrochloride (5-MeOT, Aldrich), cisapride (Janssen; free base converted into a hydrochloride salt in house), granisetron, renzapride, metoclopramide (SmithKline Beecham), 2-methyl 5-hydroxytryptamine (2-Me-5-HT), SDZ 205 557 (2-methoxy-4-amino-5-chloro-benzoic acid 2-diethylamino ethyl ester), tropisetron (ICS 205 903), (R)- and (S)-zacopride, 5-hydroxy-indalpine (5-OH-IP), 5-carboxyamidotryptamine (5-CT), (±)-8-hydroxy-2-(di-n-propyl-amino) tetralin (8-OH-DPAT, synthesized in house). BIMU 1 (endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride) and BIMU 8 (endo-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl) ethyl-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride) were a kind gift from Dr Carlo Rizzi, Boehringer Ingelheim, Italy.

All drugs were dissolved in 0.9% saline and diluted to appropriate concentrations with Krebs solution. With tropisetron and cisapride (free base only), a minimum amount of tartaric acid was used to dissolve the compounds.

Results

Preliminary experiments

In the absence of granisetron (but in the presence of methiothepin (100 nM)), concentration-response curves to 5-HT (10 pM–100 μM, $n = 4$) in the guinea-pig ileum and distal colon were biphasic in nature (Figure 1). In the distal colon, the first phase of the curve had an estimated pEC_{50} of 9.2 ± 0.2 and plateaued at $52.5 \pm 3.5\%$ of the 5-HT-evoked maximum ($n = 4$). This compares with an estimated pEC_{50} value of 7.9 ± 0.04 and maximum of $31.8 \pm 1.6\%$ of the 5-HT maximum in the ileum ($n = 4$). The second phase of the curves were selectively antagonized by granisetron (1 μM, results not shown), leaving a simple monophasic curve.

In the guinea-pig colon, the concentration-response curve to low concentrations of 5-HT (10 pM–10 nM) was

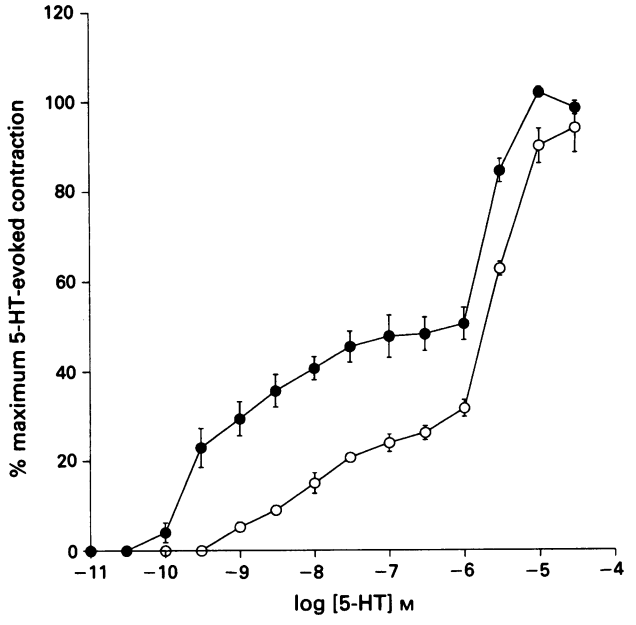


Figure 1 Concentration-response curves to 5-hydroxytryptamine (5-HT) in isolated longitudinal-muscle myenteric plexus (LMMP) strips from guinea-pig ileum (○) and colon (●). Experiments were carried out in the presence of methiothepin (100 nM), but in the absence of granisetron. Each point represents the mean ± s.e.mean of 4 observations.

Table 1 Agonist potency in the guinea-pig distal colon longitudinal muscle myenteric plexus preparation

Agonist	*Concentrations*	*pEC$_{50}$*	*Intrinsic activity*	n
5-HT	10 pM–10 nM	9.2 ± 0.08	1.0	38
5-MeOT	100 pM–1 μM	8.7 ± 0.1	1.0 ± 0.02	9
5-CT	10 nM–100 μM	5.7 ± 0.08	1.0 ± 0.02	13
2-Me-5-HT	1 μM–100 μM	3.8 ± 0.07	1.0 ± 0.03	4
Tryptamine	100 nM–300 μM	4.7 ± 0.1	0.9 ± 0.1	14
Renzapride	1 nM–10 μM	7.2 ± 0.1	1.0 ± 0.02	13
(R)-zacopride	10 nM–30 μM	5.8 ± 0.1	1.0 ± 0.05	12
(S)-zacopride	10 nM–30 μM	6.3 ± 0.08	1.0 ± 0.02	12
Cisapride base	10 nM–3 μM	6.9 ± 0.2	0.4 ± 0.1	4
Cisapride HCl	10 nM–3 μM	6.9 ± 0.1	0.9 ± 0.06	4
Metoclopramide	10 nM–30 μM	5.7 ± 0.1	0.9 ± 0.05	11
BIMU 1	10 nM–3 μM	6.7 ± 0.1	0.8 ± 0.07	4
BIMU 8	10 nM–3 μM	6.9 ± 0.08	0.5 ± 0.08	8
8-OH-DPAT	10 nM–10 μM	Inactive	NA	4
5-OH-indalpine	10 nM–10 μM	Inactive	NA	4

Each value represents the mean ± s.e.mean of a number (n) of preparations.

unaffected by granisetron (1 μM). Thus, the pEC$_{50}$ values were identical in the absence and presence of granisetron (9.2 ± 0.2 (n = 4) and 9.2 ± 0.08 (n = 38) respectively). Consequently, granisetron was added routinely in all subsequent experiments to block the second phase of the 5-HT curve. Under these conditions, the contractions evoked by 5-HT (10 pM–10 nM) were prevented by atropine (1 μM, n = 6) and TTX (300 nM, n = 4), suggesting that responses were predominantly due to stimulation of cholinergic neurones (results not shown).

Responses to 5-HT (10 pM–10 nM) were not significantly affected by cocaine (300 nM–30 μM). The respective pEC$_{50}$ and E$_{max}$ values for 5-HT were 9.3 ± 0.1, 100 ± 0 (control), 9.3 ± 0.06, 100 ± 2 (300 nM cocaine), 9.2 ± 0.08, 97 ± 3 (3 μM cocaine) and 9.3 ± 0.07, 102 ± 2 (30 μM cocaine); n = 4 each. Concentration-response curves to both 5-HT (10 pM–10 nM) and 5-methoxytryptamine (5-MeOT, 100 pM–100 nM) were unaffected by a 30 min pre-incubation with the irreversible monoamine oxidase inhibitor pargyline (100 μM). The pEC$_{50}$ and E$_{max}$ values for 5-HT in the absence and presence of pargyline were 9.3 ± 0.1, 100 ± 0 and 9.4 ± 0.1, 98 ± 3 respectively. The equivalent values for 5-MeOT were 8.1 ± 0.08, 97 ± 2 and 8.2 ± 0.05, 99 ± 4; n = 4 each. Thus, in the guinea-pig distal colon, neither neuronal uptake nor monoamine oxidase metabolism were believed to affect significantly the responses to these agonists. Hence, it was not deemed necessary to include these agents in subsequent experiments.

Agonist profile

In the guinea-pig distal colon, 5-HT evoked concentration-dependent contractions with a mean pEC$_{50}$ of 9.2 ± 0.08. Responses were slow in onset, reaching a plateau after approximately 30 s. Preliminary experiments with animals of a different age demonstrated that the sensitivity of the preparations to 5-HT was highly dependent on the size of the guinea-pigs used. Thus, with very small animals (150–200 g) the pEC$_{50}$ could be as low as 10.1, although dissection of the tissue often proved difficult due to the thinness of the LMMP. With large guinea-pigs (450–550 g), the pEC$_{50}$ of 5-HT dropped to 7.8. For this reason, the size of the guinea-pigs was tightly controlled in all further experiments (200–300 g).

Four tryptamine analogues namely, 5-MeOT, 5-CT, tryptamine itself and 2-Me-5-HT acted as full agonists in this model. Their pEC$_{50}$ values were 8.7, 5.7, 4.7 and 3.8 respectively. Results are shown in full in Figure 2 and Table 1.

Five substituted benzamides were examined. Renzapride, (S)-zacopride (R)-zacopride, cisapride (HCl) and metoclopramide were each full agonists with pEC$_{50}$s of 7.2, 6.3, 5.8, 6.9 and 5.7 respectively (Figure 3, Table 1). When cisapride was used in its original form (free base), it was a partial agonist (pEC$_{50}$ of 6.9 and an intrinsic activity of 0.4).

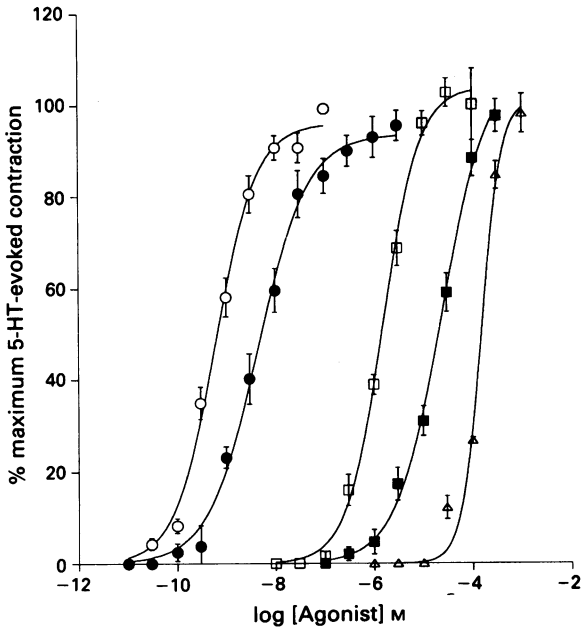


Figure 2 Concentration-response curves to 5-hydroxytryptamine (5-HT, ○), 5-methoxytryptamine (●), 5-carboxamidotryptamine (□), tryptamine (■) and 2-methyl-5-hydroxytryptamine (△) in guinea-pig distal colon LMMP. Experiments were carried out in the presence of methiothepin (100 nM) and granisetron (1 μM). Each point represents the mean ± s.e.mean of between 4 and 38 observations. For n values for each compound, see Table 1.

The benzimadazolone derivatives, BIMU 1 and BIMU 8 were also examined. Both were partial agonists relative to 5-HT with intrinsic activities of 0.5 and 0.8 respectively. The pEC$_{50}$ for BIMU 1 was estimated to be 6.9, and for BIMU 8, 6.7. (Figure 4, Table 1).

The 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT and the putative 5-HT$_{1P}$ receptor agonist 5-OH-indalpine were found to be inactive. The results for all compounds tested are summarized in Table 1.

Antagonist profile

Tropisetron (ICS 205-930, 1–10 μM, n = 7) produced a parallel rightward shift of the 5-HT concentration-response curve,

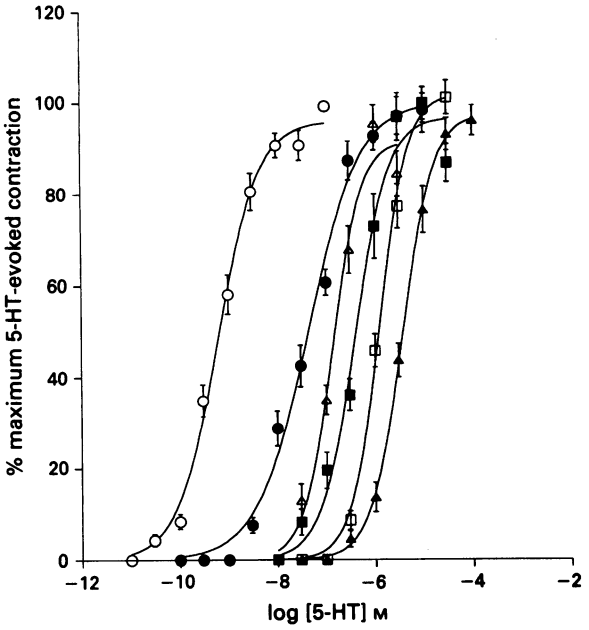


Figure 3 Concentration-response curves to 5-hydroxytryptamine (5-HT, ○), renzapride (●), (R)-zacopride (□), (S)-zacopride (■), cisapride (HCl, △) and metoclopramide (▲) in guinea-pig distal colon LMMP. Experiments were carried out in the presence of methiothepin (100 nM) and granisetron (1 μM). Each point represents the mean ± s.e.mean of between 4 and 38 observations. For *n* values for each compound, see Table 1.

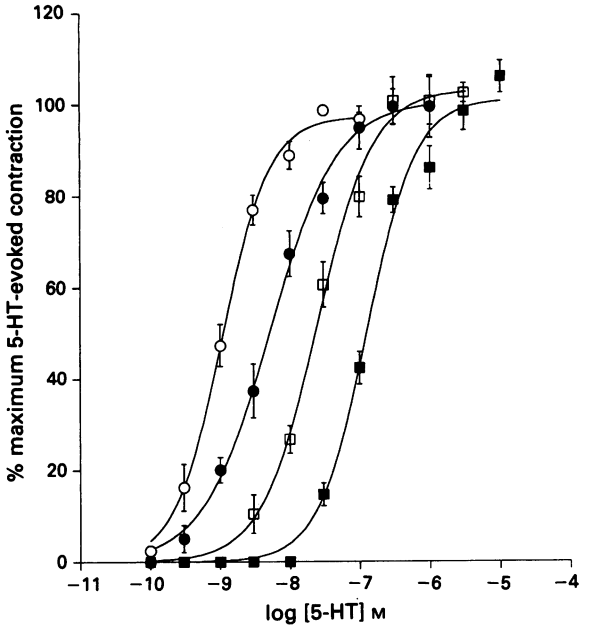


Figure 5 Concentration-response curves to 5-hydroxytryptamine (5-HT) in the absence (○) and presence of 100 nM (●), 300 nM (□) and 1 μM (■) SDZ 205 557 in guinea-pig distal colon LMMP. Experiments were carried out in the presence of methiothepin (100 nM) and granisetron (1 μM). Each point represents the mean ± s.e. mean of 6 observations.

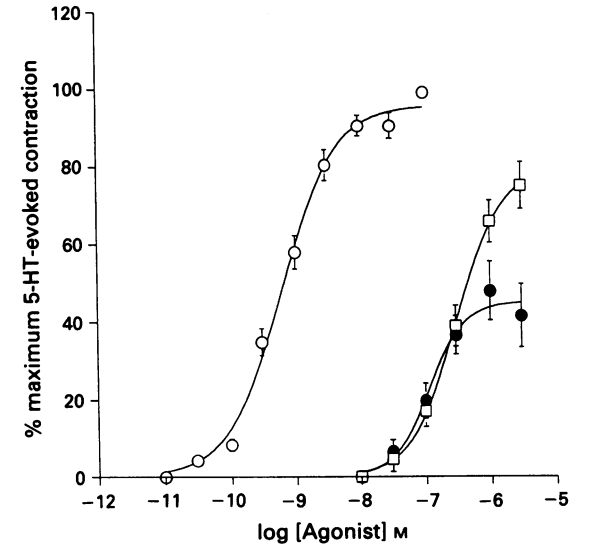


Figure 4 Concentration-response curve to 5-hydroxytryptamine (5-HT, ○), BIMU 1 (●) and BIMU 8 (□), in guinea-pig distal colon LMMP. Experiments were carried out in the presence of methiothepin (100 nM) and granisetron (1 μM). Each point represents the mean ± s.e.mean of between 4 and 38 observations. For *n* values for each compound, see Table 1.

yielding an apparent pA_2 of 6.4 ± 0.1 and a slope of the Schild plot of 1.3 ± 0.1. This value was significantly ($P<0.05$) greater than unity, but may be attributed to the fact that, at 10 μM, tropisetron reduced the maximum DMPP-evoked contraction (by 29 ± 5%; $P<0.05$) in the same preparation.

SDZ 205 557 (100 nM–1 μM, $n=6$) produced a parallel rightward shift of the 5-HT concentration-response curve (Figure 5). The slope of the Schild plot (1.06 ± 0.09) was not significantly different from unity and yielded an estimated pA_2 value of 7.8 ± 0.1. At the concentrations tested, SDZ 205 557 had no effect on DMPP (100 nM–100 μM)-evoked contractions in the same preparation.

Estimates of the pK_B value for SDZ 205 557 against several other 5-HT_4 receptor agonists were calculated to be 7.3 ± 0.2 ($n=4$) for 5-MeOT, 7.5 ± 0.09 ($n=4$) for renzapride and 7.9 ± 0.2 ($n=4$) for 5-CT.

Discussion

The present study clearly demonstrates that, in the presence of 5-HT_1, 5-HT_2 and 5-HT_3 receptor antagonists, 5-HT evokes cholinergically-mediated contractions of the guinea-pig distal colon LMMP which are mediated by the 5-HT_4 receptor.

A similar preparation, dissected from the ileum, has long been used to study the effects of 5-HT on the 'atypical' 5-HT receptor in the guinea-pig gastrointestinal tract (Buchheit *et al.*, 1985; Sanger, 1987). The receptor mediating this response is believed to be of the 5-HT_4 subtype (Eglen *et al.*, 1990). Although clearly identifiable, the response in the guinea-pig ileum is very small, making accurate analysis of its pharmacology difficult. The present paper identifies a similar response in the guinea-pig distal colon. Relative to the 5-HT_3 receptor-mediated contraction in either the ileum or colon, both responses are slow in onset and are well maintained, suggesting that they are mediated by a similar mechanism. The response in the colon, however, is significantly larger than that in the ileum, making it a better preparation in which to investigate the pharmacology of the 5-HT_4 receptor.

In the rat oesophagus (Baxter *et al.*, 1991) and human atrial (Kaumann *et al.*, 1990a,b) models of the 5-HT_4 receptor, cocaine caused a concentration-dependent shift to the left of the 5-HT concentration-effect curves. Since cocaine has been shown to block neuronal uptake mechanisms (Verbeuren *et al.*, 1983), such an observation is consistent with the idea that exogenously added 5-HT is being 'lost' through uptake systems. However, since cocaine, at a concentration ranging from 300 nM to 30 μM, had no significant effect on

the concentration-effect curves to 5-HT in the guinea-pig distal colon, it would appear that neuronal uptake mechanisms play no significant role in this preparation.

In certain isolated gastrointestinal smooth muscle preparations (e.g. rat stomach), contractions evoked by tryptamine and 5-MeOT were potentiated by inhibitors of the enzyme monoamine oxidase (MAO), whereas those produced by 5-HT were unaffected (Vane, 1959; Handschumacher & Vane, 1967). Such an observation is consistent with the idea that the lipophilic tryptamine and 5-MeOT molecules entered the cell and were metabolized, whereas the polar 5-HT molecule was excluded from the cell and hence from metabolism by the cytoplasmic MAO enzyme. In view of this observation, the irreversible monoamine oxidase inhibitor, pargyline, was investigated in the guinea-pig distal colon model. At a concentration of 100 μM, pargyline had no effect on the concentration-response curves to either 5-HT or the more lipophilic analogue, 5-MeOT, suggesting that a MAO barrier does not preclude the access of 5-HT or 5-MeOT to the 5-HT$_4$ receptors in this model. Since this is a stripped LMMP preparation, however, these results do not preclude the existence of such a barrier in another layer of the guinea-pig gastrointestinal tract. Indeed, histochemical studies in guinea-pig intestine have previously demonstrated strong MAO staining in the mucosa, but weak or negative activity in the muscle layers (Penttila, 1968). Thus, the lack of mucosa in the present preparation probably explains the lack of activity of pargyline.

Contractions evoked by 5-HT in the guinea-pig distal colon were prevented by both atropine and TTX. Such an observation suggests that, as is the case in the guinea-pig ileum (Buchheit *et al.*, 1985; Sanger & Nelson, 1989), the 5-HT$_4$ receptors in the colon are located on cholinergic neurones within the myenteric plexus. Thus, 5-HT$_4$ receptor-mediated contractions are evoked indirectly via acetylcholine release.

In comparison with other published 5-HT$_4$ receptor models (see Introduction for references), the guinea-pig distal colon is a very sensitive model to 5-HT. Thus, in the distal colon, 5-HT evokes contractions with a pEC_{50} of 9.2. This compares with published values of 7.4 (human atria), 8.2 (rat oesophagus), 7.5 (guinea-pig ascending colon), 7.5 (human adrenocortical tissue) and 8.4 (sheep pulmonary vein) in other isolated preparations. The exact mechanism underlying the high potency of 5-HT in the guinea-pig distal colon can only be speculated upon. It may be attributed to either a high density of 5-HT$_4$ receptors, or to good amplification capabilities of the tissue. Since the response to 5-HT in the guinea-pig distal colon is mediated indirectly via ACh release, a multi-step process would lend itself to a large degree of amplification. This contrasts with the less well coupled rat oesophagus model of the 5-HT$_4$ receptor, where responses are mediated directly via activation of a receptor located on the smooth muscle.

The rank order of potency of agonists has long been used as a means of characterizing receptors. For tryptamine analogues, the rank order of potency in the guinea-pig distal colon was; 5-HT > 5-MeOT > > 5-CT > tryptamine > 2-Me-5-HT. While the rank order is the same as that reported in both the guinea-pig ileum (Craig & Clarke, 1990) and the rat oesophagus (Baxter *et al.*, 1991), subtle differences do exist when results are compared with those from the other models. For example, the potency of 5-MeOT, relative to 5-HT appears to vary from one model to another. In the rat oesophagus, 5-HT and 5-MeOT were approximately equipotent (Baxter *et al.*, 1991). In the present study 5-HT was 3.4 times more potent than 5-MeOT. In the guinea-pig ileum (Craig & Clarke, 1990) and guinea-pig ascending colon (Elswood *et al.*, 1991) however, the potency ratios went up to 8 and 25 respectively. The reason for these discrepancies is unknown but may reflect varying attempts to block factors such as uptake and metabolism of the tryptamine analogues.

Substituted benzamides have been used as tools in the identification of 5-HT$_4$ receptor-mediated responses (Dumuis *et al.*, 1989; Craig & Clarke, 1990; Baxter *et al.*, 1991). Whereas in the colliculi neurones, renzapride, cisapride and zacopride have a greater intrinsic activity than 5-HT itself, in the guinea-pig ileum and rat oesophagus their intrinsic activities range from around 0.8 to 1.0. Metoclopramide, on the other hand, in all models tested to date, is a partial agonist (Dumuis *et al.*, 1989; Baxter *et al.*, 1991; Elswood *et al.*, 1991). In the guinea-pig distal colon, all substituted benzamides tested (including metoclopramide) were full agonists relative to 5-HT. The high intrinsic activity of metoclopramide in the present study may be attributed to either the effective coupling mechanism in this preparation or to a high receptor density. The rank order of potency of agonists for this group of compounds was: 5-HT > renzapride > cisapride > (S)-zacopride > (R)-zacopride > metoclopramide. Such a rank order of potency of agonists is consistent with that reported in the literature for 5-HT$_4$ receptor activation. In particular, it is significant that the 5-HT receptor in the guinea-pig distal colon discriminates between (R)- and (S)-zacopride, with the (S)-isomer being about 3 times more potent than the (R)-form. This finding is consistent with that in the guinea-pig ileum (Eglen *et al.*, 1990) and rat oesophagus (Baxter *et al.*, 1991).

The final set of agonists investigated in the guinea-pig distal colon were the benzimidazolone derivatives (Nicola *et al.*, 1990; Dumuis *et al.*, 1991). BIMU 1 and BIMU 8 had very similar potencies and were both partial agonists relative to 5-HT. At high concentrations (> 10 μM), BIMU 1 and BIMU 8 revealed bell-shaped concentration-response curves. The partial agonism seen with both of these compounds suggests that their high potency stems from their high affinity, rather than from a high intrinsic activity at the 5-HT$_4$ receptor. BIMU 1 and BIMU 8 have also been observed to be highly potent agonists in the mouse embryo colliculi neurones (Dumuis *et al.*, 1991), rat oesophagus (Baxter & Clarke, 1992), guinea-pig ileum (Rizzi *et al.*, 1992) and guinea-pig proximal colon (Grossman *et al.*, 1993). In rat oesophagus, both were partial agonists, whereas in the colliculi neurones and guinea-pig ileum, BIMU 8 displayed a higher intrinsic activity than 5-HT and 5-MeOT respectively. In the brain, at least, this high intrinsic activity observed with BIMU 8 may be due to an additional action in the CNS assay system (see Baxter & Clarke, 1992). Bearing in mind the apparent effective coupling or high receptor density in the guinea-pig distal colon model, it would have been expected that BIMU 1 and BIMU 8 would have behaved as full agonists. The fact that this was not the case is difficult to explain, but may again be due to an additional action of these compounds at high concentrations (e.g. anticholinergic activity), opposing the 5-HT$_4$ receptor-mediated contraction.

Comparing agonist potencies and intrinsic activities with those in the guinea-pig proximal colon (Elswood *et al.*, 1991), it is clear that differences are observed. While this may reflect regional differences in the receptor density or coupling mechanisms, an alternative explanation may be the size of the animals used. In the present study it was found that the sensitivity of the preparation to 5-HT was highly dependent on the age of the guinea-pigs. With very young animals, pEC_{50} values could be as low as 10.1, compared with 7.8 in very old animals. Similar findings have been reported by Buchheit *et al.* (1985) in experiments involving longitudinal muscle strips of guinea-pig ileum. In the Elswood study, larger (300–400 g) animals were used, perhaps reflecting the lower sensitivity of their preparations to 5-HT and other agonists.

From the agonist studies it can be concluded that the receptor mediating 5-HT-evoked contractions in the guinea-pig isolated distal colon is not significantly different from that in other 5-HT$_4$ receptor models. Further evidence for the existence of the 5-HT$_4$ receptor in the guinea-pig distal colon stems from studies with tropisetron and SDZ 205 557. In the present study tropisetron produced a rightward displacement

of the 5-HT concentration-effect curve, yielding an apparent pA_2 of 6.4 and a slope of the Schild plot significantly greater than unity. This value compares well with that reported in the literature for the colliculi neurones (6.0–6.3; Dumuis *et al.*, 1988a), rat oesophagus (6.7; Baxter *et al.*, 1991) and guinea-pig ascending colon (6.4; Elswood *et al.*, 1991). SDZ 205 557 has been identified as a selective competitive antagonist of the 5-HT_4 receptor-mediated contractions in the guinea-pig ileum, with a 30 fold lower affinity for the 5-HT_3 receptor (Buchheit *et al.*, 1991; 1992). In the present study, SDZ 205 557 acted as a potent competitive antagonist of 5-HT-evoked contractions in the guinea-pig distal colon, yielding a pA_2 of 7.8. Furthermore, when examined against other 5-HT_4 receptor agonists (both tryptamine analogues and benzamides), the pK_B values were found to be agonist-independent, ranging from 7.5 to 7.9. Although these values are slightly higher than those quoted in the literature (Buchheit *et al.*, 1991; 1992; Lorrain *et al.*, 1992) they are consistent with activity at the 5-HT_4 receptor.

In summary, the present results suggest that the guinea-pig distal colon contains neuronal receptors with a pharmacology similar to that of the 5-HT_4 receptor. Furthermore, this appears to be a highly sensitive 5-HT_4 receptor model and may be a useful tool in the investigation of 5-HT_4 receptor-mediated events.

We wish to thank Dr Carol Rizzi for his generous gift of BIMU 1 and BIMU 8.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonism. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BAXTER, G.S. & CLARKE, D.E. (1992). Benzimidazolone derivatives act as 5-HT_4 receptor ligands in rat oesophagus. *Eur. J. Pharmacol.*, **212**, 225–229.

BAXTER, G.S., CRAIG, D.A. & CLARKE, D.E. (1991). 5-Hydroxytryptamine$_4$ receptors mediate relaxation of the rat oesophagus tunica muscularis mucosae. *Naunyn-Schmied. Arch. Pharmacol.*, **343**, 439–446.

BOCKAERT, J., SEBBEN, M. & DUMUIS, A. (1990). Pharmacological characterisation of 5-hydroxytryptamine$_4$ (5-HT_4) receptors positively coupled to adenylate cyclase in adult guinea-pig hippocampal membranes: effect of substituted benzamide derivatives. *Mol. Pharmacol.*, **37**, 408–411.

BUCHHEIT, K.H., ENGEL, G., MUTSCHLER, E. & RICHARDSON, B. (1985). Study of the contractile effects of 5-hydroxytryptamine (5-HT) in the isolated longitudinal muscle strip from guinea-pig ileum. Evidence for two distinct release mechanisms. *Naunyn-Schmied. Arch. Pharmacol.*, **329**, 36–41.

BUCHHEIT, K.-H., GAMSE, R. & PFANNKUCHE, H.-J. (1991). SDZ 205 557, a selective antagonist at 5-HT_4 receptors in the isolated guinea-pig ileum. *Eur. J. Pharmacol.*, **200**, 373–374.

BUCHHEIT, K.-H., GAMSE, R. & PFANNKUCHE, H.-J. (1992). SDZ 205 557, a selective, surmountable antagonist for 5-HT_4 receptors in the isolated guinea-pig ileum. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 387–393.

COCKS, T.M. & ARNOLD, P.J. (1992). 5-Hydroxytryptamine (5-HT) mediates potent relaxation in the sheep isolated pulmonary vein via activation of 5-HT_4 receptors. *Br. J. Pharmacol.*, **107**, 591–596.

CRAIG, D.A. & CLARKE, D.E. (1990). Pharmacological characterisation of a neuronal receptor for 5-hydroxytryptamine in guinea-pig ileum with properties similar to the 5-hydroxytryptamine$_4$ receptor. *J. Pharmacol. Exp. Ther.*, **252**, 1378–1386.

DUMUIS, A., BOUHELAL, R., SEBBEN, M. & BOCKAERT, J. (1988a). A 5-HT receptor in the central nervous system, positively coupled with adenylate cyclase, is antagonised by ICS 205-930. *Eur. J. Pharmacol.*, **146**, 187–188.

DUMUIS, A., BOUHELAL, R., SEBBEN, M., CROY, R. & BOCKAERT, J. (1988b). A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol. Pharmacol.*, **34**, 880–887.

DUMUIS, A., GOZLAN, H., SEBBEN, M., ANSANAY, H., RIZZI, C.A., TURCONI, M., MONFERINI, E., GIRALDO, E., SCHIANTARELLI, P., LADINSKY, H. & BOCKAERT, J. (1992). Characterisation of a novel 5-HT_4 receptor antagonist of the azabicycloalkyl benzimidazolone class: DAU 6285. *Naunyn-Schmied Arch. Pharmacol.*, **345**, 264–269.

DUMUIS, A., SEBBEN, M. & BOCKAERT, J. (1989). The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT_4) positively coupled to adenylate cyclase in neurons. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 403–410.

DUMUIS, A., SEBBEN, M., MONFERINI, E., NICOLA, M., TURCONI, M., LADINSKY, H. & BOCKAERT, J. (1991). Azabicycloalkyl benzimidazolone derivatives as a novel class of potent agonists at the 5-HT_4 receptor positively coupled to adenylate cyclase in brain. *Naunyn-Schmied. Arch. Pharmacol.*, **343**, 245–249.

EGLEN, R.M., SWANK, S.R., WALSH, L.K.M. & WHITING, R.L. (1990). Characterisation of 5-HT_3 and the 'atypical' 5-HT receptors mediating guinea-pig ileal contractions *in vitro*. *Br. J. Pharmacol.*, **101**, 513–520.

ELSWOOD, C.J., BUNCE, K.T. & HUMPHREY, P.P.A. (1991). Identification of putative 5-HT_4 receptors in guinea-pig ascending colon. *Eur. J. Pharmacol.*, **196**, 149–155.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology*. Vol. 33, pp. 283–335. Berlin, Heidelberg, New York: Springer.

GROSSMAN, C.J., KILPATRICK, G.J. & BUNCE, K.T. (1993). Development of a radioligand binding assay for 5-HT_4 receptors in guinea-pig and rat brain. *Br. J. Pharmacol.*, **109**, 618–624.

HANDSCHUMACHER, R.E. & VANE, J.R. (1967). The relationship between the penetration of tryptamine and 5-hydroxytryptamine into smooth muscle and the associated contractions. *Br. J. Pharmacol.*, **29**, 105–118.

IDRES, S., DELARUE, C., LEFEBVRE, H. & VAUDRY, H. (1991). Benzamide derivatives provide evidence for the involvement of a 5-HT_4 receptor type in the mechanism of action of serotonin in frog adrenocortical cells. *Mol. Brain Res.*, **10**, 251–258.

KAUMANN, A.J., SANDERS, L., BROWN, A.M., MURRAY, K.J. & BROWN, M.J. (1990a). Human atrial 5-HT receptors: similarity to rodent neuronal 5-HT_4 receptors. *Br. J. Pharmacol.*, **100**, 319P.

KAUMANN, A.J., SANDERS, L., BROWN, A.M., MURRAY, K.J. & BROWN, M.J. (1990b). A 5-hydroxytryptamine receptor in human atrium. *Br. J. Pharmacol.*, **100**, 879–885.

LEFEBVRE, H., CONTESSE, V., DELARUE, C., FFEUILLOLEY, M., HERY, F., GRISE, P., RAYNAUD, G., VERHOFSTAD, A.A.J., WOLF, L.M. & VAUDRY, H. (1992). Serotonin-induced stimulation of cortisol secretion from human adrenocortical tissue is mediated through activation of a serotonin$_4$ receptor subtype. *Neuroscience*, **47**, 999–1007.

LORRAIN, J., GROSSET, A. & O'CONNOR, S.E. (1992). 5-HT_4 receptors, present in piglet atria and sensitive to SDZ 205 557, are absent in papillary muscle. *Eur. J. Pharmacol.*, **229**, 105–108.

NICOLA, M., TURCONI, M., SCHIAVONE, A., RIZZI, C.A., CESANA, R., MICHELETTI, R., MONFERINI, E. & LADINSKY, H. (1990). A new class of non benzamide 5-HT_3 antagonists and 5-HT_4 agonists. *XIth International Symposium on Medicinal Chemistry*, Jerusalem, Israel, Abstract P4.

PARKER, R.B. & WAUD, D.R. (1971). Pharmacological estimation of drug receptor dissociation constants. Statistical evaluation: I. Agonists. *J. Pharmacol. Exp. Ther.*, **177**, 1–12.

PENTTILA, A. (1968). Distribution and intensity of monoamine oxidase activity in the mammalian duodenum. *Acta Physiol. Scand.*, **73**, 121–127.

RIZZI, C.A., COCCINI, T., ONORI, L., MANZO, L. & TONINI, M. (1992). Benzimadazolone derivatives: a new class of 5-hydroxytryptamine$_4$ receptor agonists with prokinetic and acetylcholine releasing properties in the guinea-pig ileum. *J. Pharmacol. Exp. Ther.*, **261**, 412–419.

SANGER, G.J. (1987). Increased gut cholinergic activity and antagonism of 5-hydroxytryptamine M-receptors by BRL 24924: potential clinical importance of BRL 24924. *Br. J. Pharmacol.*, **91**, 77–87.

SANGER, G.J. & NELSON, D.R. (1989). Selective and functional 5-hydroxytryptamine$_3$ receptor antagonism by BRL 43694 (granisetron). *Eur. J. Pharmacol.*, **159**, 113–124.

VANE, J.R. (1959). The relative activities of some tryptamine analogues on the isolated rat stomach strip preparation. *Br. J. Pharmacol. Chemother.*, **14**, 87–98.

VERBEUREN, T.J., JORDAENS, F.H. & HERMAN, A.G. (1983). Accumulation and release of [^{3}H]-5-hydroxytryptamine in saphenous veins and cerebral arteries of the dog. *J. Pharmacol. Exp. Ther.*, **226**, 579–588.

VILLALON, C.M., DEN BOER, M.O., HEILIGERS, J.P.C. & SAXENA, P.R. (1990). Mediation of 5-hydroxytryptamine-induced tachycardia in the pig by the putative 5-HT$_4$ receptor. *Br. J. Pharmacol.*, **100**, 665–667.

WARDLE, K.A. & SANGER, G.J. (1992). Guinea-pig distal colon; a sensitive model of the putative 5-HT$_4$ receptor. *Br. J. Pharmacol.*, **105**, 279P.

(Received May 26, 1993
Revised August 23, 1993
Accepted August 24, 1993)

Br. J. Pharmacol. (1993), **110**, 1600–1606 © Macmillan Press Ltd, 1993

ZD1542, a potent thromboxane A_2 synthase inhibitor and receptor antagonist *in vitro*

R.P. Brownlie, N.J. Brownrigg, H.M. Butcher, R. Garcia, R. Jessup, V.J. Lee, S. Tunstall & [1]M.G. Wayne

Vascular Inflammatory and Musculoskeletal Research Department, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG

1 The thromboxane A_2 synthase (TXS) inhibitory activity and the thromboxane A_2 (TP)-receptor blocking action of ZD1542 (4(*Z*)-6-[2S,4S,5R)-2-[1-methyl-1-(2-nitro-4-tolyloxy)ethyl]-4-(3-pyridyl)-1,3-dioxan-5-yl]hex-4-enoic acid) has been evaluated *in vitro* on platelets and whole blood from a range of species including man. Antagonist activity has also been investigated in vascular and pulmonary smooth muscle preparations *in vitro*.

2 ZD1542 caused concentration-dependent inhibition of human platelet microsomal thromboxane B_2 (TXB_2) production *in vitro* ($IC_{50} = 0.016\ \mu M$); this inhibition was associated with an increase in prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ formation.

3 ZD1542 also inhibited collagen-stimulated TXS in human, rat and dog whole blood giving IC_{50} values of 0.018, 0.009 and 0.049 μM respectively. The drug did not modify platelet cyclo-oxygenase activity as inhibition of TXB_2 formation was associated with a concomitant increase in the levels of PGD_2, PGE_2 and $PGF_{2\alpha}$. ZD1542 had little if any effect against cultured human umbilical vein endothelial cell (HUVEC) cyclo-oxygenase ($IC_{50} > 100\ \mu M$) and prostacyclin (PGI_2) synthase ($IC_{50} = 18.0 \pm 8.6\ \mu M$).

4 ZD1542 caused concentration-dependent inhibition of U46619-induced aggregation responses of human, rat and dog platelets yielding apparent pA_2 values of 8.3, 8.5 and 9.1 respectively. The drug was selective as, at concentrations up to 100 μM, it did not modify 5-hydroxytryptamine (5-HT) or the primary phases of adenosine diphosphate (ADP) and adrenaline-induced aggregation. Furthermore, ZD1542 (100 μM) modified only weakly the platelet inhibitory effects of PGD_2, PGE_1 and PGI_2.

5 ZD1542 also caused concentration-dependent inhibition of U46619-mediated contractions of rat thoracic aorta, guinea-pig trachea and lung parenchyma preparations giving apparent pA_2 values of 8.6, 8.3 and 8.5 respectively. At concentrations approaching three orders of magnitude greater than those required to block U46619-mediated contractions, the drug did not affect the actions of non-prostanoid agonists or exhibit agonist activity in any of the smooth muscle preparations employed; neither did it interact at EP- or FP-receptors.

6 In conclusion, the present study demonstrates that ZD1542 is a drug that exhibits both potent, selective TXS inhibition and TXA_2 receptor antagonism.

Keywords: Thromboxane A_2; prostaglandin; TP-receptor antagonist; thromboxane synthase inhibitor; platelet

Introduction

Since the discovery of thromboxane A_2 (TXA_2, Hamberg *et al.*, 1975) and the subsequent characterization of its potent bronchoconstrictor, vasoconstrictor and platelet aggregatory activity, attempts have been made to develop agents that either inhibit its synthesis or antagonize its action. Early clinical experience with dazoxiben prompted the realisation that specific inhibition of TXS results in an attendant accumulation of prostaglandin (PG) endoperoxides (PGG_2, PGH_2) which themselves directly activate TP-receptors thereby significantly reducing the potential benefit gained from suppressing TXA_2 production. Consequently, TXS inhibitors have generally failed to realise the clinical benefit that was expected of this form of therapy (Fiddler & Lumley, 1990).

The development of TP-receptor antagonists continues to be an active area of research; however, to date there is insufficient clinical data available to enable a meaningful judgement to be made about the therapeutic value of this class of compound. It is widely expected that TXA_2 receptor antagonists will have greater clinical efficacy than TXS inhibitors, but the question remains whether such compounds can compete successfully with aspirin.

In theory, the combined administration of a TXS inhibitor with a TXA_2 antagonist should overcome the potential limitations of a single agent approach. Specific synthase inhibition would redirect arachidonic acid metabolism, increasing the formation of the endogenous platelet inhibitory prostanoids PGD_2 and PGI_2 while the receptor antagonist would ablate the TP-receptor stimulatory activity of the accumulated prostaglandin endoperoxides. The potent anti-platelet actions of PGD_2 and PGI_2 should render this approach more clinically effective than aspirin. Indeed, experimental data are accruing which clearly demonstrate synergy between TXA_2 receptor antagonism and TXA_2 synthase inhibition *in vitro* (Bertele & de Gaetano, 1982; Gresele *et al.*, 1991).

We now describe the *in vitro* pharmacological profile of ZD1542 (Figure 1), a compound that combines the properties of potent, selective TXA_2 synthase inhibition with specific TXA_2 receptor antagonism.

Methods

Human platelet microsomes

Preparation of human platelet microsomes and measurement of arachidonic acid (AA) metabolism was carried out using modifications of the procedures described by Howarth *et al.* (1982). Human venous blood was collected from an ante-

[1] Author for correspondence.

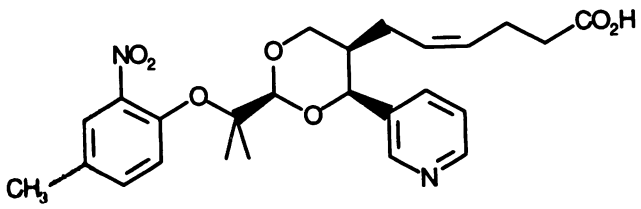


Figure 1 The chemical structure of ZD1542 (4(*Z*)-6-[2S,4S,5R)-2-[1-methyl-1-(2-nitro-4-tolyloxy)ethyl]-4-(3-pyridyl)-1,3-dioxan-5-yl]hex-4-enoic acid).

cubital vein of healthy volunteers who had been drug-free for at least 7 days, into 3.2% (w/v) trisodium citrate. Platelet rich plasma (PRP) was prepared by differential centrifugation (200 *g*, 15 min) at room temperature. Platelets were harvested by centrifugation of PRP (600 *g*, 10 min), resuspended in Tris/HCl (50 mM, pH 8.0) containing EDTA (1 mM), disrupted by three successive cycles of freeze-thawing and sonicated (4°C) at maximum energy in an ultrasonicator. A microsomal fraction was prepared by centrifugation of the disrupted platelet suspension (15,000 *g*, 20 min, 4°C) and centrifugation of the resultant supernatant (100,000 *g*, 100 min, 4°C). The microsomal pellet was resuspended in Tris/HCl (50 mM, pH 7.4) and stored in liquid nitrogen.

Measurement of AA metabolism was performed by quantitative radio chromatography. [1-^{14}C]-arachidonic acid (10 μM, 58.4 mCi mmol^{-1}) was incubated (30 min, 25°C) with human platelet microsomes (HPM) in Tris/HCl buffer (50 mM, pH 7.4) containing haematin (10 μM) and L-tryptophan (5 mM) in the presence of vehicle or compound (final volume 0.2 ml). Reactions were terminated by the addition of ethyl acetate/1 M citric acid (0.5 ml, 19:1 v/v) and the extracted radioactive metabolites separated by thin layer chromatography (t.l.c. 0.2 mm silica gel). Chromatographs were developed twice in a diethyl ether/acetic acid (9:1, v/v) solvent system and the resultant metabolites quantified in a Berthold t.l.c. linear analyser. R_F values were determined using authentic radiolabelled eicosanoids. The potency of compounds was expressed as an IC_{50} value for the inhibition of TXB_2 biosynthesis.

Human whole blood

TXA_2 formation in whole blood was assessed by measuring its metabolically stable degradation product, TXB_2 using a specific radioimmunoassay (RIA) (Bailey *et al.*, 1983). The selectivity of TXS inhibition was assessed by measurement of the immunoactivity of PGE_2, PGD_2 and $PGF_{2\alpha}$ generated in the presence of drugs. Aliquots of citrated human whole blood, obtained as described above, were incubated (2 h, 37°C) in the presence of equine collagen fibrils (100 μg ml^{-1}) in a shaking water bath after pretreatment (15 min, 37°C) with either vehicle or compound. Cellular debris was sedimented by centrifugation (25,000 *g*, 2 min) and indomethacin (1 mM) was added to the resultant cell-free plasma which was stored at − 20°C until subjected to specific RIA to determine the concentration of TXB_2, PGE_2 (Hawarth & Carey, 1986), $PGF_{2\alpha}$ (Sigma) and PGD_2 (Sigma) present in the sample. The potency of compounds was expressed as an IC_{50} value for the inhibition of TXB_2 biosynthesis.

Steroid 11β-hydroxylase

Rat adrenal mitochondria were prepared as described by Cross *et al.* (1985). Adrenal glands from Alderley Park strain male Wistar rats were homogenized in a tight-fitting Douce homogenizer in 33 volumes of medium (10 mM HEPES, 250 mM sucrose, 2 mM EGTA, pH 7.4) and centrifuged (900 *g*, 10 min, 4°C). The resultant supernatant was centrifuged (10,000 *g*, 15 min, 4°C) and the mitochondrial pellet resuspended in 10 volumes of ice cold buffer (10 mM HEPES, 250 mM sucrose, 1 mM $MgCl_2$, 5 mM KH_2PO_4, 4 mM sodium malate).

Drugs were pre-incubated (10 min, 37°C) in the presence of 4-[^{14}C]-11-deoxycorticosterone (50 μM, 0.1 μCi μM^{-1}); mitochondrial suspension (100 μl) was then added and the whole incubated (37°C, 10 min) in a shaking water bath. Incubations were terminated by the addition of CH_2Cl_2 (5 ml) and distilled water (0.5 ml) and the tubes rotated for 20 min. After centrifugation (1,000 *g*, 15 min) the upper aqueous layer was aspirated to waste, the organic layer was evaporated to dryness under N_2 following the removal of moisture by the addition of anhydrous Na_2SO_4. Extracts were resuspended in a minimal volume of CH_2Cl_2 and applied to a silica t.l.c. plate which was developed in a running solvent of $CHCl_3$/MeOH (97:3, v/v). The conversion of deoxycorticosterone to corticosterone was measured in a Berthold Linear t.l.c. Analyser and inhibitor potency expressed as an IC_{50}.

Human cultured umbilical vein endothelial cells

Human umbilical cords were obtained within 12 h of normal delivery and primary cultures of HUVECs were prepared according to the method described by Garcia *et al.* (1988). Cultured HUVECs (1st–4th passage) were seeded (5×10^4 cells/well) onto 24 well plates (16 mm, CoStar, Cambridge, MA, USA), maintained at 37°C in humidified air containing 5% CO_2 and allowed to grow to 70–80% confluence. Cell monolayers were washed twice with medium (Gibco 199), pre-incubated with vehicle or compound (37°C, 15 min), stimulated with either arachidonic acid (10 μM) or calcium ionophore (A23187, 10 μM) and incubated for a further 2 h. Incubations were terminated and cell-free medium was aspirated into indomethacin (1 mM); samples were stored at − 20°C until subjected to RIA for 6-oxo-$PGF_{1\alpha}$ (Forder & Carey, 1983).

Preparation of platelet rich plasma

Human venous blood was collected from an antecubital vein of healthy volunteers, who had been drug-free for at least 7 days, into 3.2% (w/v) trisodium citrate.

Male Alderley Park Wistar rats (250–350 g) were anaesthetized with fluothane and bled from the abdominal aorta into silicon-coated tubes containing heparin, 50 units ml^{-1}.

Alderley Park beagle dogs (17–25 kg) were bled from a jugular vein into 3.8% (w.v) trisodium citrate.

Anticoagulants were used in the ratio 1 part to 9 parts whole blood. PRP was prepared by centrifugation (15 min, 25°C) at 200 *g* for human and rat blood. To maximize the yield of dog PRP two separate centrifugations (15 min, 25°C) at 150 *g* were carried out.

Platelet aggregation studies

Aliquots (0.25 ml) of PRP in siliconized glass tubes were stirred (900 r.p.m.) and pre-incubated (37°C) for 60 s in a Payton aggregometer prior to addition of ZD1542 or vehicle. Platelets were incubated for a further 60 s before aggregation was initiated with one of a variety of agonists. Platelet aggregation was measured as an increase in light transmission, minimum and maximum light transmission having been set on a chart recorder (Kipp & Zonen) using PRP and platelet poor plasma, respectively. Each aggregation response was allowed to proceed to its maximum, the change in light transmission in arbitrary units at this point, was expressed as a percentage of the largest change achieved in the first control curve. A complete concentration-response curve was obtained to each agonist. Antagonist activity was calculated by determining concentration ratios from agonist EC_{50} values derived after a 60 s preincubation of PRP with either vehicle

or ZD1542. Where appropriate, antagonist activity was expressed in terms of 'apparent pA_2 values'.

In a separate series of experiments we assessed the effect of ZD1542 on the anti-aggregatory effects of PGE_1, PGI_2 and PGD_2. A single sub-maximal concentration of ADP (EC_{70}) was used to induce platelet aggregation. Concentrations of PGE_1, PGI_2 and PGD_2 which caused 50% inhibition of the aggregation induced by ADP were determined. Aliquots (0.25 ml) of PRP were then pre-incubated as previously described for 30 s prior to addition of vehicle or ZD1542 and incubated for a further 15 s before either PGE_1 (30 nM), PGI_2 (30 nM) or PGD_2 (20 nM) was added; aggregation was finally initiated with ADP (2 μM) 60 s later.

Isolated smooth muscle preparations

Thoracic aortae were obtained from male Wistar rats (Alderley Park strain, 200–350 g) which had been terminally anaesthetized with an excess of fluothane. After trimming away non-vascular tissue the vessels were spirally cut. Trachea, lung parenchyma strips and ileum were obtained from terminally anaesthetized male Dunkin-Hartley guinea-pigs (250–400 g). Tracheae were trimmed free of connective tissue and cut into rings. Five intact rings were tied together to form a tracheal chain (Jessup *et al.*, 1986). Eyes were obtained from female Alderley Park beagles (15–20 kg) after terminal overdose of Euthatal. The iris sphincter muscle was dissected and 1–2 cm strips used.

Smooth muscle preparations were suspended in 5 ml organ baths containing modified Krebs-Henseleit solution (composition mM: NaCl 118, glucose 11, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2 and $CaCl_2$ 2.5) to which indomethacin (10 μM) was added, maintained at 37°C and bubbled with a 95% O_2 plus 5% CO_2 mixture. Aorta and ileum preparations were subjected to an initial resting tension of 1 g and trachea, lung and iris to a tension of 0.5 g. Responses were recorded isotonically throughout.

Agonist concentration-response curves were constructed either cumulatively (rat aorta, guinea-pig trachea and lung, dog iris) or by single dose of increasing concentration with wash out between doses (guinea-pig ileum) in the presence of vehicle or ZD1542. The increase in tension developed by the tissue was expressed as a percentage of the maximum control response. Antagonist activity was calculated by determining concentration-ratios derived from the agonist EC_{50} values obtained in the absence and 30 min after the addition of ZD1542 to the bathing solution. Antagonist activity was expressed in terms of pA_2 values.

Drugs used

ZD1542, and Dazoxiben were synthesized by Zeneca Pharmaceuticals, were solubilized in dimethylsulphoxide (DMSO) at 100 mM and diluted directly into assay buffer to give less than 0.1% DMSO final concentration. All reagents used were analytical grade and obtained from Sigma as were calcium ionophore (A23187), indomethacin, aspirin, arachidonic acid, TXB_2, 6-oxo$PGF_{1\alpha}$, and prostaglandins D_2, E_2 and $F_{2\alpha}$. All radioligands were purchased from Amersham together with RIA kits for PGD_2 and $PGF_{2\alpha}$. U46619 (15S)-hydroxy 11α,9α-(epoxymethano)-prosta 5Z, 13E-dienoic acid) (Bundy, 1975) was obtained from Cayman Chemicals. Equine collagen fibrils were purchased from Hormon Chemie (Munchen, Germany).

Expression of results

It proved impractical to generate data suitable for Schild analysis on all smooth muscle preparations; concentration-ratios were derived by comparing EC_{50} values obtained in the presence and absence of ZD1542 and antagonist potency expressed as an apparent pA_2. Data are expressed as the arithmetic mean value ± s.e.mean from (n) experiments.

Results

Inhibition of human TXA_2 synthase

ZD1542 (0.001 μM–10 μM), when incubated with human platelet microsomes, caused concentration-dependent inhibition of TXB_2 formation giving an IC_{50} value of 0.016 ± 0.006 μM ($n = 4$). This effect was due to selective inhibition of TXA_2 synthase as ZD1542, at a final concentration of 10 μM did not cause significant inhibition of PGE_2 and $PGF_{2\alpha}$ formation. Indeed, the inhibition of TXB_2 synthesis by ZD1542 was associated with a concentration-dependent increase in $PGE_2/PGF_{2\alpha}$ levels. Indomethacin and dazoxiben proved to be potent inhibitors of platelet cyclo-oxygenase and TXA_2 synthase respectively, giving IC_{50} values of 0.17 ± 0.8 μM ($n = 4$) and 0.09 ± 0.01 μM ($n = 18$). Indomethacin caused equipotent inhibition of TXB_2, PGE_2 and $PGF_{2\alpha}$, whilst dazoxiben exhibited a profile identical to that observed with ZD1542 (Table 1).

In human, rat and dog whole blood, ZD1542 (0.001 μM–10 μM) caused concentration-dependent inhibition of collagen-stimulated thromboxane production, giving IC_{50} values of 0.018 ± 0.003 μM ($n = 8$), 0.009 ± 0.003 μM ($n = 4$) and 0.049 ± 0.01 μM ($n = 4$) respectively. In human blood, indomethacin and dazoxiben also caused concentration-dependent inhibition of collagen-stimulated TXB_2 formation giving IC_{50} values of 0.26 ± 0.015 μM ($n = 4$) and 1.07 ± 0.25 μM ($n = 28$) respectively.

Inhibition of platelet cyclo-oxygenase, as achieved with aspirin (10 μM) and indomethacin (1 μM), resulted in a reduction in plasma TXA_2, PGE_2, $PGF_{2\alpha}$ and PGD_2 levels. In contrast, incubation of human whole blood with a selective TXA_2 synthase inhibitor (ZD1542, 1 μM or dazoxiben, 10 μM) produced profound inhibition of TXA_2 production and a concomitant increase in levels of PGE_2, $PGF_{2\alpha}$ and PGD_2 (Figure 2). Thus, ZD1542 (1 μM) caused a reduction in TXA_2 synthesis (173.9 ± 27.6 to 5.0 ± 0.9 ng ml^{-1}; $n = 6$) which was associated with an increase in PGE_2 (5.6 ± 0.7 to 188.1 ± 19.3 ng ml^{-1}; $n = 6$), $PGF_{2\alpha}$ (2.8 ± 0.3 to 48.1 ± 2.5 ng ml^{-1}; $n = 6$) and PGD_2 levels (1.0 ± 0.2 to 16 ± 2.6 ng ml^{-1}; $n = 6$).

Selectivity

Deoxycorticosterone was readily hydrolyzed to corticosterone on incubation with rat adrenal mitochondria. ZD1542 (0.1–100 μM) did not modify the adrenal cytochrome P-450 enzyme steroid 11β-hydroxylase activity of the preparation

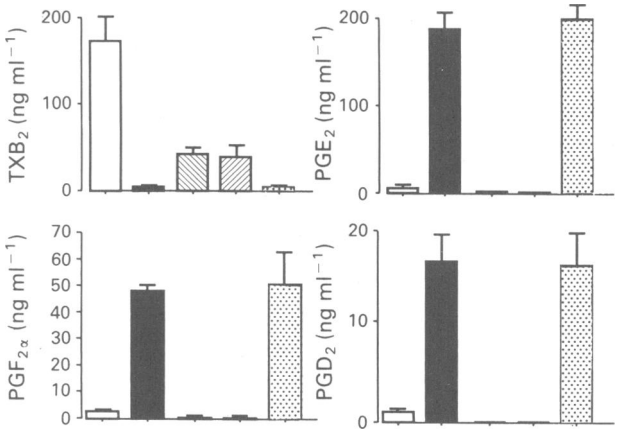


Figure 2 The effect of vehicle (□), ZD1542 1 μM (■), aspirin 10 μM (▨), indomethacin 1 μM (▨) and dazoxiben 10 μM (▨) on collagen (100 μg ml^{-1})-stimulated eicosanoid production in citrated human whole blood *in vitro*. Data are expressed as the mean (with s.e.mean) measurement from 6 individuals.

Table 1 The potency of ZD1542, dazoxiben and indomethacin upon [1-^{14}C]-arachidonic acid metabolism by human platelet microsomes

Concentration (μmol)	*ZD1542* (% inhibition)		*Dazoxiben* (% inhibition)		*Indomethacin* (% inhibition)	
	TXB_2	$PGE_2/PGF_{2\alpha}$	TXB_2	$PGE_2/PGF_{2\alpha}$	TXB_2	$PGE_2/PGF_{2\alpha}$
0.001	0.4 ± 0.4	−72.6 ± 25.4	2.5 ± 0.9	−5.3 ± 4.6		
0.01	46.0 ± 7.4	−370.3 ± 117.8	20.5 ± 2.6	−63.6 ± 11.2	17.6 ± 6.3	7.2 ± 2.0
0.1	72.7 ± 4.2	−507.0 ± 205.0	55.5 ± 1.7	−186.2 ± 14.1	44.0 ± 11.8	42.7 ± 4.1
1	74.3 ± 4.9	−497.8 ± 208.3	76.4 ± 1.1	−264.8 ± 19.9	78.2 ± 3.0	68.5 ± 5.7
10			81.3 ± 1.1	−278.1 ± 22.6	86.6 ± 4.5	83.1 ± 3.3

Data shown are the mean ± s.e.mean percentage inhibition of eicosanoid biosynthesis, measured as TXB_2, PGE_2 and $PGF_{2\alpha}$ by quantitative radio chromatography, in at least four individual preparations of HPM. Negative values represent percentage potentiation.

giving an IC_{50} value of $>100\,\mu M$ ($n=4$). Clotrimazole however, under identical circumstances gave an IC_{50} value of $0.34 \pm 0.04\,\mu M$ ($n=9$).

Human umbilical vein endothelial cell monolayers (1–4 passage) spontaneously produced PGI_2 (9 ± 2.2 pg/10^3 cells; $n=5$). In contrast, cells that were passaged more frequently (>10 times) produced predominantly PGE_2 (202 ± 22.6 pg/10^3 cells; $n=5$). These multi-passaged cells were used to evaluate the effect of ZD1542 on endothelial cell cyclooxygenase. Following the incubation of HUVECs (1–4 passage) with either vehicle, arachidonic acid or calcium ionophore, 9, 280 and 116 pg 6-oxo$PGF_{1\alpha}$ per 1,000 cells was detected in the surrounding medium. Pretreatment of HUVECs with indomethacin ($0.01\,\mu M - 10\,\mu M$) resulted in a concentration-dependent inhibition of spontaneous, arachidonic acid and calcium ionophore stimulated release of PGI_2 giving IC_{50} values of 0.05 ± 0.01, 0.08 ± 0.03 and $0.03 \pm 0.002\,\mu M$ ($n=4$) respectively.

Unlike indomethacin, ZD1542 did not potently inhibit spontaneous, arachidonic acid or calcium ionophore-stimulated release of PGI_2 from endothelial cells giving IC_{50} values of 18.0 ± 8.6, 20.5 ± 3.5 and $20.2 \pm 4.5\,\mu M$ ($n=4$) respectively. Furthermore ZD1542, at a final concentration of $100\,\mu M$, did not cause a statistically significant ($P>0.05$, Student's t test) inhibition of PGE_2 formation ($n=5$) from multi-passaged HUVECs.

Activity of ZD1542 on human platelets

In vitro ZD1542 ($0.005-1{,}000\,\mu M$) did not itself induce shape change or aggregation of human, rat or dog platelets.

ZD1542 ($0.005-0.05\,\mu M$) caused concentration-dependent parallel shifts to the right of U46619 aggregation curves in human PRP. Evaluation of the data gave an 'apparent pA_2 value' of 8.3 ± 0.05 ($n=4$). ZD1542, when added to human PRP *in vitro* at a concentration greater than $0.05\,\mu M$, caused insurmountable blockade of U46619 responses (Figure 3). The compound also blocked U46619-induced aggregation of rat and dog platelets with 'apparent pA_2 values' of 8.5 ± 0.05 and 9.1 ± 0.1 ($n=4$) respectively.

ZD1542 ($0.1 \pm 10\,\mu M$) caused concentration-dependent inhibition of collagen-induced aggregation of human platelets. The drug ($0.1-10\,\mu M$) also inhibited the secondary, but not the primary phase of adrenaline and ADP aggregation. ZD1542 ($100\,\mu M$) did not inhibit 5-hydroxytryptamine (5-HT)-induced aggregation of human platelets (Table 2).

PGI_2 (30 nM), PGE_1 (30 nM), and PGD_2 (20 nM) caused approximately 50% inhibition of human platelet aggregation induced by a single sub-maximal concentration of ADP (EC_{70}; $2\,\mu M$). ZD1542 ($100\,\mu M$) did not profoundly ($P>0.05$, Student's t test, paired data) affect PGE_1, PGI_2 or PGD_2 inhibition of the primary phase of ADP-induced human platelet aggregation giving mean inhibitions of $1.3 \pm 2.0\%$ and $8.9 \pm 7.2\%$; $n>=4$) for PGE_1 and PGD_2 respectively. ZD1542 ($100\,\mu M$) did cause minimal, but statistically significant ($P<0.05$, Student's t test, paired data) inhibition of PGI_2-mediated inhibition of ADP-induced aggregation ($14.7 \pm 1.7\%$; $n=4$).

Vascular smooth muscle pharmacology

ZD1542 (1 mM) did not modify the resting tone of any of the vascular smooth muscle preparations used.

ZD1542 caused concentration-dependent parallel shifts to the right of U46619 response curves on rat thoracic aorta preparations. Analysis (Arunlakshana & Schild, 1959) of the data gave a pA_2 value of 8.6 ± 0.2 ($n=24$) with a Schild plot slope of 1.03 which was not significantly different from unity.

The compound was selective; it did not inhibit those contractions of vascular smooth muscle induced by non-prostanoid agonists. Thus, on rat thoracic aorta when tested at a concentration of $10\,\mu M$ the compound yielded an 'apparent pA_2 value' of <5.0 against both noradrenaline ($n=4$) and 5-HT ($n=4$)-induced contractions.

Pulmonary smooth muscle

ZD1542 did not affect the resting tone of guinea pig pulmonary smooth muscle preparations at a concentration of $10\,\mu M$.

The compound caused concentration-dependent parallel shifts to the right of U46619 response curves on both guinea-pig tracheal chain and lung parenchyma preparations.

This potent antagonist activity was reflected in 'apparent pA_2 values' of 8.3 ± 0.07 ($n=8$) and 8.5 ± 0.1 ($n=4$) respectively.

ZD1542 ($10\,\mu M$) did not cause significant inhibition of histamine-induced contractions of guinea-pig trachea or lung parenchyma nor did it inhibit PGE_2-induced relaxation of histamine ($10\,\mu M$)-precontracted tracheal preparations giving 'apparent pA_2 values' of 5.1 ± 0.1 ($n=4$), <5.0 ($n=4$) and <5.0 ($n=4$), respectively.

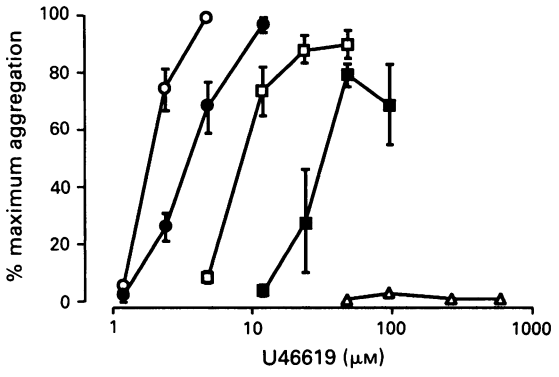


Figure 3 The effect of vehicle (○) and ZD1542; $0.005\,\mu M$ (●), $0.025\,\mu M$ (□), $0.05\,\mu M$ (■) and $0.1\,\mu M$ (△) on U46619-induced human platelet aggregation *in vitro*. Curves are the mean ± s.e.mean from 4 experiments.

Table 2 The potency and selectivity of ZD1542 at human platelet receptor

Agonist	*EC_{50}* (mean ± s.e.)	Antagonist concentration (μM)	*Concentration ratio* (mean ± s.e.)	n
U46619	0.91 ± 0.06 μM	0.005	1.79 ± 0.2	4
		0.025	4.64 ± 0.47	4
		0.05	15.0 ± 2.25	4
		0.01	>250	4
Collagen	0.35 ± 0.13 μg ml^{-1}	0.1	3.40 ± 1.3	4
		1.0	16.0 ± 4.8	4
		10	25.8 ± 10.3	4
ADP (primary phase)	1.2 ± 0.2 μM	10	1.25 ± 0.07^{NS}	4
Adrenaline (primary phase)	1.1 ± 0.3 μM	10	1.25 ± 0.16^{NS}	6
5-HT	2.1 ± 0.5 μM	100	1.37 ± 0.04^{NS}	4

Concentration ratios are the mean ± s.e.mean from at least four experiments and were determined by comparing agonist EC_{50} values in the presence of vehicle or ZD1542.
^{NS}Not statistically significant ($P<0.05$, Student's t test).

ZD1542 (10 μM) did not alter the resting tone of guinea-pig ileum preparations nor did it significantly modify contractions induced by PGE_2, acetylcholine, or histamine (Table 3).

ZD1542 (10 μM) did not exhibit intrinsic agonist activity at dog iris PGF receptors. Furthermore, ZD1542 (10 μM) did not potently modify $PGF_{2\alpha}$-induced contractions of dog iris preparations giving an 'apparent pA_2 value' of 5.0 ± 0.2 ($n = 4$).

Discussion

The present study has shown that ZD1542 exhibits the pharmacological profile and biochemical properties of a potent specific, TP-receptor antagonist and selective TXA_2 synthase inhibitor. The two activities of the compound are expressed over the same concentration range *in vitro* in the blood of several species; the IC_{50} for TXA_2 synthase inhibition and TXA_2 receptor antagonism in human platelets being 18 nM and 5 nM respectively. This close association of the two activities is in contrast to the profile of other TXA_2 synthase inhibitor/antagonists such as R 68 070 (De Clerk *et al.*, 1989) in which the potency, in comparbale systems, of TXA_2 synthase inhibition (IC_{50}, 15 nM) is two orders of magnitude greater than its receptor antagonist activity (IC_{50}, 2,000 nM); this represents a limitation with this compound. It has been reported (Hoet *et al.*, 1990) that R 68 070 is less effective than aspirin in inhibiting the cyclo-oxygenase dependent component of collagen-induced platelet aggregation *in vitro*. The authors claim that this observation is a function of the weak, competitive nature of the TXA_2 receptor antagonism exhibited by R 68 070.

The activity of ZD1542 as a specific TXS inhibitor is exemplified by data generated in several experiments. The drug causes concentration-related reduction in TXB_2 formation from human platelet microsomes incubated with arachidonic acid; a concomitant increase in the production of PGE_2 and $PGF_{2\alpha}$ is also observed indicating that the compound causes redirection of endoperoxide metabolism and does not inhibit human platelet cyclo-oxygenase. When incubated with human whole blood, ZD1542 reduces plasma levels of TXB_2 and causes an increase in the quantities of immunoreactive PGD_2, PGE_2 and $PGF_{2\alpha}$. Inhibition of plasma TXB_2 can also be achieved by blockade of cyclo-oxygenase using aspirin or indomethacin; however, in these instances, a parallel inhibition of PGD_2, PGE_2 and $PGF_{2\alpha}$ is observed. In these respects, the profile of ZD1542 is similar to that of other specific TXA_2 synthase inhibitors (Uotila & Matintalo, 1984; Ambler *et al.*, 1985; Terashita *et al.*, 1986).

Primary cultures of human umbilical vein endothelial cells spontaneously synthesize eicosanoids, the predominant product being PGI_2. When HUVECs are subjected to repeated passage the profile of eicosanoids secreted progressively alters; production of PGI_2 decreases whilst PGE_2 synthesis

Table 3 The potency and selectivity of ZD1542 on isolated smooth muscle preparations

Preparation	*Agonist*	*Agonist EC_{50}* (μM, mean ± s.e.mean)	*Apparent pA_2* (mean ± s.e.)	n
Rat thoracic aorta	U-46619	0.11 ± 0.02	8.51 ± 0.15	24
	5-HT	1.6 ± 0.2	<5.0*	4
	Noradrenaline	0.04 ± 0.01	<5.0*	8
Guinea-pig trachea	U-46619	0.3 ± 0.05	8.3 ± 0.07	8
	PGE_2	0.2 ± 0.03	<5.0*	4
	Histamine	0.3 ± 0.05	5.1 ± 0.1	4
Guinea-pig lung parechyma	U-46619	0.7 ± 0.1	8.5 ± 0.1	4
	Histamine	1.9 ± 0.2	<5.0*	4
Guinea-pig ileum	PGE_2	0.05 ± 0.03	5.8 ± 0.1	4
	Histamine	0.14 ± 0.02	<5.0*	8
	5-HT	0.2 ± 0.02	6.5 ± 0.1	8
	Acetylcholine	0.06 ± 0.01	<5.0*	4
Dog iris	$PGF_{2\alpha}$	0.08 ± 0.02	5.0 ± 0.2	4

Apparent pA_2 values are the mean ± s.e.mean from at least four experiments and were determined by comparing the agonist EC_{50} values in the presence of vehicle or ZD1542.
*Concentration-ratio <2 in the presence of ZD1542 (10 μM).

increases. ZD1542 causes inhibition of HUVEC PGI_2 synthase; however this activity is only expressed at a concentration (IC_{50} 18 μM) that is three orders of magnitude greater than that required for TXS inhibition. In multi-passaged HUVECs, ZD1542 (100 μM) does not inhibit PGE_2 biosynthesis suggesting that the weak inhibitory activity observed with the drug in the early passaged cells is directed specifically against PGI_2 synthase and not cyclo-oxygenase. In both primary and multi-passaged cultured HUVECs, indomethacin (IC_{50} 0.08 μM) caused potent dose-related reduction of AA-induced PGI_2 and PGE_2 biosynthesis.

TXS is a cytochrome P-450, haeme-containing enzyme (Ullrich & Haurand, 1983). Certain classes of TXS inhibitor have been shown to lack selectivity against cytochrome P450 enzymes in particular those present in the liver and adrenals (Cross *et al.*, 1985). To demonstrate the selectivity of ZD1542 the compound was assessed against a variety of enzymes including the cytochrome P-450 enzyme, steroid 11β-hydroxylase. ZD1542, at a final concentration of 100 μM, was without effect in a series of isolated enzyme systems including; 5-lipoxygenase, 11β-hydroxylase, prolyl hydroxylase and aldose reductase (data not shown).

In addition to its selective TXA_2 synthase inhibitory properties, ZD1542 is a selective antagonist at TP-receptors causing blockade of U46619 response in rat, dog and human platelets, rat vasculature and bronchial smooth muscle from the guinea-pig. Adrenaline and ADP-induced platelet aggregation is characterized by an initial phase known to be unaffected by inhibitors of cyclo-oxygenase and TXA_2 synthase, and TXA_2 receptor antagonists. The secondary, irreversible phase of adrenaline and ADP aggregation is, however, associated with, and dependent on, cyclo-oxygenase product release. Similarly, cyclo-oxygenase products of arachidonic acid metabolism are known to contribute to collagen-induced human platelet aggregation responses *in vitro*. ZD1542 blocks the cyclo-oxygenase-dependent component of collagen, arachidonic acid, ADP and adrenaline-induced platelet aggregation but does not affect 5-HT-evoked responses or the primary, cyclo-oxygenase-independent phase of ADP or adrenaline. PGI_2, PGE_1 and PGD_2 are potent antiplatelet agents *in vitro* which exert their activity by receptor-mediated stimulation of adenylate cyclase, resulting in elevation of intraplatelet adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels. ZD1542 does not modify the antiplatelet activity of these prostanoids at concentrations approaching three orders of magnitude greater than those required to produce a threshold blockade of the TP-receptor.

In isolated vascular and respiratory smooth muscle preparations, ZD1542 caused selective concentration-dependent inhibition of all TP-receptor-mediated responses. *In vitro*, guinea-pig ileum and dog iris preparations have been shown to be preferentially sensitive to PGE_2 and $PGF_{2\alpha}$ respectively and as such have been designated as EP and FP-receptor models (Kennedy *et al.*, 1982). In contrast to its potent TP-receptor blocking activity, ZD1542 was without effect at both EP and FP-receptors.

Aspirin was one of the first effective platelet inhibitors to become widely available and today it is the most extensively used and clinically successful antiplatelet drug. By inhibiting cyclo-oxygenase, aspirin blocks the formation of not only those eicosanoids that activate platelet TP-receptors such as PGG_2, PGH_2 and TXA_2, but also those which inhibit platelet activation namely PGD_2 and PGI_2. Attempts have been made to identify a dose of aspirin which would spare endothelial cell PGI_2 synthesis (Patrono, 1989; Chiabrando *et al.*, 1992). It has been calculated that 95% inhibition of platelet TXA_2 synthase is required to maintain an optimal antithrombotic effect (Reilly & Fitzgerald, 1987). It has proved impossible to achieve this degree of TXA_2 synthase inhibition with aspirin without compromising vascular PGI_2 synthesis. Chiabrando *et al.* (1992) administered aspirin (30 mg day^{-1}) to healthy subjects for eight days and reported an 80% reduction of urinary of 2,3-dinor TXB_2 which was associated with a 45% reduction in 2,3-dinor-6-oxo–$PGF_{1\alpha}$ levels. This study demonstrates that even with sub-optimal doses of aspirin, PGI_2 biosynthesis is compromised. Moreover, if one subscribes to the hypothesis that platelet derived endoperoxides can be utilized by the vessel wall to synthesize PGI_2, then even a vessel wall-sparing low-dose aspirin regimen would decrease PGI_2 production at sites of increased platelet-vessel wall interaction (Kyrle *et al.*, 1987).

There are several pharmacological approaches to the manipulation of AA metabolism which have the potential to overcome the problems associated with aspirin whilst maintaining the beneficial effects attributed to reduction of TXA_2 biosynthesis. These include TXS inhibition, TXA_2 receptor antagonism and a combination of these two activities. TXA_2 is the predominant metabolite of PGH_2 in activated platelets; inhibition of TXS indirectly increases the local formation of other platelet-derived prostaglandins such as PGE_2, $PGF_{2\alpha}$ and PGD_2. Moreover, TXS inhibitors, when administered to normal human subjects increase PGI_2 formation 2–3 fold (Reilly *et al.*, 1986). PGI_2 and PGD_2 both inhibit platelet function by stimulating adenylate cyclase. However, the attendant accumulation of PG endoperoxides that results from TXS inhibition activates TP-receptors which suppresses the activity of adenylate cyclase thereby negating the anti-aggregatory potential of PGI_2 and PGD_2. PGH_2 is a highly effective TP-receptor agonist and this action is in part thought to be responsible for the disappointing efficacy of TXS inhibitors in early clinical trials (Fiddler & Lumley, 1990).

TXA_2 receptor antagonists block the action of PGG_2, PGH_2 and TXA_2 at TP-receptors on platelets and vasculature but do not modify the spectrum of arachidonic acid metabolites. In preliminary clinical trials, TP-receptor antagonists have yielded promising results (Fiddler & Lumley, 1990); however, these drugs preserve rather than increase the endogenous production of inhibitory prostaglandins and hence only have a limited potential to exhibit greater efficacy than that observed with aspirin (Lumley *et al.*, 1991).

In theory, co-administration of an inhibitor of TXS with a TXA_2 receptor antagonist should overcome the limitations of either agent alone. The TXS inhibitor would redirect AA metabolism thus enhancing the levels of the anti-aggregatory prostanoids PGD_2 and PGI_2, while the TXA_2 antagonist would block the activity of PGH_2 and TXA_2 at the TP-receptor thereby unmasking adenylate cyclase-mediated platelet inhibition. Experimental data *in vitro* support this hypothesis; increased cyclic AMP levels are observed in AA-stimulated human platelets pretreated with a TXS inhibitor/antagonist combination; this increase does not occur in quiescent platelets or in platelets pretreated with either agent alone (Gresele *et al.*, 1987). The synergistic effects of the combination of these two classes of drug can be readily abolished by the simultaneous administration of a cyclo-oxygenase inhibitor. There is an increasing body of evidence that *in vivo* the combination of these two classes of drug exhibits greater therapeutic efficacy than either agent alone. These data have been generated in a wide range of animal models including platelet-mediated thromboembolism in mouse lung (Gresele *et al.*, 1990), canine coronary thrombus formation (Bush & Smith, 1987), thrombin-induced sudden death in the rabbit (Zimmerman & Olsen, 1987) and AA-induced bronchoconstriction in the cat (Underwood *et al.*, 1987). These results appear to validate the rationale for combining TXS inhibitors with TXA_2 receptor antagonists. However, the co-administration of two drugs with potentially different kinetic profiles is clearly impractical, especially for long term prophylaxis. Therefore, an agent such as ZD1542 which offers both these activities at similar potencies has the potential of becoming a most useful therapeutic agent.

References

AMBLER, J., BUTLER, K.D., KU, E.C., MAGUIRE, E.D., SMITH, J.R. & WALLIS, R.B. (1985). CGS 12970: a novel, long acting thromboxane synthetase inhibitor. *Br. J. Pharmacol.*, **86**, 497–504.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BAILEY, I.A., CAREY, F., HAWORTH, D. & SMITH, H.J. (1983). Effects of vascular trauma and transient myocardial ischaemia on coronary venous prostaglandin levels in the dog. *Cardiovasc. Res.*, **17**, 127–131.

BERTELE, V. & DE GAETANO, G. (1982). Potentiation by dazoxiben, a thromboxane synthetase inhibitor, of platelet aggregation inhibitory activity of a thromboxane antagonist and of prostacyclin. *Eur. J. Pharmacol.*, **85**, 331–333.

BUNDY, G.L. (1975). The synthesis of prostaglandin endoperoxide analogues. *Tetrahedron Lett.*, **24**, 1975–1960.

BUSH, L.P. & SMITH, S.G. (1987). Comparative and combined effects of U63557A, a TXA_2 synthetase inhibitor and BM13.177 a TXA_2 receptor antagonist, on cyclic flow reductions in stenosed canine coronary arteries. *Fed. Proc.*, **46**, 1316 (abstr.).

CHIABRANDO, C., RIVOLTELLA, L., MARTELLI, L., VALZACCHI, S. & FANELLI, R. (1992). Urinary excretion of thromboxane and prostacyclin metabolites during chronic low dose aspirin: evidence for an extrarenal origin of urinary thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ in healthy subjects. *Biochim. Biophys. Acta*, **1133**, 247–254.

CROSS, P.E., DICKENSON, R.P., PARRY, J.M. & RANDALL, M.J. (1985). Selective thromboxane synthetase inhibitors. 1. 1-[(Aryloxy)alkyl]-1H-imidazoles. *J. Med. Chem.*, **28**, 1427–1432.

DE CLERK, F., BEETENS, J., DE CHAFFOY DE COURCELLES, D., FREYNE, E. & JANSSEN, P.A.J. (1989). R 68 070: thromboxane A_2 synthetase inhibition and thromboxane A2/prostaglandin endoperoxide receptor blockade combined in one molecule–1. Biochemical profile in vitro. *Thromb. Haemostatis.*, **61**, 35–42.

FIDDLER, G.I. & LUMLEY, P. (1990). Preliminary clinical studies with thromboxane synthase inhibitors and thromboxane receptor blockers. A review. *Circulation*, **81**, Suppl. I, 69–78.

FORDER, R.A. & CAREY, F. (1983). Measurement of human venous plasma prostacyclin and metabolites by radioimmunoassay: a reappraisal. *Prostaglandins Leukot. Med.*, **12**, 313–346.

GARCIA, J.G.N., AZGHANI, A., CALLAHAN, K.S. & JOHNSON, A.R. (1988). Effect of platelet activating factor on leukocyte-endothelial cell interactions. *Thromb. Res.*, **51**, 83–96.

GRESELE, P., ARNOUT, J., DECKMYN, H., HUYBRECHTS, E., PIETERS, G. & VERMYLEN, J. (1987). Role of proaggregatory and antiaggregatory prostaglandins in hemostatis studies with combined thromboxane synthase inhibition and thromboxane receptor antagonism. *J. Clin. Invest.*, **80**, 1435–1445.

GRESELE, P., CORONA, C., ALBERTI, P. & NENCI, G.G. (1990). Picotamide protects mice from death in a pulmonary embolism model by a mechanism independent from thromboxane suppression. *Thromb. Haemostasis*, **64**, 80–86.

GRESELE, P., DECKMYN, H., NENCI, G.G. & VERMYLEN, J. (1991). Thromboxane synthase inhibitors, thromboxane antagonists and duel blockers in thrombotic disorders. *Trends Pharmacol. Sci.*, **12**, 158–163.

HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2994–2998.

HAWORTH, D. & CAREY, F. (1986). Thromboxane synthase inhibition: implications for endoperoxide metabolism. *Prostaglandins*, **31**, 33–45.

HAWORTH, D., FISHER, R.W. & CAREY, F. (1982). Inverse coupling of lipoxygenase and cyclo-oxygenase in human microsomal fractions. *Biochem. Soc. Trans.*, **10**, 239–240.

HOET, B., FALCON, C., DE REYS, S., ARNOUT, J., DECKMYN, H. & VERMYLEN, J. (1990). R68070, a combined thromboxane/endoperoxide receptor antagonist and thromboxane synthase inhibitor, inhibits human platelet activation in vitro and in vivo: a comparison with aspirin. *Blood*, **75**, 646–653.

JESSUP, C.L., JESSUP, R. & WAYNE, M. (1986). Pharmacological action of ICI 180080, a novel thromboxane receptor antagonist. *J. Pharm. Pharmacol.*, **38**, 754–757.

KENNEDY, I., COLEMAN, R.A., HUMPHREY, P.P.A., LEVY, G.P. & LUMLEY, P. (1982). Studies on the characterisation of prostanoid receptors: a proposed classification. *Prostaglandins*, **24**, 667–689.

KYRLE, P.A., EICHLER, H.G., JAGER, U. & LECHNER, K. (1987). Inhibition of prostacyclin and thromboxane A_2 generation by low-dose aspirin at the site of plug formation in man *in vivo*. *Circulation*, **75**, 1025–1029.

LUMLEY, P., KEERY, R.J., WHARTON, K.A., WHITE, B.P. & WATTS, I.S. (1991). Maximal blockade of TXA_2 without compromised PGI_2 formation; a superior profile of the TXA_2 receptor blocking drug GR32191 over aspirin. In *Advances in Prostaglandin, Thromboxane and Leukotriene Research.* ed. Samuelsson, B., Dahlén, S., Fritsch, J. & Hedqvist, P. Vol. 21, pp. 603–606. New York: Raven Press.

PATRONO, C. (1989). Aspirin and human platelets from clinical trials to acetylation of cyclooxygenase and back. *Trends Pharmacol. Sci.*, **10**, 453–458.

REILLY, I.A.G., DORAN, J., SMITH, B. & FITZGERALD, G.A. (1986). Increased thromboxane biosynthesis in a human preparation of platelet activation: biochemical and functional consequences of selective inhibition of thromboxane synthase. *Circulation*, **73**, 1300–1306.

REILLY, I.A.G. & FITZGERALD, G.A. (1987). Inhibition of thromboxane formation in vivo and ex vivo implications for therapy with platelet inhibitory drugs. *Blood*, **69**, 180–186.

TERASHITA, Z., IMURA, Y., TANABE, M., KAWAZOE, K., NISHIKAWA, K., KATO, K. & TERAO, S. (1986). CV-4151 – a potent, selective thromboxane A_2 synthetase inhibitor. *Thromb. Res.*, **41**, 223–237.

UNDERWOOD, D.C., MCNAMARA, D.B., KNOOP, S.M., HYMAN, A.L. & KADOWITZ, P.J. (1987). Differential effects of thromboxane synthesis and receptor blockade on thromboxane formation and activity in the cat airway. *Fed. Proc.*, **46**, 691 (abstr.)

ULLRICH, V. & HAURAND, M. (1983). Thromboxane synthase as a cytochrome P450 enzyme. In *Advances in Prostaglandin, Thromboxane and Leukotriene Research.* ed. Samuelsson, B., Paoletti, R. & Ramwell, P.W. Vol. 11, pp. 105–110. New York: Raven Press.

UOTILA, P. & MATINTALO, M. (1984). Inhibition of thromboxane synthetase by OKY-1581 stimulates the formation of PGE_2, $PGF_{2\alpha}$, PGD_2 and 6-keto-$PGF_{1\alpha}$ in human platelets. *Prostaglandins Leukot. Med.*, **14**, 41–46.

ZIMMERMAN, M.B. & OLSEN, R.W. (1987). Synergism between a thromboxane receptor antagonist and thromboxane synthetase inhibitors, but not phosphodiesterase inhibitors, in a model of thrombin-induced sudden death. *Cardiovasc. Drugs Ther.*, **1**, 306 (abstr.)

(Received July 29, 1993
Revised August 20, 1993
Accepted August 25, 1993)

Br. J. Pharmacol. (1993), **110**, 1607–1613 © Macmillan Press Ltd, 1993

Antinociceptive activity of NK_1 receptor antagonists: non-specific effects of racemic RP67580

[1]N.M.J. Rupniak, S. Boyce, A.R. Williams, G. Cook, J. Longmore, G.R. Seabrook, M. Caeser, S.D. Iversen & R.G. Hill

Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR

1 Release of substance P in the dorsal horn is considered a primary event in the perception of pain. The profile of racemic RP67580, a non-peptide antagonist at the NK_1 (substance P) receptor, was examined in a range of antinociception tests on rodents.

2 At doses up to 30 mg kg^{-1}, s.c. racemic RP67580 exhibited antinociceptive activity in writhing and formalin paw tests in mice and gerbils. Acetic acid induced writhing and the licking response to formalin were reduced to 40–50% of the level observed in vehicle-treated animals ($P<0.05$). However, this agent was not active in mouse tail flick, rat paw pressure or rat and guinea-pig formalin paw tests.

3 Like racemic RP67580, the calcium channel blockers nifedipine (30 mg kg^{-1}, i.p.) and verapamil (10 or 20 mg kg^{-1}, s.c.) inhibited the response to formalin by approximately 60% in gerbils ($P<0.05$ compared with vehicle-treated animals).

4 Evidence for calcium channel antagonist activity of RP67580 was obtained *in vitro*. Racemic RP67580 inhibited calcium entry into depolarized strips of guinea-pig ileum longitudinal muscle myenteric plexus (apparent $K_B = 587 \pm 115$ nM), inhibited [^{3}H]-diltiazem binding to rabbit skeletal membranes ($IC_{50} = 298$ nM) and depressed high threshold calcium currents in neurones cultured from rat cortex (10% inhibition at 10 μM).

5 These findings indicate that the acute antinociceptive effects of RP67580 may not be attributable to a specific interaction with NK_1 receptors and may be mediated via calcium channel blockade.

Keywords: Antinociception; substance P; antinociceptive activity in rat, mouse, gerbil, guinea-pig; calcium channel

Introduction

Substance P has been suggested as a primary initiator of nociception. Release of this peptide in the dorsal horn of the spinal cord occurs in response to intense or prolonged noxious thermal, mechanical or chemical stimulation (Duggan *et al.*, 1987; McCarson & Goldstein, 1991) and intrathecal application of substance P causes hyperalgesia to thermal stimulation (Yashpal *et al.*, 1982). Conversely, chronic treatment of rats with capsaicin, which depletes tachykinins in the primary afferent terminals in the spinal cord, decreases sensitivity to noxious thermal or chemical stimuli applied to the paws (Hayes & Tyers, 1980), and in some laboratories intrathecal administration of peptide substance P antagonists blocks nociception in tail flick and hot plate tests in mice (Vaught & Scott, 1987). However, the antinociceptive properties of peptide tachykinin receptor antagonists are often nonspecific and accompanied by motor impairment (Rodriguez *et al.*, 1983). The recent development of nonpeptide NK_1 receptor antagonists such as CP-96,345 has provided new research tools with which to investigate the role of tachykinins in nociception.

The observations described above suggest that conventional antinociception tests should be appropriate to demonstrate analgesic effects of such compounds. Consistent with the proposal that selective NK_1 antagonists may represent a novel class of analgesics, CP-96,345 was active in the rat formalin paw test (Yamamoto & Yaksh, 1991; Birch *et al.*, 1992) and mouse acetic acid-induced writhing (Nagahisa *et al.*, 1992b) and hot plate tests (Lecci *et al.*, 1991). However, whilst rats and mice are usually employed for such screens, they are not the most appropriate species for evaluation of CP-96,345 which binds with 70 fold higher affinity to the NK_1 receptor present in human, gerbil and guinea-pig brain than to that present in rat and mouse brain (Beresford *et al.*, 1991; Gitter *et al.*, 1991; Fong *et al.*, 1992). Demonstrations of nonspecific cardiovascular depressant effects (Lembeck *et al.*, 1992) and calcium channel antagonist activity (Schmidt *et al.*, 1992) raised the possibility that the antinociceptive effects of CP-96,345 were not mediated via a specific interaction with NK_1 receptors. This has been confirmed recently by the lack of stereoselectivity of CP-96,345 in reversing inflammation-induced hyperalgesia in rats (Nagahisa *et al.*, 1992a).

A second structurally unrelated NK_1 antagonist, RP67580, which binds with high affinity to the rat NK_1 receptor ($K_i = 4.16$ nM, Garret *et al.*, 1991; Fardin *et al.*, 1992), also exhibited antinociceptive activity in mice, antagonizing phenylbenzoquinone-induced writhing and the formalin paw response ($ED_{50} = 0.07$ mg kg^{-1} and 3.7 mg kg^{-1}, respectively; Garret *et al.*, 1991). We have used racemic RP67580 as a more appropriate compound with which to characterize further the antinociceptive effects of an NK_1 antagonist using rat and mouse screens sensitive to conventional analgesic drugs. In order to determine whether the antinociceptive effects of RP67580 reflect the species differences in NK_1 receptor pharmacology revealed with CP-96,345 the writhing and formalin paw tests were modified for use with gerbils and guinea-pigs. The potential calcium channel blocking effects of racemic RP67580 were also investigated.

Methods

Antinociceptive profile of racemic RP67580

Subjects Male Sprague-Dawley rats (130–190 g for paw pressure experiments, 200–300 g for formalin paw test; Bantin & Kingman), BKTO mice (20–37 g, Bantin & Kingman), Mongolian gerbils (50–70 g, Leeds University) and Dunkin Hartley guinea-pigs (200–240 g, Interfauna) were housed separately in groups of 4–6 under standard labora-

[1] Author for correspondence.

tory conditions (22 ± 1°C; 12 h light/dark cycle). Animals were acclimatized to the testing room at least 2 h before behavioural testing. For formalin paw and writhing experiments subjects were habituated to individual perspex observation boxes for a minium of 30 min. Behaviours were rated by an experimenter who was blind to treatment. Experiments conformed to ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and the intensity of noxious stimuli were the minimum possible with which to demonstrate reliable effects of standard analgesic agents. Each animal was used once only and was humanely killed immediately following completion of testing. Groups of 8 animals received each drug treatment or vehicle.

Mouse tail flick Mice were examined for latency to withdraw their tails from a noxious thermal stimulus using a Socrel tail flick meter (model DS20). The bulb voltage was adjusted to achieve a mean tail flick latency of 3–4 s in normal animals. To avoid tissue damage following treatment with analgesic agents trials were terminated if the animals did not respond within 10 s.

Rat paw pressure Responses to mechanical pressure were assessed in rats with a modified Ugo Basile algesiometer. The animal's hind paw was positioned over a convex surface (radius 2.5 mm) and gradually compressed until the animal vocalized. Values were taken for both paws and the mean calculated for each animal before (baseline) and after the appropriate pretreatment period with test drugs. Differences from baseline scores were compared with values obtained in vehicle-treated animals.

Acetic acid-induced writhing The number of discrete abdominal constrictions was counted for 10 min during the time of peak effect either immediately (gerbils) or commencing 5 min after (mice) intraperitoneal injection of 0.6% acetic acid. The injection volume was 10 ml kg^{-1}.

Formalin paw test The duration of licking and biting following subcutaneous injection of formalin in the dorsal (mouse, gerbil and guinea-pig) or intraplantar (rat) surface of the hind paw was recorded. The concentration and injection volume varied according to species: 50 µl of 5% formalin in rats; 20 µl of 0.2% (early phase) or 1% (late phase) solution in mice; 20 µl of 1 or 2% solution in gerbils and 50 µl of 1% solution in guinea-pigs. The early phase response was recorded immediately thereafter for 5 min (rat and mouse) or 10 min (gerbil and guinea-pig). Late phase responses were recorded in separate animals commencing 15 min after injection of formalin and continued for 15 (mouse) or 25 min (rat).

Drug administration Unless otherwise stated all test substances were administered 30 min before behavioural observation as follows: morphine sulphate (3 or 5 mg kg^{-1}, dissolved in 0.9% saline: 60 min pretreatment in rats; May and Baker Ltd., U.K.), racemic RP67580 ((3a**R**, 7a**R** and 3a**S**, 7a**S**)-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl]perhydroisoindol-4-one) hydrochloride (3–30 mg kg^{-1}, dissolved in distilled water; synthesized in the Department of Medicinal Chemistry, MSD Harlow, U.K.), nifedipine (20 or 30 mg kg^{-1}, suspended in 0.5% methocel; Sigma, U.K.) and verapamil hydrochloride (10 or 20 mg kg^{-1}; Cordilox ampoules, Abbott, U.K.). All test compounds were injected as solutions subcutaneously except for nifedipine which was administered intraperitoneally as a suspension. Initial doses of racemic RP67580 were based on the minimum effective dose of the active enantiomer in the mouse formalin paw test (Garret *et al.*, 1991). In mice and gerbils a larger injection volume (ml kg^{-1}) could be administered than in rats and guinea-pigs; because of solubility problems above 10 mg kg^{-1} (pH 4) the maximum dose of racemic RP67580 examined was therefore slightly lower in larger rodents. Doses of calcium channel antagonists were based on their ability to inhibit the behavioural response to formalin in rats (Miranda *et al.*, 1992). Each dose of test drug or vehicle was administered to 8 animals. All doses are expressed as base weight of drug.

Statistical analysis Where necessary data were subjected to logarithmic or square root transformation to achieve normality and homogeneity of variance prior to ANOVA. In cases where F values were associated with $P<0.05$, groups were compared with control animals using Newman-Keuls' multiple comparison test.

Calcium channel antagonist activity of RP67580 in vitro

Guinea-pig ileum myenteric plexus longitudinal muscle strip Strips of ileum longitudinal muscle/myenteric plexus (LM/MP) were obtained from male Dunkin-Hartley guinea-pigs (300–450 g). The strips were mounted for isometric tension recording in organ baths containing Krebs physiological salt solution (PSS) (mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.1) aerated with 95% O_2:5% CO_2, maintained at 37°C, pH 7.4. A resting tension of 0.5 g was applied. Following a 60 min equilibration period, tissues were placed (for 20 min) in a modified Krebs PSS containing zero calcium (0 mM $CaCl_2$), double Mg ($MgSO_4$ 2.4 mM, to maintain tissue viability) and high KCl (84.9 mM, to depolarize the tissue and open voltage-operated calcium channels). It is possible that depolarized conditions may lead to the release of neurotransmitters and degranulate mast cells; therefore the experiments were carried out in the presence of atropine (1 µM), indomethacin (1 µM), mepyramine (1 µM) and methysergide (1 µM). A concentration-effect curve to calcium was obtained by adding $CaCl_2$ (0.03–30 nM) directly to the tissue bath (control). The tissues were washed with normal Krebs PSS and equilibrated (60 min) with either (±)-RP67580 (1 µM), (±)-CP96,345 ((2**R**, 3**R**)-cis-2-(diphenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo [2.2.2]octan-3-amine];100 nM), nifedipine (1 nM) or verapamil (30 nM). The tissues were then transferred to the modified Krebs PSS (containing the appropriate antagonist) for 20 min and the concentration-effect curve to calcium was repeated.

Determination of K_B values The response to each concentration of calcium was expressed as a percentage of the maximum of the control curve. Dose-ratios (DR) were obtained and used to calculate the dissociation constant (apparent K_B) using the following equation where apparent K_B = [concentration of antagonist tested]/(DR − 1). Dose-response curves were fitted to the data by least-squares analysis of variance to the equation $Y = Y_{max}/(1 + (EC_{50}/\text{agonist concentration})^{nH})$, where EC_{50} is the half maximally effective concentration and nH is the Hill coefficient, using an iterative procedure on a VAX computer with Research System 1 Software (BBN Software Products Corporation).

Inhibition of [^{3}H]-diltiazem binding at the L-type calcium channel in skeletal muscle membranes Tissue preparation was by a modification of the method described by Fernandez *et al.* (1980). Briefly, the hind leg muscle from a New Zealand white rabbit (Froxfield Farm) was dissected and washed in cold saline. Approximately 250 g of muscle homogenized in 4 volumes (w/v) of 0.3 M sucrose, 20 mM Tris-maleate pH 7.0, in a Waring blender for 50 s. The homogenate was centrifuged at 3000 g/10 min, and the supernatant removed and recentrifuged at 10,000 g for 20 min. The supernatant was then filtered through several layers of cheese cloth and solid KCl added to the filtrate to a final concentration of 0.5 M. This was centrifuged at 150,000 g for 30 min, the supernatant discarded and the resultant pellet resuspended in a minimum volume of sucrose buffer (see above) using a glass-teflon homogeniser at 200 r.p.m. and recentrifuged at

150,000 g/30 min. This step was repeated once more. The pellet was taken and resuspended in 25% sucrose 20 mM Tris-HCl pH 7.4 in a glass teflon homogenizer; 6 ml of homogenate was then carefully layered on top of 28 ml of 27.5% sucrose, 20 mM Tris-HCl pH 7.4 buffer using thin wall polypropylene tubes and centrifuged for 16 h at 22,000 r.p.m. in a type SW28 swing-out rotor. The supernatant was discarded and the pellet resuspended into 50 ml 50 mM Tris-HCl pH 7.4 in a glass teflon homogenizer at 600 r.p.m. This was recentrifuged at 20,000 r.p.m. for 20 min. The supernatant was discarded and the pellet resuspended in a minimum volume of 50 mM Tris-HCl pH 7.4; 500 µl aliquots were frozen at − 20°C for subsequent use. Protein content of the membranes was determined by the method of Lowry *et al.* (1951).

On the day of the experiment, the following were added to each assay tube: 100 µl [^{3}H]-diltiazem (NEN, specific activity 60–87 Ci mmol^{-1}) at a final concentration of 10 nM, 50 µl of test compound in 50 mM Tris-HCl pH 7.0, 10 µM $CaCl_2$, 10 µM $MgCl_2$, 0.2% bovine serum albumin and 100 µl rabbit skeletal muscle membranes (300 µg protein/assay tube). Non-specific binding was defined with 10 µM (±)-diltiazem.

Tubes were incubated for 3 h at room temperature and the contents filtered through GF/C filters (pre-soaked in 0.5% PEI for 2 h) and washed with 3 × 3 ml ice-cold 10 mM HEPES-Tris buffer pH 7.0. Filters were counted for radioactivity in a scintillation counter. The concentrations of diltiazem and RP67580 to inhibit specific binding of radiolabelled diltiazem by 50% (IC_{50}) were determined by non-linear least squares regression analysis using RS1 (BBM Software Products Corporation).

Depression of high threshold calcium currents in neurones cultured from rat cortex Neocortical cells were maintained in culture as described previously (Priestley *et al.*, 1990). Whole cell voltage-clamp recordings were made from neocortical neurones grown in tissue culture for 7 to 14 days by methods similar to those described for recording sodium currents (Caeser *et al.*, 1993). Cells were voltage-clamped at − 80 mV and currents were evoked by 100 ms depolarizing command pulses to O mV at a stimulation frequency of 0.1 Hz. In all experiments, external Ca^{2+} was substituted by Ba^{2+}. Patch pipettes were filled with (mM): $CsMeSO_4$ 115, ETGA 11, $MgCl_2$ 2, HEPES 10, Mg-ATP 2, leupeptin 0.1, adjusted to pH 7.3. Cells were continuously superfused with a solution containing (in mM): NaCl 120, CsCl 5, tetraethylammonium 15, 4-aminopyridine 5, $MgCl_2$ 2, $BaCl_2$ 4, glucose 25, HEPES 10, tetrodotoxin 0.001, adjusted to pH 7.3 at 22°C. Whole cell calcium channel currents were measured in the absence and presence of RP67580 (10 µM) and compared with the inhibition caused by racemic CP-96,345 (Schmidt *et al.*, 1992) applied to the bath.

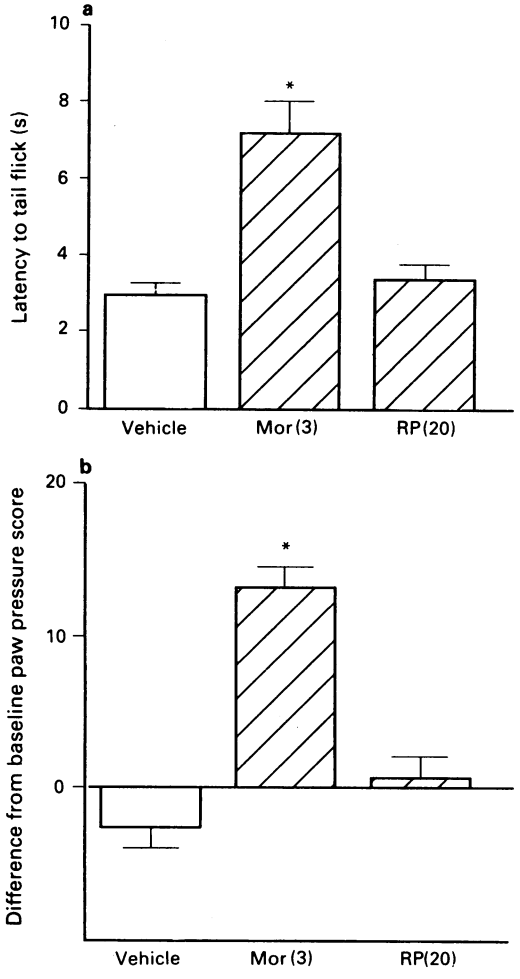


Figure 1 (a) Latency to withdraw the tail from noxious thermal stimulation in mice following treatment with morphine (3 mg kg^{-1}, s.c., 30 min previously) or racemic RP67580 (RP; 20 mg kg^{-1}, s.c., 30 min previously) and (b) thresholds for vocalization following application of mechanical pressure to the hind paw in rats after treatment with morphine (Mor; 5 mg kg^{-1}, s.c., 60 min previously) or racemic RP67580 (RP; 20 mg kg^{-1}, s.c., 30 min previously). Data were subjected to ANOVA followed by Newman Keuls multiple comparison test. *$P \leq 0.05$ compared with vehicle treatment.

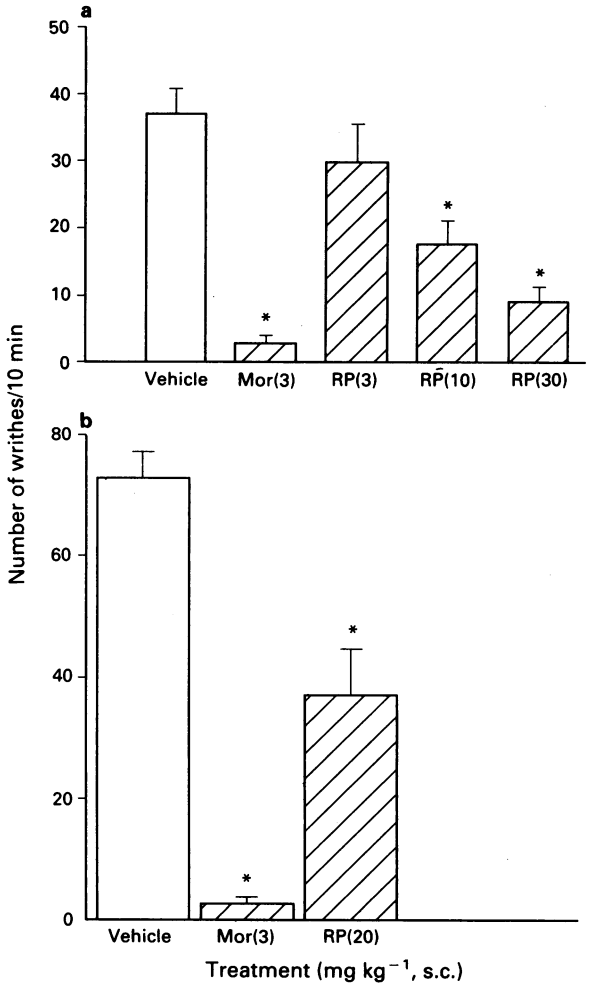


Figure 2 Inhibition of acetic acid (0.6%) induced writhing in mice (a) and gerbils (b) following treatment with morphine (3 mg kg^{-1}, s.c.) or racemic RP67580 (RP; 3–30 mg kg^{-1}, s.c.) 30 min previously. *$P \leq 0.05$ compared with vehicle-treated animals.

Results

Antinociceptive effects of racemic RP67580

Mouse tail flick test Treatment with morphine (3 mg kg^{-1} s.c.; 30 min previously) increased tail flick latencies compared to vehicle-treated mice. In contrast, treatment with racemic RP67580 (20 mg kg^{-1}, s.c.; 30 min previously) did not alter tail flick latency (Figure 1a). In this and other experiments RP67580 appeared to cause a local irritant effect evidenced by scratching directed at the site of injection.

Rat paw pressure test Treatment with morphine (5 mg kg^{-1}, s.c., 60 min previously) caused an approximate doubling in paw pressure thresholds compared with baseline values. Responses of rats treated with racemic RP67580 (20 mg kg^{-1}, s.c., 30 min previously) did not differ from those of vehicle-treated animals (Figure 1b).

Table 1 Summary of effects of racemic RP67580 in formalin paw test in different species (early phase)

Species	*Morphine* (3 or 5 mg kg^{-1}, s.c.)	*Racemic RP67580* (20 or 30 mg kg^{-1}, s.c.)
Mouse	+	+
Rat	+	–
Gerbil	+	+
Guinea-pig	+	–

(+ = active; – = inactive.

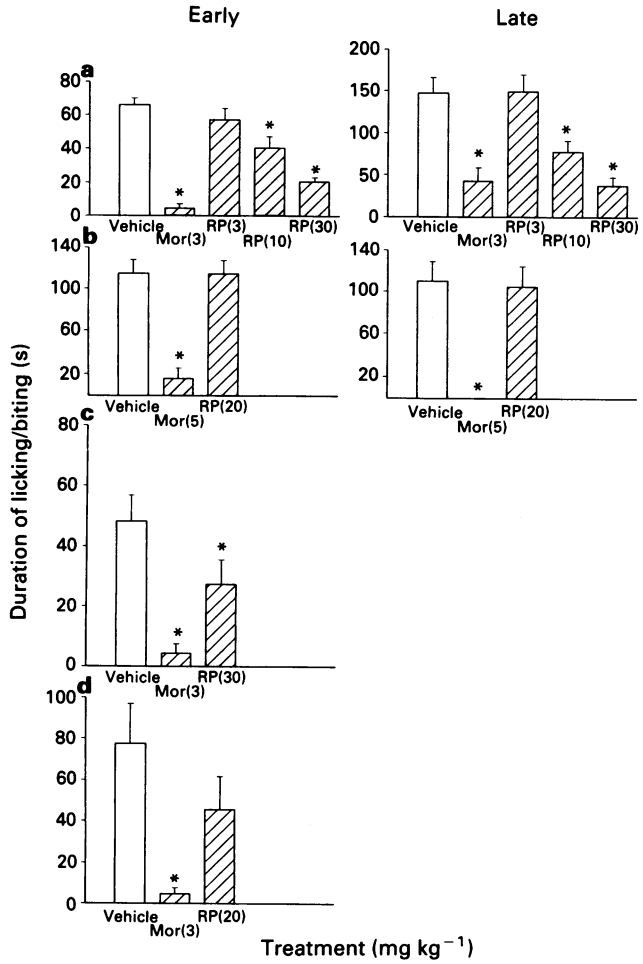


Figure 3 Inhibition of licking and biting induced by injection of formalin into the hind paw in mice (a), rats (b), gerbils (c) and guinea-pigs (d) following treatment with morphine (Mor; 3 or 5 mg kg^{-1}, s.c.) or racemic RP67580 (RP; 3–30 mg kg^{-1}, s.c.) 30 min previously. *$P \leq 0.05$ compared with vehicle treated animals.

Acetic acid-induced writhing In mice, treatment with morphine (3 mg kg^{-1}, s.c.) or racemic RP67580 (10 or 30 mg kg^{-1}, s.c.) 30 min previously caused a 50% or greater inhibition of writhing induced by 0.6% acetic acid i.p. The lowest dose of racemic RP67580 examined (3 mg kg^{-1}) did not attenuate writhing. Similarly in gerbils writhing was inhibited by pretreatment with morphine (3 mg kg^{-1}, s.c.) or racemic RP67580 (20 mg kg^{-1}, s.c.; Figure 2).

Formalin paw test In mice, treatment with morphine (3 mg kg^{-1}, s.c.) or racemic RP67580 (10 or 30 mg kg^{-1}, s.c.) reduced the duration of licking and biting of the hind paw during both the early (first 5 min) and late (15–30 min) phase responses following injection of formalin. In contrast, although morphine (5 mg kg^{-1}, s.c.) blocked the early and late phase response in rats, racemic RP67580 did not. In gerbils and guinea-pigs there was an early, but no late, phase of licking and biting following injection of formalin. In both species morphine (3 mg kg^{-1}, s.c.) inhibited this behaviour. In gerbils, but not guinea-pigs, racemic RP67580 (20 or 30 mg kg^{-1}, s.c.) reduced licking and biting (Figure 3). These findings are summarised in Table 1.

Antinociceptive effect of calcium channel antagonists in gerbil formalin paw test (early phase) The selective calcium channel antagonist nifedipine (30 mg kg^{-1}, i.p.) and the nonselective antagonist verapamil (10 or 20 mg kg^{-1}, s.c.) inhibited formalin-induced licking and biting in gerbils (Figure 4).

Calcium channel antagonist activity of RP67580 in vitro

Guinea-pig ileum muscle strip Racemic RP67580 exerted an inhibitory effect on calcium entry into depolarized strips of guinea-pig ileum LM/MP smooth muscle. There was a rightward shift in the position of the calcium dose-response curve with little or no effect on the maxima of the curve (Figure 5). The apparent K_B value for racemic RP67580 was 587 ± 115 nM. The apparent K_B value for (±)-CP-96,345 was 54 ± 18 nM, nifedipine was 0.3 ± 0.05 nM and verapamil was 15.0 ± 2.0 nM ($n = 4$ in each case).

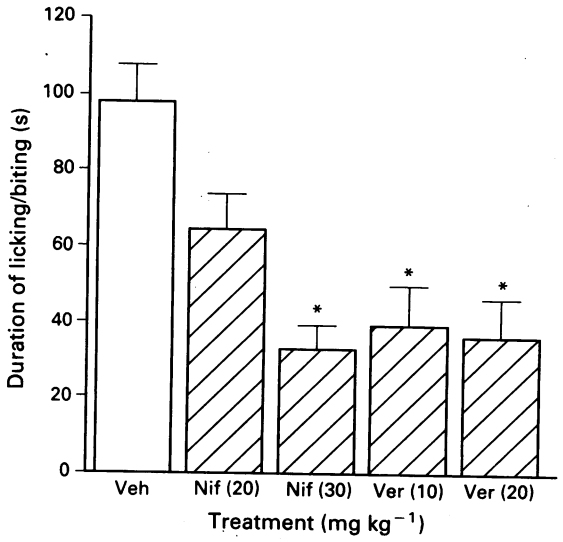


Figure 4 Inhibition of licking and biting induced by injection of formalin into the hind paw of gerbils following treatment with nifedipine (Nif; 20 or 30 mg kg^{-1}, i.p.) or verapamil (Ver; 10 or 20 mg kg^{-1}, s.c.) 30 min previously. *$P \leq 0.05$ compared with vehicle-treated animals.

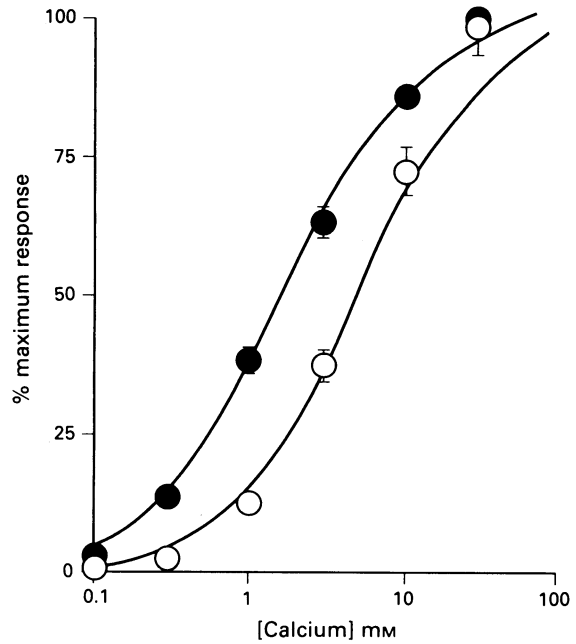


Figure 5 Contractile responses to calcium in depolarized strips of guinea-pig ileum LM/MP obtained in the absence (●) and presence (○) of racemic RP67580 (1 μM). Values are the mean ± 1 s.e.mean. Responses were expressed as a % of the maximum response evoked by calcium. Curves were fitted by least squares analysis of variance.

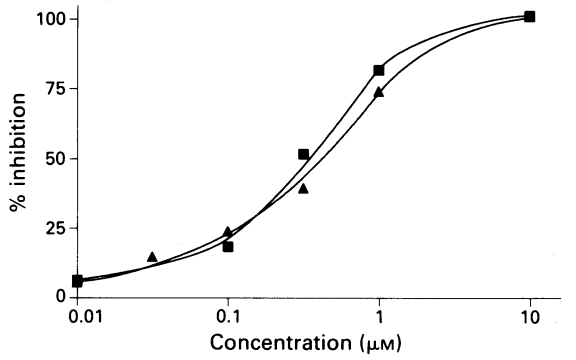


Figure 6 Inhibition of [^{3}H]-diltiazem binding to rabbit skeletal muscle membranes by diltiazem (▲) and racemic RP67580 (■).

Inhibition of [^{3}H]-diltiazem binding Inhibition of [^{3}H]-diltiazem binding to rabbit skeletal muscle membranes by diltiazem and RP67580 produced monophasic competitive curves with Hill coefficients close to unity. Concentration-response curves for diltiazem and racemic RP67580 against [^{3}H]-diltiazem receptor binding to rabbit skeletal muscle membranes are shown in Figure 6. The concentration of diltiazem to inhibit 50% of specific binding (IC_{50}) of [^{3}H]-diltiazem was 0.37 μM. The IC_{50} for RP67580 was 0.29 nM.

Depression of calcium currents in neurones cultured from rat cortex High threshold calcium currents were reversibly depressed by 10 μM racemic RP67580 ($n = 6$). At 10 μM (±)-RP67580 decreased calcium currents by 10 ± 2% of control values. Similarly, (±)-CP-96,345 reversibly depressed peak currents by 33 ± 6% at a concentration of 10 μM ($n = 6$).

Discussion

Racemic RP67580 exhibited a selective antinociceptive profile in writhing and formalin paw tests but was not active in thermal or mechanical screens. These findings confirm and extend those of Garret *et al.* (1991) and Moussaoui *et al.* (1992). The doses required to induce antinociceptive effects in the mouse writhing and formalin paw tests in the present study were somewhat higher (10 mg kg^{-1}, s.c.) than those reported by previous investigators (0.07–3.5 mg kg^{-1}; Garret *et al.*, 1991). This apparent difference in dose-range is likely to reflect our use of the racemic mixture rather than the active enantiomer and the more stringent criterion of a statistically significant active dose rather than an ED_{50} value estimated by regression analysis for the writhing assay.

Broadly speaking RP67580 was active in antinociception tests sensitive to nonsteroidal anti-inflammatory drugs; there is also evidence for anti-inflammatory activity of this agent in rat models of neurogenic plasma extravasation and substance P-induced plasma extravasation (Garret *et al.*, 1991). However, our observations indicate that the antinociceptive effects of RP67580 are not mediated via a specific interaction with the rat-type NK_1 receptor. Whereas this agent inhibited the early and late phase response to injection of formalin in the paw of mice, neither phase was blocked in rats. This is in contrast with the enantiomeric selectivity of CP-96,345 in antagonizing the late phase response in rats (Yamamoto & Yaksh, 1991) and is particularly surprising in view of the reported increase in substance P-like immunoreactivity in the dorsal horn of the spinal cord induced during the early and late phase components of the response to formalin in rats (McCarson & Goldstein, 1990) and the ability of RP67580 to block facilitation of a nociceptive spinal reflex in the rat (Laird *et al.*, 1993). Interestingly marked, and as yet unexplained, species differences have also been reported in the effects of intrathecal administration of peptide substance P antagonists; neurotoxicity and paralysis occurred in rats, but not mice (Vaught & Scott, 1987). A further anomaly was that RP67580 was able to attenuate the response to formalin in gerbils, but not guinea-pigs, both species bearing the human-type NK_1 receptor as defined using CP-96,345 (see Table 1). Similarly acetic acid-induced writhing was antagonized by RP67580 in both mice and gerbils at similar doses. These findings would not have been predicted given the higher potency expected for this agent in mice and rats compared with gerbils. This study is the first direct comparison of the *in vivo* effects of an NK_1 receptor antagonist in a range of rodent species in which differences in receptor pharmacology and molecular biology have been demonstrated.

More widespread antinociceptive effects of RP67580 extending to mechanical and thermal screens would be predicted from the association of tachykinin release in the dorsal horn with noxious stimulation in these modalities. Failure to detect these effects using conventional tests in conscious animals may be attributable to the transient application of noxious stimuli, since in anaesthetized animal studies prolonged, intense noxious stimulation was required to increase tachykinin release in the spinal cord (Duggan *et al.*, 1987). Thus CP-96,345 selectively blocked the prolonged excitatory postsynaptic potential evoked by intense mechanical compression or heating of the skin or intense electrical stimulation of C fibres in anaesthetized cats but did not affect the response to transient stimuli (Radhakrishnan & Henry, 1991; de Konink & Henry, 1991). These observations may implicate tachykinins in the development of hyperalgesia associated with inflammation or tissue damage rather than acute pain. However, neither the anti-inflammatory nor the antinociceptive effects of CP-96,345 show clear enantiomeric selectivity and are therefore unlikely to be mediated via a specific interaction with the NK_1 receptor (Nagahisa *et al.*, 1992a).

Other pharmacological actions, such as blockade of calcium channels, might explain anomalies such as the antinociceptive activity of CP-96,345, but not RP67580, in the mouse hot plate test (Lecci *et al.*, 1991; Moussaoui *et al.*, 1992). We therefore investigated the possible interaction of RP67580 with calcium channels *in vitro*. Racemic RP67580 inhibited calcium entry into depolarized strips of guinea-pig

ileum LM/MP, most likely reflecting the blockade of voltage-operated calcium channels. Racemic CP-96,345 exhibited a similar effect. In this preparation it is possible that addition of calcium chloride could cause the release of endogenous tachykinins, which in turn could evoke a contractile response that is susceptible to blockade by NK_1-receptor antagonists. However, it seems unlikely that antagonism of the effects of endogenously released tachykinins accounts for the inhibitory effects of racemic RP67580 on calcium entry. The apparent K_B value for inhibition of calcium entry by racemic RP67580 was 587 nM and for inhibition of NK_1-receptor-mediated contractions in guinea-pig ileum LM/MP was 100 nM (unpublished observations, this laboratory). A similar value for NK_1-receptor blockade by RP67580 was reported by Carruete *et al.* (1992). In contrast, there is a clear dissociation between the ability of racemic CP-96,345 to inhibit calcium entry (apparent $K_B = 54$ nM) and its ability to antagonise NK_1-receptor mediated contractions in guinea-pig ileum LM/MP ($K_B = 0.5$ nM, Razzaque *et al.*, 1993). Moreover, the ability of racemic RP67580 to displace [^{3}H]-diltiazem binding from skeletal muscle membranes *in vitro* with an affinity similar to the L-channel calcium antagonist, diltiazem and to depress high threshold calcium currents in cortical neuronal cultures provides direct evidence for calcium channel antagonist activity. Similar evidence for calcium channel activity of racemic RP67580 has been obtained by other investigators (inhibition of [^{3}H]-desmethoxyverapamil binding to rat heart membranes, $IC_{50} = 210 \pm 16.6$ nM; McLean *et al.*, unpublished observations). The affinity of RP67580 for ion channels may be responsible for the depressor effects on systemic blood pressure reported following i.v. administration to anaesthetized rats (Laird *et al.*, 1993). Finally, the calcium channel antagonists nifedipine and verapamil were extremely effective inhibitors of calcium entry and also exhibit a selective antinociceptive profile in rodents identical to that of CP-96,345 and RP67580, namely; formalin paw, writhing and, under certain conditions, hot plate tests (Miranda *et al.*, 1992; present study).

The value of local anaesthetic agents, which block ion channels, in relieving pain in the clinic is being increasingly recognised (Swerdlow, 1988). It is not known whether calcium channel blockers primarily exert their antinociceptive actions centrally or peripherally. The transduction of noxious thermal and chemical stimuli at polymodal nociceptive terminals appears to involve activation of voltage-dependent ion channels. Thus, the response of polymodal nociceptors in the cornea was markedly attenuated following topical application of the calcium channel blockers cadmium and diltiazem (Pozo *et al.*, 1992). Diltiazem-sensitive calcium channels have also been identified in dorsal root ganglion cells (Konnerth *et al.*, 1987).

Our findings and those of others do not provide evidence for tachykinin-operated activation of NK_1 receptors by noxious stimuli applied at threshold or near threshold intensities as are used in nociception tests in awake animals. However, an important therapeutic role for NK_1 anagonists in clinical pain remains a possibility which requires further investigation using more appropriate animal models. Thus, in anaesthetized rats RP67580, but not the inactive (+)-enantiomer, RP68651, inhibited the facilitation of a spinal flexor reflex induced by noxious conditioning stimuli (Laird *et al.*, 1993) and inhibited neurogenic inflammation of the dura induced by stimulation of the trigeminal ganglion (Shepheard *et al.*, 1993).

We wish to thank Dr P. Hutson for providing the [^{3}H]-diltiazem binding data.

References

BERESFORD, I.J.M., BIRCH, P.J., HAGAN, R.M. & IRELAND, S.J. (1991). Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist, CP96,345. *Br J. Pharmacol.*, **104**, 292–293.

BIRCH, P.J., HARRISON, S.M., HAYES, A.G., ROGERS, H. & TYERS, M.B. (1992). The non-peptide NK_1 receptor antagonist, (±)-CP96,345, produces antinociceptive and anti-oedema effects in the rat. *Br. J. Pharmacol.*,**105**, 508–510.

CAESER, M., SEABROOK, G.R. & KEMP, J.A. (1993). Block of voltage-dependent sodium currents by the substance P receptor antagonist (±)-CP-96,345 in neurones cultured from rat cortex. *Br. J. Pharmacol.*, **109**, 918–924.

CARRUETE, A., MOUSSOUI, S.M., CHAMPION, A., COTTEZ, O., GONIOT, P. & GARRETT, C. (1992). Comparison in different tissue preparations of the *in vitro* pharmacological profile of RP67580, a new non-peptide substance P antagonist. *Neuropeptides*, **23**, 245–250.

DE KONINK, Y. & HENRY, J.L. (1991). Substance P – mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons *in vivo* by noxious stimulation. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11344–11348.

DUGGAN, A.W., MORTON, C.R., ZHAO, Z.Q. & HENDRY, I.A. (1987). Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. *Brain Res.*, **403**, 345–349.

FARDIN, V., FOUCAULT, F., BOCK, M.D. & GARRET, C. (1992). Binding profile of RP67580, a new non-peptide sustance P antagonist: comparison with CP96,345. *Br. J. Pharmacol.*, **105**, 80P.

FERNANDEZ, J.L., ROSEMBLATT, M. & HIDALGO, C. (1980). Highly purified sarcoplasmic reticulum membrane vesicles are devoid of Ca^{2+} independent ATPase activity. *Biochim. Biophys. Acta*, **599**, 552–568.

FONG, T.M., YU, H. & STRADER, C.D. (1992). Molecular basis for the species selectivity of the neurokinin-1 receptor antagonists CP96,345 and RP67580. *J. Biol. Chem.*, **267**, 25668–25671.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., PEYRONNEL, J.-F., BLANCHARD, J.-C. & LADURON, P.M. (1991). Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 10208–10212.

GITTER, B.D., WATERS, D.C., BRUNS, R.F., MASON, N.R., NIXON, J.A. & HOWBERT, J.J. (1991). Species differences in affinities of non-peptide antagonists for substance P receptors. *Eur. J. Pharmacol.*, **197**, 237–238.

HAYES, A.G. & TYERS, M.B. (1980). Effects of capsaicin on nociceptive heat, pressure and chemical thresholds and on substance P levels in the rat. *Brain Res.*, **189**, 561–564.

KONNERTH, A., LUX, H.D. & MORAD, M. (1987). Proton-induced transformation of calcium channel in chick dorsal root ganglion cells. *J. Physiol.*, **386**, 603–633.

LAIRD, J.M.A., HARGREAVES, R.J. & HILL, R.G. (1993). Effect of RP67580, a non-peptide neurokinin$_1$ receptor antagonist, on facilitation of a nociceptive spinal flexion reflex in the rat. *Br. J. Pharmacol.*, **109**, 713–718.

LECCI, A., GUILIANI, S., PATACCHINI, R., VITI, G. & MAGGI, C.A. (1991). Role of NK_1 tachykinin receptors in thermonociception: effect of (±)-CP-96,345, a non-peptide substance P antagonist, on the hot plate test in mice. *Neurosci. Letts*, **129**, 299–302.

LEMBECK, F., CONNERER, J., TSUCHIYA, M. & NAGAHISA, A. (1992). The non-peptide tachykinin antagonist, CP96,345, is a potent inhibitor of neurogenic inflammation. *Br. J. Pharmacol.*, **105**, 527–530.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, A.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MCCARSON, K.E. & GOLDSTEIN, B.D. (1990). Time course of the alteration in dorsal horn substance P levels following formalin: blockade by naloxone. *Pain*, **41**, 95–100.

MCCARSON, K.E. & GOLDSTEIN, B.D. (1991). Release of substance P into the superficial dorsal horn following nociceptive activation of the hind paw of the rat. *Brain Res.*, **568**, 109–115.

MIRANDA, H.F., BUSTAMANTE, D., KRAMER, V., PELISSIER, T., SAAVEDRA, H., PAEILE, C., FERNANDEZ, E. & PINARDI, G. (1992). Antinociceptive effects of Ca^{2+} channel blockers. *Eur. J. Pharmacol.*, **217**, 137–141.

MOUSSAOUI, S.M., CARRUETTE, A., MONTIER, F. & GARRET, C. (1992). RP67580, a non-peptide substance P antagonist, inhibits neurogenic inflammation and possesses antinociceptive activities in rodents. *Neuropeptides*, **22**, 46.

NAGAHISA, A., ASAI, R., KANAI, Y., MURASE, A., TSUCHIYA-NAKAGAKI, M., NAKAGAKI, T., SHIEH, T.-C. & TANIGUCHI, K. (1992a). Non-specific activity of (±)-CP96,345 in models of pain and inflammation. *Br. J. Pharmacol.*, **107**, 273–275.

NAGAHISA, A., KANAI, Y., SUGA, O., TANIGUCHI, K., TSUCHIYA, M., LOWE, J.A. & HESS, H.-J. (1992b). Antiinflammatory and analgesic activity of a non-peptide substance P receptor antagonist. *Eur. J. Pharmacol.*, **217**, 191–195.

POZO, M.A., GALLEGO, R., GALLAR, J. & BELMONTE, C. (1992). Blockade by calcium antagonists of chemical excitation and sensitization of polymodal nociceptors in the cat's cornea. *J. Physiol.*, **450**, 179–189.

PRIESTLEY, T., HORNE, A.L., McKERNAN, R.M. & KEMP, J.A. (1990). The effect of NMDA receptor glycine site antagonists on hypoxia-induced neurodegeneration of rat cortical cell cultures. *Brain Res.*, **531**, 183–188.

RADHAKRISHNAN, V. & HENRY, J.L. (1991). Novel substance P antagonist, CP-96,345 blocks responses of cat spinal dorsal horn neurones to noxious cutaneous stimulation and to substance P. *Neurosci. Letts*, **132**, 39–43.

RAZZAQUE, Z., LONGMORE, J. & HILL, R.G. (1993). Comparison of the effect of (±) CP96,345 and L-668,169 on neurokinin receptor mediated responses in rat and guinea-pig isolated tissues. *Neuropeptides*, in press.

RODRIGUEZ, R.E., SALT, T.E., CAHUSAC, P.M.B. & HILL, R.G. (1983). The behavioural effects of intrathecally administered [D-PRO^2, D-TRP^{7,9}]-substance P, an analogue with presumed antagonist actions in the rat. *Neuropharmacology*, **22**, 173–176.

SCHMIDT, A.W., McLEAN, S. & HEYM, J. (1992). The substance P receptor antagonist CP-96,345 interacts with calcium channels. *Eur. J. Pharmacol.*, **215**, 351–352.

SHEPHEARD, S.L., WILLIAMSON, D.R., HILL, R.G. & HARGREAVES, R.J. (1993). The non-peptide neurokinin$_1$ receptor antagonist, RP 67580 blocks neurogenic plasma extravasation in the dura mater of rats. *Br. J. Pharmacol.*, **108**, 11–12.

SWERDLOW, M. (1988). The use of local anaesthetics for chronic pain. *The Pain Clinic*, **2**, 3–6.

VAUGHT, J.L. & SCOTT, R. (1987). Species difference in the behavioural toxicity produced by intrathecal substance P antagonists: relationship to analgesia. *Life Sci.*, **40**, 175–181.

YAMAMOTO, T. & YAKSH, T.L. (1991). Stereospecific effects of a nonpeptide NK$_1$ selective antagonist, CP96,345: Antinociception in the absence of motor dysfunction. *Life Sci.*, **49**, 1955–1963.

YASHPAL, K., WRIGHT, D.M. & HENRY, J.L. (1982). Substance P reduces tail-flick latency: implications for chronic pain syndromes. *Pain*, **14**, 155–167.

ZIMMERMANN, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*, **16**, 109–110.

(Received May 28, 1993
Revised August 19, 1993
Accepted August 26, 1993)

Br. J. Pharmacol. (1993), **110**, 1614–1620 © Macmillan Press Ltd, 1993

Profile of capsaicin-induced mouse ear oedema as neurogenic inflammatory model: comparison with arachidonic acid-induced ear oedema

[1]Hideo Inoue, Nobuyuki Nagata & *Yasuko Koshihara

Research Laboratory, Minophagen Pharmaceutical Co., 2-5233, Komatsubara, Zama, Kanagawa 228, Japan and *Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, Sakaecho, Itabashi-ku, Tokyo 173, Japan

1 We have investigated the mechanism of capsaicin-induced mouse ear oedema compared with that of arachidonic acid (AA)-induced ear oedema, and evaluated the possible involvement of neuropeptides in the development of capsaicin-induced oedema.

2 Topical application of capsaicin (0.1–1.0 mg per ear) to the ear of mice produced immediate vasodilatation and erythema followed by the development of oedema which was maximal at 30 min after the treatment. This oedema was of shorter duration with less swelling than AA-induced oedema (2.0 mg per ear).

3 Capsaicin-induced ear oedema was unaffected when inhibitors of arachidonate metabolites including platelet activating factor (PAF) were administered before capsaicin (250 μg per ear) application, while these agents significantly prevented AA-induced oedema. Dexamethasone, histamine H_1 and/or 5-hydroxytryptamine (5-HT) antagonists, and substance P (SP) antagonists were effective in inhibiting both models. Furthermore, a Ca^{2+}-channel blocker and the capsaicin inhibitor, ruthenium red, were effective inhibitors of capsaicin oedema but had no effect on AA-induced oedema.

4 Phosphoramidon (50 μg kg^{-1}, i.v.), an endopeptidase inhibitor, markedly ($P<0.001$) enhanced only capsaicin-induced ear oedema, but bestatin (0.5 mg kg^{-1}, i.v.), an aminopeptidase, failed to enhance oedema formation.

5 Neuropeptides (1–100 pmol per site) such as rat calcitonin gene-related peptide (CGRP), SP, neurokinin A (NKA), and vasoactive intestinal peptide (VIP), which are released from capsaicin-sensitive neurones, caused ear oedema by intradermal injection. Furthermore, a synergistic effect of CGRP (10 fmol per site) and SP (10 pmol per site) on oedema formation was observed.

6 The oedema induced by neuropeptides was significantly ($P<0.05$ or $P<0.001$) inhibited when cyproheptadine (20 mg kg^{-1}, p.o.), a histamine H_1 and 5-HT antagonist, was administered before injection. In contrast, nifedipine (50 mg kg^{-1}, p.o.), a Ca^{2+}-channel blocker, and indomethacin (10 mg kg^{-1}, p.o., except for NKA), a cyclo-oxygenase inhibitor, had little effect on neuropeptide-induced oedema.

7 These results suggest that the mechanism of capsaicin-induced ear oedema is different from that of AA-induced oedema and suggest that the development of capsaicin-induced ear oedema is primarily mediated by neuropeptides. The neuropeptides released after activation of sensory nerves cause an increase of vascular permeability by interactions with endothelial cells and by histamine (and 5-HT) release from mast cells.

Keywords: Capsaicin; ear oedema; ruthenium red; calcitonin gene-related peptide; substance P; vasoactive intestinal peptide; neurokinin A; phosphoramidon

Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the primary pungent ingredient of red peppers in the genus *Capsicum*, is well known to evoke neurogenic acute inflammatory responses such as axon reflex vasodilatation, plasma extravasation, coughing, and painful sensitization (see review by Holzer, 1991). With repeated administration, protective actions characterized by an inhibition of inflammation and antinociception have been found. Various actions of capsaicin appear as a result of selective stimuli of a subset of capsaicin-sensitive afferent neurones which contain neuropeptides as neurotransmitters (Holzer, 1988; Maggi & Meli, 1988 for reviews). Neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), and neurokinin A (NKA), which are released by capsaicin from peripheral endings of afferent neurones, have been considered as the chemical mediators of skin inflammation (Holzer, 1988; 1991; Maggi & Meli, 1988; Saria *et al.*, 1988). Other studies have shown that inflammatory responses including oedema formation, vasodilatation and wheal are evoked in response to intradermal injection of neuropeptides (Williams, 1982; Devillier *et al.*, 1986; Brain & Williams, 1988). Further, neuropeptides have been found in the synovial fluid of patients with rheumatoid arthritis (Larsson *et al.*, 1989; Marshall *et al.*, 1990) and suggested to play a role in skin diseases including psoriasis (Farber *et al.*, 1986), blister, burn (Wallengren *et al.*, 1986), dermatographism and cold urticaria (Wallengren *et al.*, 1987).

Recently, it has been reported that capsaicin can induce acute inflammation in the mouse ear (Mantione & Rodriguez, 1990; Gábor & Rázga, 1992) and rat paw (De & Ghosh, 1990). However, direct evidence for the participation of mediators in this inflammatory response is lacking. In this paper the authors discuss the mechanism of capsaicin-induced mouse ear oedema compared with arachidonic acid (AA)-induced ear oedema which is predominantly mediated via the arachidonate pathway (Young *et al.*, 1984; Carlson *et al.*, 1985; Opas *et al.*, 1985; Chang *et al.*, 1986; Inoue *et al.*, 1988) and the possible involvement of neuropeptides in the capsaicin-induced ear oedema.

[1] Author for correspondence.

Methods

Assay for capsaicin- and AA-induced mouse ear oedema

Six week old male ddY mice weighing 30–35 g (Japan SLC, Japan) were used for the experiments. Animals were conscious when tested. Induction of mouse ear oedema was based on the method of Mantione & Rodriguez (1990) for capsaicin and of Inoue *et al.* (1988) for AA. Capsaicin and AA were dissolved in acetone at a concentration of 12.5 mg ml^{-1} and 100 mg ml^{-1}, respectively and 20 μl (capsaicin; 250 μg per ear, AA; 2 mg per ear) was then applied topically to both surfaces of an ear of each mouse. The development of capsaicin-induced ear oedema was observed for 3 h following application. Pharmacological agents were dissolved in acetone or ethanol for topical application, and were suspended with 1% polyoxyethylene sorbitan monooleate (Tween 80, Tokyo Kasei Chemical Industry, Japan) in saline (0.9% NaCl) for oral and intravenous administration. Topical and oral administration were given 30 min and 60 min before capsaicin application, respectively. For AA-induced ear oedema, both treatments were given 30 min before AA application. Intravenous administration of agents was performed 15 min before the irritant treatment. Mice in the control group received the vehicle only. Ear thickness was measured 30 min and 60 min after capsaicin and AA treatment with dial calipers (Ozaki Factory, Japan) measuring to an accuracy of 0.01 mm. Ear oedema was expressed as an increase in ear thickness. Erythema was assessed with the naked eye compared with the untreated ear.

Intradermal injection of neuropeptides into the ear

Intradermal injections into the ear of male ddY mice (6 weeks old) were made with a hypodermic needle (0.28 × 18.0 mm) and a repeating dispenser (Hamilton Co., U.S.A.). The outer surface of the ear of mice anaesthetized with ether (Showa Ether Co., Japan) received an injection of 5 μl saline containing SP, CGRP, NKA, or VIP at various concentrations. Control mice received saline. Ear oedema was examined 30 min after peptide-treatment with dial calipers.

Materials

Pharmacological agents were purchased as follows: aspirin, Nakarai Chemical Co., Japan; AA, capsaicin, SP, αCGRP (rat), VIP (porcine), NKA, nordihydroguaiaretic acid (NDGA), indomethacin, pyrilamine, dexamethasone, cyproheptadine, chlorpheniramine, nifedipine, verapamil, diltiazen, nicardipine, cinnarizine, flunarizine, ruthenium red, [D-Pro^2,D-$Trp^{7,9}$]-SP, [D-Arg^1,D-$Trp^{7,9}$,Leu^{11}]-SP (spantide), [D-P-Cl-Phe^6,Leu^{17}]-VIP, CGRP fragment $_{8-37}$, bestatin, D-Arg,-[Hyp^3,D-Phe^7]-bradykinin (NPC 567), pimozide, Nα-adamantaneacetyl-D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin, [$Thi^{5,8}$,D-Phe^7]-bradykinin, [Leu^8, des Arg^9]-bradykinin, tetracaine and phosphoramidon, Sigma Chemical Co., U.S.A.; gossypol and CV 3988 (rac-3-CN-n-octadecyl carbamoyloxy)-2-methoxypropyl-2-thiazolioethyl-phosphate), Biomol Research Laboratories, U.S.A.; methysergide, amiloride and loperamide, Research Biochemicals Incorporated, U.S.A.; PGAS 385 (H_2N-Lys-Pro-Arg-Arg-Pro-Tyr-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2, VIP antagonist) and [Ac-Tyr^1,D-Phe^2]-GRF (1–29) amide, Bachem Fine Chemicals Inc., U.S.A.; AA 861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone) was a generous gift from Takeda Chemical Industry, Japan. AC 5-1 (3,4,2′,4′-tetrahydroxy-2-geranyldihydrochalcone) was prepared by Dr Y. Fujimoto, Faculty of Pharmaceutical Sciences, Nihon University.

Statistics

The statistical analysis system (Meancomp) package (Institute of Endocrinology, Gunma University, Japan) was used for data analysis. Results are expressed as mean ± s.e.mean. Statistical significance of differences between control and test groups was determined by Student's *t* test for unpaired data or the Cochran-Cox test.

Results

Development of mouse ear oedema induced by a topical application of capsaicin

The ear oedema induced by a single application of capsaicin at various doses was examined for 3 h by measuring ear thickness. Skin reactions such as erythema due to vasodilatation appeared immediately and oedema formation became detectable at 5 min after treatment (Figure 1). As previously found (Mantione & Rodriguez, 1990; Gábor & Rázga, 1992), the ear oedema developed rapidly and reached a maximum at 30 min after capsaicin treatment. Then oedema gradually decreased with time but erythema was attenuated at 2 h. A dose-dependent enhancement of oedema development was not observed with increased concentrations of capsaicin (100–1000 μg). In the subsequent experiments, induction of ear oedema was performed by treatment with capsaicin at a dose of 250 μg, which is the same dose as previously studied (Mantione & Rodriguez, 1990).

Effect of pharmacological agents on capsaicin- and AA-induced mouse ear oedema

The influence of various agents on both ear oedema induced by a single application of 250 μg capsaicin and 2 mg AA is summarized in Table 1a,b. AA-induced ear oedema reached a maximum level at 60 min after the treatment (Young *et al.*, 1984; Chang *et al.*, 1986; Inoue *et al.*, 1988). The capsaicin-induced ear oedema was less than AA-induced oedema, and an increase in ear thickness at 30 and 60 min after capsaicin and AA was 0.132 ± 0.023 and 0.308 ± 0.021 mm (66 experiments, $n = 6$), respectively. A PAF antagonist (0.5 or 1.0 mg per ear) and 5-lipoxygenase inhibitors (1.0 mg per ear) such as NDGA, AC-5, and AA 861 significantly inhibited AA-induced mouse ear oedema while these inhibitors did not suppress capsaicin-induced ear oedema. Dexamethasone (0.1 mg per ear), a steroid anti-inflammatory drug; chlorpheniramine (25 mg kg^{-1}, p.o.), a histamine H_1 blocker; methysergide (4 mg kg^{-1}, i.v.), a 5-HT antagonist; and cyproheptadine (25 mg kg^{-1}, p.o.), a histamine H_1 and 5-HT antagonist; were very effective in both models. The Ca^{2+}-channel blockers, nifedipine, verapamil, diltiazen, loperamide, nicardipine, and flunarizine significantly inhibited capsaicin-induced ear oedema at doses of less than 50 mg kg^{-1} (p.o.). However, these compounds had no effects on AA-induced

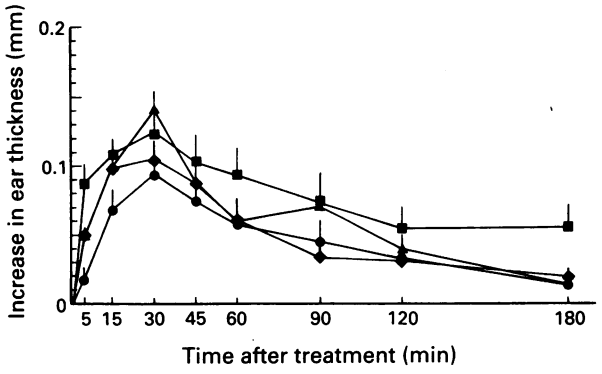


Figure 1 The time course of ear oedema formation induced by capsaicin. Mouse ears were treated by topical application of capsaicin at various doses. Ear oedema is the mean ear thickness of test animals minus that of animals before treatment. Each point represents the mean ± s.e.mean value from 7 animals; (●) 100 μg; (▲) 250 μg; (◆) 500 μg; (■) 1000 μg.

Table 1a Effects of pharmacological agents on capsaicin- and arachidonic acid (AA)-induced mouse ear oedema

Class	Dose (mg kg^{-1}, ear)	Route	Inhibition (%) Capsaicin oedema	AA oedema
Inhibitor of AA pathway				
Indomethacin	10	p.o.	− 20 ± 17	24 ± 6*
	1.0	t.a.	− 2 ± 16	57 ± 8***
Aspirin	200	p.o.	− 22 ± 25	19 ± 7
	1.0	t.a.	− 4 ± 20	− 12 ± 6
NDGA	1.0	t.a.	− 1 ± 20	44 ± 3***
AC-5	1.0	t.a.	− 5 ± 18	66 ± 6***
AA 861	1.0	t.a.	− 10 ± 13	53 ± 7***
Tetracaine	1.0	t.a.	33 ± 6	53 ± 8**
Dexamethasone	0.1	t.a.	76 ± 4***	56 ± 5***
PAF antagonist				
Gossypol	1.0	t.a.	2 ± 10	55 ± 7***
CV 3988	0.5	t.a.	− 38 ± 10	33 ± 8**
Histamine and 5-HT antagonist				
Cyproheptadine	12.5	p.o.	33 ± 14	
	25		35 ± 11*	64 ± 4***
	50		52 ± 4***	70 ± 3***
Histamine antagonist				
Chlorpheniramine	12.5	p.o.	7 ± 17	
	25		37 ± 7**	31 ± 9*
	50		54 ± 4**	26 ± 5*
Pyrilamine	50	p.o.	− 7 ± 14	13 ± 4*
5-HT antagonist				
Methysergide	2	i.v.	31 ± 13	35 ± 12*
	4		53 ± 6**	52 ± 11**
Ca^{2+}-channel blocker				
Nifedipine	12.5	p.o.	42 ± 5	
	25		64 ± 8*	− 2 ± 4
	50		71 ± 6**	3 ± 4
Verapamil	12.5	p.o.	44 ± 8	
	25		61 ± 5*	− 10 ± 3
	50		69 ± 3*	6 ± 6
Diltiazen	25	p.o.	13 ± 7	
	50		46 ± 6**	− 1 ± 1
Loperamide	25	p.o.	37 ± 12	− 1 ± 4
	50		56 ± 6***	7 ± 5
Nicardipine	25	p.o.	− 2 ± 18	− 7 ± 2
	50		58 ± 2**	6 ± 4
Cinnarizine	50	p.o.	19 ± 11	4 ± 4
Pimozide	50	p.o.	25 ± 14	18 ± 5
Amiloride	50	p.o.	21 ± 11	3 ± 4
Flunarizine	25	p.o.	2 ± 12	− 1 ± 3
	50		53 ± 4**	6 ± 5

Test compounds were applied topically (t.a.) 30 min (but dexamethasone was given 3 h) before irritant treatment. Oral administration (p.o.) was performed 30 min for AA oedema and 60 min for capsaicin oedema before treatment. Intravenous administration was 15 min before treatment. Values are expressed as mean ± s.e.mean of 6–7 animals. Statistical significance from the control at *$P<0.05$, **$P<0.01$ and ***$P<0.001$.

oedema. Ruthenium red, a reported capsaicin inhibitor (Amann & Maggi, 1991 for review), inhibited capsaicin-induced oedema dose-dependently with an ED_{50} value of 0.40 (95% limit; 0.27–0.61) mg kg^{-1} (i.v.) (Figure 2). Of the neuropeptide receptor antagonists, the SP antagonist, spantide and [D-Pro^2,D-Trp^{7,9}]-SP (0.25 or 0.50 mg kg^{-1}, i.v.), significantly inhibited both models. Three kinds of VIP antagonists showed a slight effect, but no significant suppression, of capsaicin-induced ear oedema. $CGRP_{8-37}$ (CGRP antagonist) had liitle effect in either model. Bradykinin (BK) antagonists of B_1 and B_2 receptors were ineffective in both types of ear oedema. The ear oedema induced by capsaicin was significantly ($P<0.001$) enhanced by the intravenous administration of phosphoramidon (endopeptidase inhibitor) at a dose of 50 μg kg^{-1}. This compound had no effect on AA-induced ear oedema at less than 500 μg kg^{-1} (Table 2). Phosphoramidon itself did not have any effect on the ear, and bestatin (500 μg kg^{-1}, i.v.), an aminopeptidase inhibitor, did not enhance the inflammatory response.

Direct effect of neuropeptides given by intradermal injection into mouse ear

As phosphoramidon enhanced the capsaicin-induced ear oedema, participation of neuropeptides such as CGRP, SP, NKA, and VIP on oedema formation was examined by their intradermal injection into the ear. The ear injected with CGRP, SP, NKA, and VIP (10 or 100 pmol per site) swelled significantly ($P<0.05$ or $P<0.01$) (Figure 3). NKA caused marked ear oedema ($P<0.01$) even when injected at a low concentration (1 pmol per site). Furthermore, we examined the possibility of interaction of neuropeptides in the development of ear oedema (Figure 4). CGRP (1 pmol per site) and SP (10 pmol per site) themselves did not produce significant oedema: there were no differences in ear thickness from the control. However, ear oedema was induced ($P<0.001$) when SP and CGRP were injected together into ear and additionally SP (10 pmol per site) promoted oedema formation ($P<0.001$) in the presence of CGRP (10 fmol per site). But

Table 1b Effects of pharmacological agents on capsaicin- and arachidonic acid (AA)-induced mouse ear oedema

Class	*Dose* (mg kg^{-1}, ear)	*Route*	*Inhibition* (%) *Capsaicin oedema*	*AA oedema*
Capsaicin antagonist				
Ruthenium red	0.5	i.v.	49 ± 7*	
	1.0		70 ± 8**	10 ± 5
Substance P antagonist				
[D-Pro^2,D-Trp^{7,9}]-SP	0.25	i.v.	− 9 ± 18	− 3 ± 7
	0.50		53 ± 7**	41 ± 2***
[D-Arg^1,D-Trp^{7,9},Leu^{11}]-SP (Spantide)	0.25	i.v.	19 ± 7	38 ± 7**
	0.50		40 ± 4*	59 ± 4***
VIP antagonist				
[D-P-Cl-Phe^6,Leu^{17}]-VIP	0.50	i.v.	32 ± 19	21 ± 7
[Ac-Tyr^1,D-Phe^2]-GRF (1-29) amide	0.50	i.v.	27 ± 4	not tested
PGAS 385	0.50	i.v.	25 ± 9	1 ± 5
CGRP antagonist				
CGRP fragment$_{8-37}$	0.50	i.v.	− 1 ± 14	3 ± 1
Bradykinin antagonist				
Nα-adamantaneacetyl-D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK	0.50	i.v.	38 ± 8	− 4 ± 6
D-Arg,[Hyp^3,D-Phe^7]-BK	0.50	i.v.	21 ± 13	− 9 ± 4
[Thi^{5,8},D-Phe^7]-BK	0.50	i.v.	21 ± 12	− 19 ± 6
[Leu^8,desArg^9]-BK	0.50	i.v.	32 ± 6	2 ± 4

Test compounds were administered intravenously 15 min before irritant treatment. Values are expressed as mean ± s.e.mean of 6–7 animals. Statistical significance from the control at $*P<0.05$, $**P<0.01$ and $***P<0.001$.

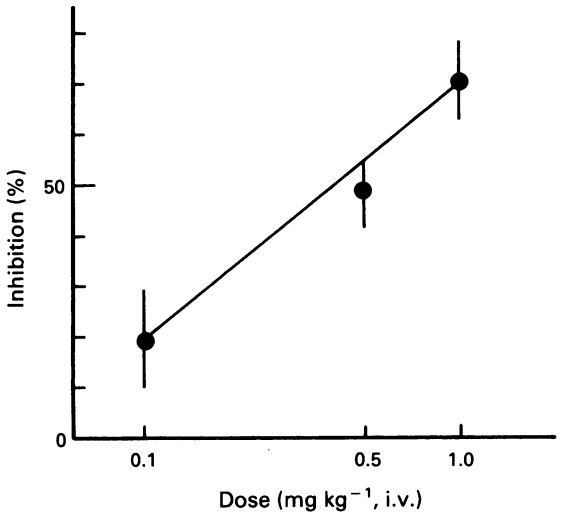


Figure 2 The inhibitory effect of ruthenium red on capsaicin-induced mouse ear oedema. The dye was administered 15 min (i.v.) before capsaicin treatment (250 μg per ear). Ear oedema was measured 30 min after treatment. Each point represents the mean ± s.e.mean value from 4–5 experiments ($n = 6$).

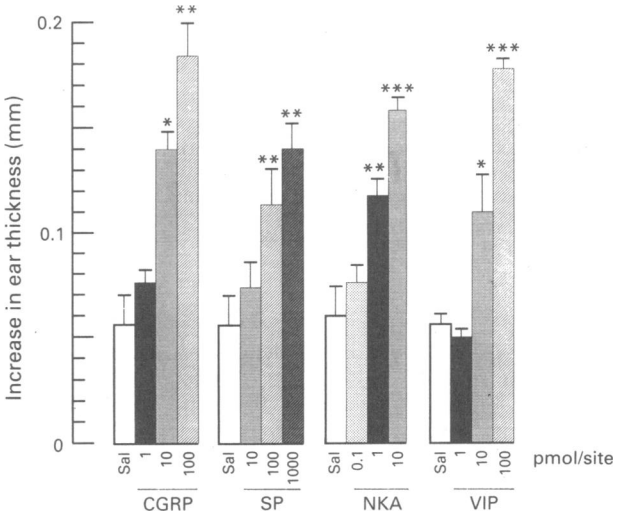


Figure 3 The effect of neuropeptides on oedema formation by intradermal injection. Ear oedema is expressed as increase in ear thickness of test animals at 30 min after treatment. Each column represents mean value (with s.e.mean) from 6 animals. Statistical significance at $*P<0.05$, $**P<0.01$, and $***P<0.001$.

Table 2 Enhancement by phosphoramidon of capsaicin-induced ear oedema

	Dose (μg kg^{-1})	*Increase in ear thickness* (mm) *Capsaicin oedema*	*AA oedema*
Control		0.134 ± 0.008	0.319 ± 0.021
Phosphoramidon	25	0.120 ± 0.013	
	50	0.203 ± 0.009***	
	100	0.330 ± 0.013***	
	500		0.307 ± 0.015
Control		0.107 ± 0.004	not tested
Bestatin	250	0.115 ± 0.005	
	500	0.104 ± 0.008	

Compounds were administered intravenously 15 min before irritant treatment. Values are expressed as mean ± s.e.mean of 6–7 animals. Statistical significance at $***P<0.001$.

concomitant injection of CGRP (1 pmol per site) together with SP, at a dose of less than 1 pmol per site, failed to produce oedema. This synergistic effect of CGRP and SP on oedema formation was observed at much lower doses than those previously reported in the rat (Gamse & Saria, 1985; Cruwys *et al.*, 1992).

Effect of indomethacin, nifedipine, and cyproheptadine on neuropeptide-induced ear oedema

We examined some pharmacological agents on mouse ear oedema induced by neuropeptides such as SP, CGRP, NKA, and VIP to clarify whether these peptides cause oedema directly or indirectly by promoting the release of chemical mediators leading to the inflammatory response. Active agents were given 30 min before peptide treatment. Indomethacin, a cylco-oxygenase inhibitor, significantly ($P<0.01$)

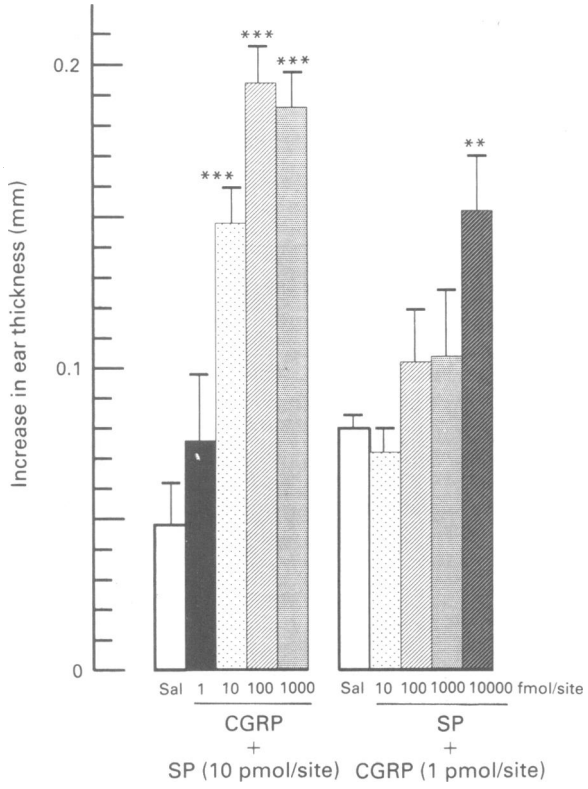


Figure 4 The synergistic effect of calcitonin gene-related peptide (CGRP) and substance P (SP) on oedema formation by concomitant injection. Oedema was induced by co-injected SP (10 pmol) with various concentrations of CGRP (1–1000 fmol) per site or co-injected CGRP (1 pmol) with various concentrations of SP (10–10000 fmol) per site. Ear oedema is expressed as increase in ear thickness of test animals at 30 min after treatment. Each column represents mean value with s.e.mean from 6 animals. Statistical significance from the control (Sal) at $^{**}P<0.01$ and $^{***}P<0.001$.

suppressed NKA-induced ear oedema at a dose of 10 mg kg^{-1} (p.o.) but CGRP-, SP-, and VIP-induced oedema were not affected (Figure 5). Nifedipine (50 mg kg^{-1}, p.o.), a Ca^{2+}-channel blocker, had little effect on any oedema. In contrast, cyproheptadine (20 mg kg^{-1}, p.o.), a histamine H_1 and 5-HT antagonist, strongly inhibited ear oedema induced by neuropeptides (1–100 pmol per site). Of the peptide antagonists (0.5 mg kg^{-1}, i.v.), spantide was a potent inhibitor ($P<0.001$) of SP oedema, and PGAS 385 also prevented ($P<0.05$) VIP-induced oedema. However, $CGRP_{8-37}$ did not suppress CGRP-induced oedema.

Discussion

The topical application of capsaicin to rat skin leads to excitation of afferent neurones (Kenins, 1982), increases in skin blood flow (Jancsó, 1968), and vasodilatation (Lynn *et al.*, 1992). In our experiment, the capsaicin-treated mouse ear became erythematous and oedematous, reaching a maximum effect at 30 min. On the other hand, a single application of AA (2 mg per ear) to the mouse ear resulted in a maximal peak of oedema formation 1 h after treatment. It is well known that the products of arachidonate pathway primarily participate in the AA-induced ear oedema, and the synthesis of prostaglandin E_2 (PGE_2), leukotriene C_4 (LTC_4) and LTD_4 is induced immediately after AA treatment (Young *et al.*, 1984; Opas *et al.*, 1985; Chang *et al.*, 1986; Inoue *et al.*, 1988).

In the present study, inhibitors of arachidonate metabolites were very effective in suppressing AA-induced ear oedema. These compounds, however, had little effect on capsaicin-induced mouse ear oedema. In addition, indomethacin (cyclo-oxygenase inhibitor) failed to prevent oedema induced by neuropeptides, except for NKA. These findings suggest that prostaglandins and leukotrienes of arachidonate metabolites cannot primarily mediate the development of capsaicin-induced ear oedema. It is also unlikely that the participation of PAF is essential for the development of capsaicin-induced ear oedema, although PAF is a candidate for mediation of AA-induced oedema (Merlos *et al.*, 1991). In contrast, histamine and 5-HT are involved in capsaicin- and AA-induced ear oedema, since their antagonists were inhibitors of both. Others have demonstrated that antagonists of histamine H_1 and SP can inhibit capsaicin-induced rat paw oedema (De & Ghosh, 1990). Similarly, SP antagonists such as [D-Pro^2,D-Trp^{7,9}]-SP and spantide are effective inhibitors of oedema induced by both agents suggesting that SP release is contributing to inflammatory response in both models.

Ruthenium red, an inorganic dye, suppressed capsaicin-induced ear oedema dose-dependently, while AA-induced oedema was unaffected. Other studies have shown that the dye significantly inhibits capsaicin-evoked release of neuropeptides (Maggi *et al.*, 1988; Amann *et al.*, 1989) by blocking the opening of the cation channel coupled to the capsaicin receptor (Maggi *et al.*, 1989a; Dray *et al.*, 1990). It is established that capsaicin evokes an influx of extracellular Ca^{2+} (Jancsó *et al.*, 1984; Zernig *et al.*, 1984) and acts by stimulating sensory C-fibre afferents through an effect on cation channels (Marsh *et al.*, 1987; review by Maggi & Meli, 1988). We have also found that some Ca^{2+}-channel blockers are able to inhibit capsaicin-induced oedema. Release of neurotransmitter is generally considered to require the presence of extracellular Ca^{2+}, but capsaicin-evoked release of neuropeptides was not reduced by blockade of voltage-sensitive Ca^{2+}-channels (Marsh *et al.*, 1987; Wood *et al.*, 1988; Maggi *et al.*, 1989b). Nevertheless, in our study, ruthenium red and Ca^{2+}-channel blockers with L-type effects such as nifedipine and verapamil were very effective in preventing the development of capsaicin oedema. In addition to this, nifedipine did not affect the oedema induced by intradermal administration of neuropeptides. Thus our data suggest that the inhibitory actions of the dye and Ca^{2+}-channel blockers on capsaicin oedema would either interrupt the coupling of the capsaicin recognition site or an increase in membrane permeability to cations, or some other way with the activation of the channels. Furthermore, it is possible that Ca^{2+} plays an important role as a second messenger in the initial stage of capsaicin-induced ear oedema, although this is a subject of debate.

The administration of phosphoramidon, a specific inhibitor of endopeptidase-24,11, resulted in a remarkable enhancement of the capsaicin-induced ear oedema. This enzyme can hydrolyze a variety of neuropeptides such as SP and NKA (Matsas *et al.*, 1984). Previous studies have shown that phosphoramidon enhanced tachykinin-induced behavioural responses of mice (Sakurada *et al.*, 1990) and that levels of SP and NKA released by capsaicin increased in guinea-pig lungs superfused with endopeptidase inhibitor (Martins *et al.*, 1991). This evidence suggests that phosphoramidon enhances the inflammatory response of capsaicin oedema by inhibiting endogenous endopeptidase in mouse ear, and the short duration of ear oedema is due to the rapid metabolism of neuropeptides by endopeptidase(s) in the ear.

We have shown that SP, CGRP, NKA, and VIP in pmol doses can produce ear oedema which is substantially reduced by cyproheptadine (a histamine H_1 and 5-HT antagonist) and certain specific neuropeptide antagonists. However, the CGRP antagonist, $CGRP_{8-37}$, was ineffective in inhibiting both capsaicin- and CGRP-induced ear oedema. This result suggests that CGRP-induced development of mouse ear oedema is dependent on CGRP-induced mast cell activation and independent of the $CGRP_1$ receptor. Furthermore, our

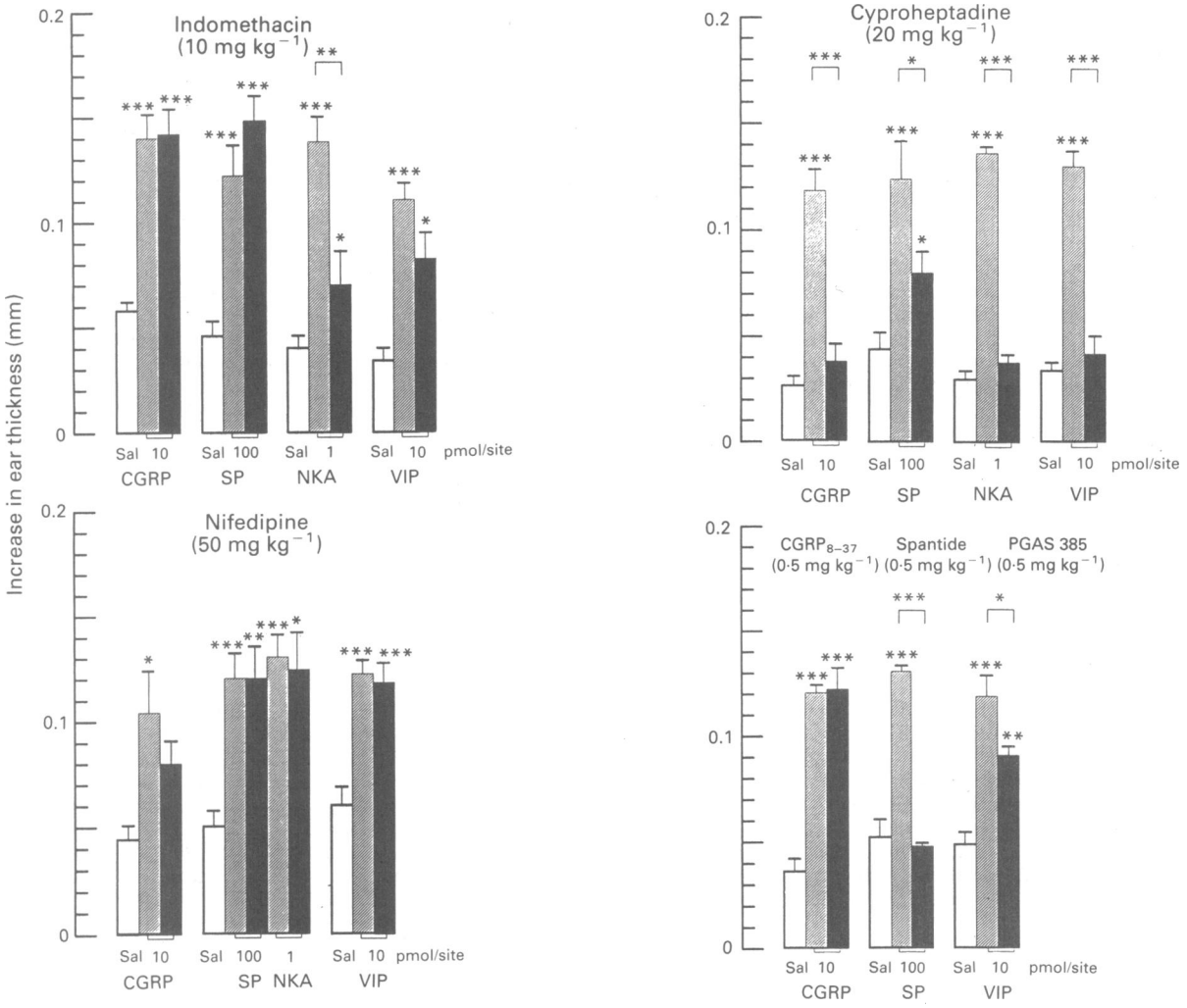


Figure 5 The effect of indomethacin, nifedipine, and cyproheptadine and peptide antagonists on neuropeptide-induced ear oedema. Ear oedema is expressed as increase in ear thickness of test animals at 30 min after intradermal injection. Test compounds were administered (p.o.) 30 min before injection except for peptide antagonists which were given at 15 min (0.5 mg kg^{-1}, i.v.) before injection. Open columns: saline (control); hatched columns: neuropeptide alone; solid columns: neuropeptide plus test compound treatment. Each column represents mean value (with s.e.mean) from 6 animals. Statistical significance at $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

studies confirmed that the synergistic effect of neuropeptides such as CGRP and SP in fmol could be participating in the development of ear oedema. Neuropeptides have been reported to release histamine from mast cells (Piotrowski & Foreman, 1985; 1986; Devillier *et al.*, 1986), but capsaicin did not cause histamine release from mast cells directly (data not shown). Additionally, mast cells are numerous around blood vessels and nerves (Nilsson *et al.*, 1990); this is further support for the suggestion that the neuropeptides evoked by capsaicin through activation of sensory nerves are able to release histamine (and 5-HT) from mast cells as the chemical mediator, and this response is an essential pathway in the development of capsaicin-induced ear oedema. In addition to this, it is very likely that the neuropeptides caused oedema formation by increasing vascular permeability through the activation of endothelial cells, as reported by others (Iwamoto & Nadel, 1989).

In conclusion, our studies demonstrate that the mechanism of capsaicin-induced mouse ear oedema is quite different from that of AA-induced oedema and that neuropeptides such as CGRP, SP, VIP, and NKA can induce oedema formation, and suggest that neuropeptides play important roles of releasing histamine (and 5-HT) as the secondary mediator of increased vascular permeability at inflammatory sites. Thus it is likely that the capsaicin-induced ear oedema is primarily mediated by neuropeptides evoked by capsaicin through activation of sensory nerves in the mouse skin. However, it is also possible that other peptides and inflammatory mediators are involved in the development of capsaicin-induced oedema.

The authors are indebted to Dr Charles R. Mantione, Nova Pharmaceutical Corporation and Prof. Terumi Nakajima, Faculty of Pharmaceutical Science, The University of Tokyo, for their helpful advices. We also express our special thanks to Dr Shoji Shibata and Dr Kensuke Shimura, Minophagen Research Laboratory, for their kind cooperation.

References

AMANN, R., DONNERER, J. & LEMBECK, F. (1989). Ruthenium red selectively inhibits capsaicin-induced release of calcitonin gene-related peptide from the isolated perfused guinea pig lung. *Neurosci. Lett.*, **101**, 311–315.

AMANN, R. & MAGGI, C.A. (1991). Ruthenium red as a capsaicin antagonist. *Life Sci.*, **49**, 849–856.

BRAIN, S.D. & WILLIAMS, T.J. (1988). Substance P regulates the vasodilator activity of calcitonin gene-related peptide. *Nature*, **335**, 73–75.

CARLSON, R.P., O'NEILL-DAVIS, L., CHANG, J. & LEWIS, A.J. (1985). Modulation of mouse ear edema by cyclooxygenase and lipoxygenase inhibitors and other pharmacologic agents. *Agents Actions*, **17**, 197–204.

CHANG, J., CARLSON, R.P., O'NEILL-DAVIS, L., LAMB, B., SHARMA, R.N. & LEWIS, A.J. (1986). Correlation between mouse skin inflammation induced by arachidonic acid and eicosanoid synthesis. *Inflammation*, **10**, 205–214.

CRUWYS, S.C., KIDD, B.L., MAPP, P.I., WALSH, D.A. & BLAKE, D.R. (1992). The effects of calcitonin gene-related peptide on formation of intra-articular oedema by inflammatory mediators. *Br. J. Pharmacol.*, **107**, 116–119.

DE, A.K. & GHOSH, J.J. (1990). Comparitive studies on the involvement of histamine and substance P in the inflammatory response of capsaicin in rat paw. *Phytother. Res.*, **4**, 42–44.

DEVILLIER, P., REGOLI, D., ASSERAF, A., DESCOURS, B., MARSAC, J. & RENOUX, M. (1986). Histamine release and local responses of rat and human skin to substance P and other mammalian tachykinins. *Pharmacology*, **32**, 340–347.

DRAY, A., FORBES, C.A. & BURGESS, G.M. (1990). Ruthenium red blocks the capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory neurones as well as the activation of peripheral nociceptors in vitro. *Neurosci. Lett.*, **110**, 52–59.

FARBER, E.M., NICKOLOFF, B.J., RECHT, B. & FRAKI, J.E. (1986). Stress, symmetry and psoriasis: possible role of neuropeptides. *J. Am. Acad. Dermatol.*, **14**, 305–311.

GABÓR, M. & RÁZGA, Z. (1992) Development and inhibition of mouse ear oedema induced with capsaicin. *Agents Actions*, **36**, 83–86.

GAMSE, R. & SARIA, A. (1985). Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. *Eur. J. Pharmacol.*, **114**, 61–66.

HOLZER, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience*, **24**, 739–768.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.*, **43**, 143–201.

INOUE, H., MORI, T. & KOSHIHARA, Y. (1988). Sulfidopeptide-leukotrienes are major mediators of arachidonic acid-induced mouse ear edema. *Prostaglandins*, **36**, 731–739.

IWAMOTO, I. & NADEL, J.A. (1989). Tachykinin receptor subtype that mediates the increase in vascular permeability in guinea pig skin. *Life Sci.*, **44**, 1089–1095.

JANCSÓ, G., KARCSÚ, S., KIRÁLY, E., SZEBENI, A., TÓTH, L., BÁCSY, E., JOÓ, F. & PÁRDUCZ, Á. (1984). Neurotoxin induced nerve cell degeneration: possible involvement of calcium. *Brain Res.*, **295**, 211–216.

JANCSÓ, N. (1968). Desensitization with capsaicin and related acylamides as a tool for studying the function of pain receptors. In *Pharmacology of Pain*. ed. Lim, R.K.S., Armstrong, D. & Pardo, E.G. pp. 33–35. Oxford: Pergamon.

KENINS, P. (1982). Responses of single nerve fibers to capsaicin applied to the skin. *Neurosci. Lett.*, **29**, 83–88.

LARSSON, J., EKBLOM, A., HENRIKSSON, K., LUNDEBERG, T. & THEODORSSON, E. (1989). Immunoreactive tachykinins, calcitonin gene-related peptide and neuropeptide Y in human synovial fluid from inflamed knee joints. *Neurosci. Lett.*, **100**, 326–330.

LYNN, B., YE, W. & COTSELL, B. (1992). The actions of capsaicin applied topically to the skin of the rat on C-fiber afferents, antidromic vasodilation and substance P levels. *Br. J. Pharmacol.*, **107**, 400–406.

MAGGI, C.A. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19**, 1–43.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GIULIANI, S., BIANCO, E.D., GEPPETTI, P. & MELI, A. (1989a). The efferent function of capsaicin-sensitive nerves: ruthenium red discriminates between different mechanisms of activation. *Eur. J. Pharmacol.*, **170**, 167–177.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., PARLANI, M., ASTOLFI, M., PRADELLES, P., PATACCHINI, R. & MELI, A. (1988). The antagonism induced by ruthenium red of the actions of capsaicin on the peripheral terminals of sensory neurons: further studies. *Eur. J. Pharmacol.*, **154**, 1–10.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., PARLANI, M., ASTOLFI, M., BIANCO, E.D., PATACCHINI, R., GIULIANI, S. & MELI, A. (1989b). The effect of calcium free medium and nifedipine on the release of substance P-like immunoreactivity and contractions induced by capsaicin in the isolated guinea-pig and rat bladder. *Gen. Pharmacol.*, **20**, 445–456.

MANTIONE, C.R. & RODRIGUEZ, R. (1990). A bradykinin $(BK)_1$ receptor antagonist blocks capsaicin-induced ear inflammation in mice. *Br. J. Pharmacol.*, **99**, 516–518.

MARSHALL, K.W., CHIU, B. & INMAN, R.D. (1990). Substance P and arthritis: analysis of plasma and synovial fluid levels. *Arthritis Rheum.*, **33**, 87–90.

MARSH, S.J., STANSFELD, C.E., BROWN, D.A., DAVEY, R. & MCCARTHY, D. (1987). The mechanism of action of capsaicin on sensory C-type neurons and their axons in vitro. *Neuroscience*, **23**, 275–289.

MARTINS, M.A., SHORE, S.A. & DRAZEN, J.M. (1991). Capsaicin-induced release of tachykinins: effects of enzyme inhibitors. *J. Appl. Physiol.*, **70**, 1950–1956.

MATSAS, R., KENNY, A.J. & TURNER, A.J. (1984). The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins; tachykinins and their analogues, by endopeptidase-24,11. *Biochem. J.*, **223**, 433–440.

MERLOS, M., GÓMEZ, L.A., GIRAL, M., VERICAT, M.L., GARCIA-RAFANELL, J. & FORN, J. (1991). Effects of PAF-antagonists in mouse ear oedema induced by several inflammatory agents. *Br. J. Pharmacol.*, **104**, 990–994.

NILSSON, G., ALVING, K., AHLSTEDT, S., HÖKFELT, T. & LUNDBERG, J.M. (1990). Peptidergic innervation of rat lymphoid tissue and lung: relation to mast cells and sensitivity to capsaicin and immunization. *Cell Tissue Res.*, **262**, 125–133.

OPAS, E.E., BONNEY, R.J. & HUMES, J.L. (1985). Prostaglandin and leukotriene synthesis in mouse ears inflamed by arachidonic acid. *J. Invest. Dermatol.*, **84**, 253–256.

PIOTROWSKI, W. & FOREMAN, J.C. (1985). On the actions of substance P, somatostatin, and vasoactive intestinal polypeptide on rat peritoneal mast cells and in human skin. *Naunyn-Schmied. Arch. Pharmacol.*, **331**, 364–368.

PIOTROWSKI, W. & FOREMAN, J.C. (1986). Some effects of calcitonin gene-related peptide in human skin and on histamine release. *Br. J. Dermatol.*, **114**, 37–46.

SAKURADA, T., TAN-NO, K., YAMADA, T., SAKURADA, S. & KISARA, K. (1990). Phosphoramidon potentiates mammalian tachykinin-induced biting, licking, and scratching behaviour in mice. *Pharmacol. Biochem. Behav.*, **37**, 779–783.

SARIA, A., MARTLING, C.-R., YAN, Z., THEODORSSON-NORHEIM, E., GAMSE, R. & LUNDBERG, J.M. (1988). Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *Am. Rev. Respir. Dis.*, **137**, 1330–1335.

WALLENGREN, J., EKMAN, R. & MÖLLER, H. (1986). Substance P and vasoactive intestinal peptide in bullous and inflammatory skin disease. *Acta Derm. Venereol. (Stockh.)*, **66**, 23–28.

WALLENGREN, J., MÖLLER, H. & EKMAN, R. (1987). Occurrence of substance P, vasoactive intestinal peptide, and calcitonin gene-related peptide in dermographism and cold urticaria. *Arch. Dermatol. Res.*, **279**, 512–515.

WILLIAMS, T.J. (1982). Vasoactive intestinal polypeptide is more potent than prostaglandin E_2 as a vasodilator and oedema potentiator in rabbit skin. *Br. J. Pharmacol.*, **77**, 505–509.

WOOD, J.N., WINTER, J., JAMES, I.F., RANG, H.P., YEATS, J. & BEVAN, S. (1988). Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neuroscience*, **8**, 3208–3220.

YOUNG, J.M., SPIRES, D.A., BEDORD, C.J., WAGNER, B., BALLARON, S.J. & DEYOUNG, L.M. (1984). The mouse ear inflammatory response to topical arachidonic acid. *J. Invest. Dermatol.*, **82**, 367–371.

ZERNIG, G., HOLZER, P. & LEMBECK, F. (1984). A study of the mode and site of action of capsaicin in guinea-pig heart and rat uterus. *Naunyn-Schmied. Arch. Pharmacol.*, **326**, 58–63.

(Received April 19, 1993
Revised August 23, 1993
Accepted August 31, 1993)

Br. J. Pharmacol. (1993), **110**, 1621–1629 © Macmillan Press Ltd, 1993

Evidence that RU 24969-induced locomotor activity in C57/B1/6 mice is specifically mediated by the 5-HT_{1B} receptor

[1]S.C. Cheetham & D.J. Heal

Boots Pharmaceuticals Research Department, Nottingham, NG2 3AA

1 The behavioural effects of the 5-HT_{1B} receptor agonists, RU 24969 and CGS 12066B, have been investigated in C57/B1/6 mice.

2 RU 24969 (1–30 mg kg^{-1}) produced intense and prolonged hyperlocomotion and other behavioural changes.

3 CGS 12066B caused similar effects, but they were much less pronounced, inconsistent and transient irrespective of whether this drug was given i.p. (1–15 mg kg^{-1}) or i.c.v. (0.2–40 μg). However, CGS 12066B (7.5 and 15 mg kg^{-1}) caused a dose-related inhibition of RU 24969 (7.5 mg kg^{-1})-induced hyperlocomotion indicating that the former is a 5-HT_{1B} partial agonist.

4 RU 24969 (7.5 mg kg^{-1} i.p.)-induced hyperlocomotion was inhibited by the (−)-, but not (+)-isomers of pindolol (4 mg kg^{-1}) and propranolol (20 mg kg^{-1}) but not by metoprolol (10 mg kg^{-1}) or ICI 118,551 (5 mg kg^{-1}), consistent with an involvement of 5-HT_{1A} or 5-HT_{1B} receptors.

5 The response was not altered by the selective 5-HT_{1A} receptor antagonist, WAY 100135 (5 mg kg^{-1}, s.c.), the 5-HT_{2A}/5-HT_{2C} receptor antagonist, ritanserin (0.1 mg kg^{-1}), the selective 5-HT_3 receptor antagonist, ondansetron (1 mg kg^{-1}) or the non-selective 5-HT receptor antagonists methysergide (3 mg kg^{-1}) and metergoline (3 mg kg^{-1}).

6 Although spiroxatrine (0.1 mg kg^{-1}) and ketanserin (1 mg kg^{-1}) inhibited RU 24969-induced hyperlocomotion, these effects were probably due to antagonism of dopamine D_2 receptors and α_1-adrenoceptors respectively.

7 Taken together, these results indicate that RU 24969-induced hyperlocomotion results specifically from activation of central 5-HT_{1B} receptors.

8 Lesioning of 5-HT neurones with 5,7-dihydroxytryptamine (75 μg, i.c.v.) or depletion with *p*-chlorophenylalanine (200 mg kg^{-1}, i.p. for 14 days) had no effect on RU 24969-induced hyperlocomotion demonstrating that the 5-HT_{1B} receptors involved are postsynaptic and that they do not show supersensitivity.

9 The involvement of other monoamine neurotransmitter systems in RU 24969-induced hyperlocomotion was also examined. The response was inhibited by the α_1-adrenoceptor antagonist, prazosin (1 mg kg^{-1}), the dopamine D_1 receptor antagonist, SCH 23390 (0.05 mg kg^{-1}) and the dopamine D_2 receptor antagonist, BRL 34778 (0.03 mg kg^{-1}), but not by the α_2-adrenoceptor antagonist, idazoxan (1 mg kg^{-1}). Lesioning noradrenergic neurones with N-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (100 mg kg^{-1}) markedly attenuated this behaviour. These results show that the hyperlocomotion is expressed via noradrenergic and dopaminergic neurones acting on α_1-adrenoceptors, D_1 and D_2 receptors.

10 RU 24969 decreased brain concentrations of 5-hydroxyindoleacetic acid whilst simultaneously increasing 5-HT, consistent with the reduction of 5-HT neuronal activity by activation of 5-HT_{1A} and 5-HT_{1B} autoreceptors. RU 24969 increased brain 3-methoxy-4-hydroxyphenylglycol, but not noradrenaline, concentrations which supports the involvement of noradrenergic neurones in the expression of hyperlocomotion. RU 24969 did not alter dopamine, dihydroxyphenylacetic acid or homovanillic acid concentrations in the nucleus accumbens suggesting that the dopaminergic neurones terminating there are not directly involved.

Keywords: RU 24969; CGS 12066B; hyperlocomotion; locomotor activity in mice; 5-HT_{1B} receptors; behavioural model; dopamine; noradrenaline

Introduction

5-Hydroxytryptamine (5-HT) receptors were first classified as 5-HT_1 and 5-HT_2 by Peroutka *et al.* (1981) based on their respective affinities for [^{3}H]-5-HT and [^{3}H]-spiperone. It is now well established that there are at least five subtypes of 5-HT_1 receptor (1A, 1B, 1D, 1E, 1F) (reviewed by Humphrey *et al.*, 1993). RU 24969 (5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole succinate) was initially described as a selective 5-HT_1 receptor agonist (Hunt & Oberlander, 1981; Green *et al.*, 1984) and subsequent evaluation revealed that RU 24969 has high and approximately equal affinity for 5-HT_{1B} and 5-HT_{1A} receptors *in vitro* (Hoyer *et al.*, 1985). The most obvious behavioural consequence of RU 24969 administration to mice or rats is the induction of intense locomotor activity without production of either the 5-HT_{1A}-mediated 5-HT syndrome or 5-HT_{2A}-mediated head-twitch behaviour (Green *et al.*, 1984; Nisbet & Marsden, 1984). RU 24969-induced hyperlocomotion has been pharmacologically characterized extensively in rats where evidence for specific mediation by central 5-HT_{1B} receptors is poor and conflicting (Goodwin & Green, 1985; Tricklebank *et al.*, 1986). By contrast, although the early investigations in mice appeared to be more promising (Green *et al.*, 1984; Goodwin & Green, 1985), this behavioural response was not studied further. In view of this, work has now been carried out to characterize RU 24969-induced hyperlocomotion in mice using a wide range of 5-HT receptor subtype-selective antagonists. In addition the synaptic location of the receptors mediating this

[1] Author for correspondence.

response has been determined by the use of selective 5-HT neuronal lesioning and neurotransmitter depletion techniques. The involvement of other monoamines in the expression of the locomotor behaviour has also been elucidated with selective antagonists, lesioning and neurotransmitter depletion and turnover methods.

Recently, CGS 12066B (7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2a]quinoxaline dimaleate) has been claimed to have selective 5-HT_{1B} receptor agonist activity (Neale *et al.*, 1987) and the behavioural consequences of administering this compound to mice have also been studied.

Methods

Animals

Adult male C57/Bl/6Ola mice (Olac) weighing 22–30 g were used. They were housed in groups of 8–10 on a 12 h light/dark cycle (commencing 06.00 h) at a temperature of 21°C and 55% humidity. Mice were allowed food and water *ad libitum*. Experiments were performed between 08.00 and 13.00 h.

Behavioural studies

Effect of RU 24969 on locomotor activity Locomotor activity was measured using perspex boxes (48 × 26.5 × 30 cm) with a wire grid floor. Three horizontal infrared photocell beams were located along the short axis of the cage and one along the long axis approximately 4.5 cm above the floor. Photocell beam interruptions were recorded by an on-line microcomputer (BBC Master Series). This system was designed, built and programmed in-house by the Bioelectronics Group, Boots Pharmaceuticals Research Department.

As in the previously described protocol by De Souza *et al.* (1986) for the measurement of hyperlocomotion after intracerebroventricular (i.c.v.) injection of RU 24969, pairs of mice were used. These were allowed 60 min habituation to the new environment and then administered RU 24969 (1–30 mg kg^{-1}), CGS 12066B (1–15 mg kg^{-1} or 0.2–40 μg, i.c.v.) or vehicle. Antagonists or the appropriate vehicle were given 30 min prior to RU 24969 (7.5 mg kg^{-1}) or saline to determine the effects on hyperlocomotion and control activity. Antagonist doses are based on results of previous behavioural experiments. Locomotor activity was recorded for 60 min after the final injection and data are expressed as the accumulated number of photocell interruptions during this period.

The dose of RU 24969 required to produce 50% of the maximum response was determined by linear regression analysis following log transformation of the data. Comparisons between antagonist and vehicle-treated groups were made by Student's *t* test (two-tailed, unpaired).

Comparison of the behavioural effects of RU 24969 and CGS 12066B Pairs of mice were administered RU 24969 (7.5 or 75 mg kg^{-1}) and CGS 12066B (10 or 25 mg kg^{-1}). Fifteen minutes later mice were assessed for the following behavioural parameters: locomotor activity (including pattern of activity), posture, sniffing, grooming, piloerection, Straub tail, forepaw treading, hindlimb abduction, head-bobbing, head-twitching, tremor and shivering and jumping. Each behavioural parameter was rated as absent, mild, moderate or intense with the exception of posture which was described as normal, lowered or hunched. Rectal temperature was measured before and 60 min after drug administration and is expressed as decrease in °C.

Neuronal lesioning and monoamine depletion experiments Depletion of brain 5-HT concentrations was achieved either by destruction of 5-HT neurones with the neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), or by inhibition of 5-HT synthesis with *p*-chlorophenylalanine (PCPA).

For lesioning experiments, mice were pretreated with desipramine (25 mg kg^{-1}) to protect central noradrenaline-containing neurones. Fifteen minutes later, the animals were anaesthetized with hexobarbitone (50 mg kg^{-1}). After a further 15 min, 5,7-DHT (75 μg) dissolved in ice-cold saline containing 0.4 mg ml^{-1} ascorbic acid was administered i.c.v. in a volume of 4 μl by use of the stereotaxic injector described by Heal (1984). Sham-lesioned mice were injected with vehicle (4 μl, i.c.v.) Mice were then housed individually and allowed to recover for 14 days. The synthesis inhibitor PCPA (200 mg kg^{-1}) was given once daily for 14 days. Control mice received saline.

Verification of substantial 5-HT depletion was initially performed by measurement of 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeODMT)-induced head-twitches because there is a good correlation between this response and the extent of 5-HT denervation (Heal *et al.*, 1985). In the PCPA experiments, the head-twitch responses were determined 6 h prior to the final dose of PCPA and in the 5,7-DHT experiments on day 15. Mice showing satisfactory responses (75% increase in the head-twitch response to 5-MeODMT above mean control value) were allowed 24 h to recover before locomotor testing.

Destruction of brain noradrenergic neurones was carried out with the selective neurotoxin, N-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4). Mice were pretreated with zimeldine (5 mg kg^{-1}) to protect central 5-HT-containing neurones and DSP-4 (100 mg kg^{-1}) was administered 30 min later. Sham-lesioned mice received saline. Mice were then allowed to recover for 7 days.

Depletion of brain noradrenaline, dopamine and 5-HT concentrations was achieved by inhibition of neurotransmitter uptake into the storage vesicles by reserpine (0.25–2 mg kg^{-1} in 50 mg ml^{-1} ascorbic acid). Control mice were injected with vehicle. Mice were then allowed to recover for 18 h.

Verification of the selectivity and extent of monoamine depletion was determined by measurement of brain 5-HT, noradrenaline and dopamine concentrations by high performance liquid chromatography (h.p.l.c.) with electrochemical detection by methods described below.

Comparisons between appropriate vehicle and 5,7-DHT, PCPA or DSP-4 treated groups were made with Student's *t* test (two-tailed, unpaired). Statistical comparisons between vehicle- and reserpine-treated groups were made by analysis of variance (Armitage & Berry, 1987) followed by Williams' test (Williams, 1972).

Biochemical studies

Brain monoamine and metabolite concentrations were determined 60 min after RU 24969 (3.75–10 mg kg^{-1}) by h.p.l.c. with electrochemical detection.

5-HT, 5-hydroxyindoleacetic acid (5-HIAA), noradrenaline, dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in whole brain (5-HT, 5-HIAA and noradrenaline) or nucleus accumbens (dopamine, DOPAC, and HVA). Tissue was homogenized in 0.1 M perchloric acid containing 0.4 mM sodium metabisulphite (antioxidant) and 0.8 μM isoprenaline (internal standard). Following centrifugation at 1100 *g* for 15 min at 4°C and 15000 *g* for 5 min at 4°C, 30 μl of the resulting supernatant was injected onto a 5 μm 25 cm Hypersil ODS 1 column maintained at 45°C. The mobile phase was 0.1 M sodium dihydrogen orthophosphate-orthophosphoric acid buffer, pH 3.2 containing 16% v/v methanol, 2.8 mM 1-octanesulphonic acid sodium salt and 0.7 mM EDTA. Monoamines and metabolites were detected with a BAS LC-4A detector (+0.75 V versus an Ag/AgCl reference electrode). Full details are given in Heal *et al.* (1991).

3-Methoxy-4-hydroxyphenylglycol (MHPG) was measured

in whole brain minus cerebellum. Tissue was homogenized in 5 volumes (w/v) of 0.1 M perchloric acid containing 0.1 μM iso-MHPG (internal standard) and 0.4 mM sodium metabisulphite (antioxidant). After centrifugation, 1100 *g* for 15 min at 4°C and 11600 *g* for 5 min at room temperature, MHPG and iso-MHPG were extracted in ethyl acetate, 0.08 M potassium bicarbonate was added and the organic phase evaporated to dryness at 40°C. The residues were dissolved in mobile phase (0.1 M sodium acetate/citric acid buffer, pH 4.4 containing 8% v/v methanol and 4.6 mM 1-octanesulphonic acid sodium salt) and injected onto a 5 μm 25 cm Spherisorb ODS 1 column maintained at 45°C. MHPG and iso-MHPG were detected with a BAS LC-4B detector (+0.75 V versus an Ag/AgCl reference electrode). Full details are given in Heal *et al.* (1989).

Statistical comparisons between control and RU 24969-treated groups were made by analysis of variance (Armitage & Berry, 1987) followed by Williams' test (Williams, 1972).

Drugs and reagents

Drugs were obtained from the following sources (in parentheses): l(−)-ascorbic acid (FSA, Loughborough); PCPA, desipramine HCl, 5,7-DHT, hexobarbitone, 5-MeODMT, metoprolol tartrate, (±)-pindolol, (±)-propranolol HCl, (+)- and (−)-propranolol HCl, reserpine (Sigma, Poole); 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrolo[1,2a]-quinoxaline dimaleate (CGS 12066B), DSP-4, idazoxan HCl, prazosin HCl, ritanserin, (R)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH 23390), spiroxatrine (Research Biochemicals Incorporated, St. Albans); ketanserin tartrate (Janssen Pharmaceuticals, Beerse); metergoline (Farmitalia, Milan); methysergide maleate, (+)- and (−)-pindolol (Sandoz, Horsforth); ondansetron (Medicinal Chemistry, Boots Pharmaceuticals Research Department); 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole succinate (RU 24969) (Roussel-Uclaf, Romainville); exo-4-amino-5-chloro-2-methoxy-N-[9-(4-fluorophenyl methyl)-9-azabicyclo(3.3.1)non-3-yl]-benzamide (BRL 34778) (SmithKline Beecham Pharmaceuticals, Harlow); erytho-(±)-1-(7-methylindan-4-yloxy)-3-isopropyl-aminobutan-2-ol (ICI 118,551) (ICI, Macclesfield) and (+)-N-tert-butyl-3-4-(2-methoxyphenyl)piperazin-1-yl-2-phenylpropanamide 2 HCl ((+)-enantiomer of WAY 100135).

Drugs were dissolved in 0.9% w/v sodium chloride solution (saline) whenever possible. (±)-Pindolol and its enantiomers, BRL 34778, CGS 12066B, ICI 118,551, metergoline, ritanserin and spiroxatrine were dissolved in a few drops of either glacial acetic acid or hydrochloric acid and made up to volume with saline. Prazosin was dissolved in distilled water. All drugs were injected intraperitoneally (i.p.), unless otherwise stated, in weight-related doses (0.1 ml 10 g^{-1} body weight). Control mice were given the appropriate vehicle.

Drugs and reagents for h.p.l.c. analysis were of the highest purity available and were obtained from the following sources: dopamine HCl, 5-hydroxytryptamine creatinine sulphate (5-HT), isoprenaline HCl, noradrenaline HCl (Sigma, Poole); perchloric acid, orthophosphoric acid, potassium bicarbonate, sodium metabisulphite (BDH, Poole); 1-octanesulphonic acid sodium salt, sodium dihydrogen orthophosphate, citric acid, sodium hydroxide (FSA, Loughborough); ethylenediaminetetraacetic acid disodium salt (EDTA; Aldrich, Gillingham); ethyl acetate, methanol (Rathburn Chemicals, Walkerburn) and 3-hydroxy-4-methoxyphenylglycol hemipiperazine (iso-MHPG; Medicinal Chemistry, Boots Pharmaceuticals Research Department). All water was distilled and deionised before use.

Results

When pairs of mice were injected with RU 24969 (1–75 mg kg^{-1}, i.p.) marked hyperlocomotion occurred in bursts accompanied by sniffing, grooming, infrequent head-bobbing and Straub tail (Figure 1, Table 1). The maximum locomotor effects of RU 24969 were observed at 10 mg kg^{-1} and the ED_{50} for this response was 2.6 mg kg^{-1} (Figure 1). Doses

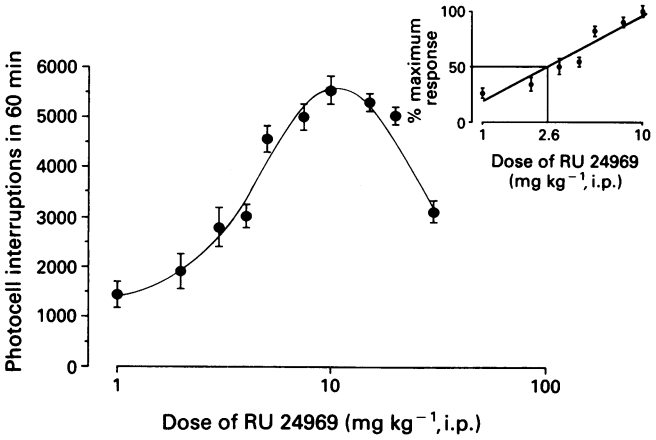


Figure 1 Dose-response relationship for RU 24969-induced hyperlocomotion in C57/Bl/6 mice. Pairs of mice were injected with RU 24969 (1–30 mg kg^{-1}, i.p.). Results are plotted as mean ± s.e.mean; $n = 9–12$ groups of mice. $ED_{50} = 2.6$ mg kg^{-1} (95% confidence limits = 1.9–4.3 mg kg^{-1}). Correlation coefficient = 0.97.

Table 1 Comparison of the behavioural effects of RU 24969 and CGS 12066B

Behavioural parameter	*RU 24969* 7.5 mg kg^{-1}	75 mg kg^{-1}	*CGS 12066B* 10 mg kg^{-1}	25 mg kg^{-1}
Locomotor activity	Moderate	Intense	Mild	Mild
Pattern of activity	Intermittent and co-ordinated. Random pattern in cage	Constant, side to side locomotion. Random pattern in cage	Intermittent and co-ordinated. Random pattern in cage	Intermittent and co-ordinated. Random pattern in cage
Posture	Hunched	Normal	Hunched	Hunched
Sniffing	Moderate	Intense	Mild	Moderate
Grooming	Moderate	Mild	Moderate	Absent
Piloerection	Absent	Absent	Moderate	Moderate
Straub tail	Mild	Mild	Absent	Mild
Forepaw treading	Absent	Absent	Absent	Absent
Hindlimb abduction	Absent	Absent	Absent	Absent
Head-bobbing	Mild	Moderate	Absent	Absent
Head-twitching	Absent	Absent	Absent	Absent
Tremor and shivering	Absent	Moderate	Moderate	Moderate
Jumping	Absent	Absent	Absent	Absent
Temperature decrease	1.9°C	5.6°C	1.6°C	3.2°C

greater than 10 mg kg^{-1} induced smaller increases in locomotor activity which was increasingly displaced by the stereotyped behavioural components of sniffing, head-bobbing and tremor (Table 1). RU 24969 did not induce head-twitches or any component of the 5-HT syndrome (forepaw treading, hindlimb abduction or flat body posture) at any dose. This 5-HT$_1$ agonist did reduce the core body temperature of the mice and this effect was marked at high dosage (Table 1).

CGS 12066B produced a similar pattern of behavioural changes to those observed with RU 24969 (Table 1). However, the behavioural components were less intense than those induced by RU 24969 and the locomotor effect was small and inconsistent when observed after either i.p. (1–15 mg kg^{-1}) or i.c.v. (0.2–40 μg) injection (Figure 2).

Administration of CGS 12066B (7.5 and 15 mg kg^{-1}) 10 min prior to RU 24969 (7.5 mg kg^{-1}) inhibited the hyperlocomotor response induced by the latter drug by 30% and 54%, respectively (Figure 3). The saline response of control mice was not altered by pretreatment with CGS 12066B (15 mg kg^{-1}, data not shown).

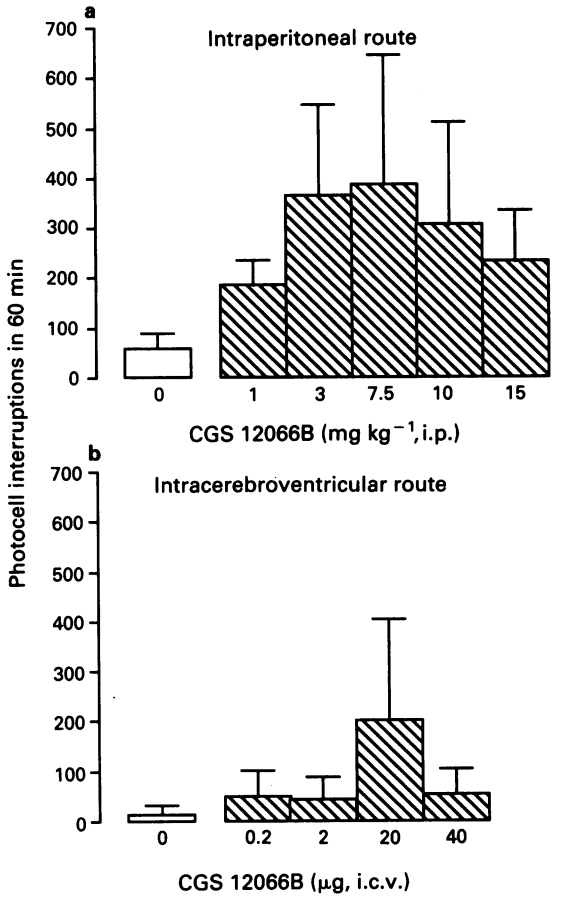


Figure 2 Dose-response relationship for CGS 12066B-induced locomotor activity in C57/B1/6 mice. Pairs of mice were injected with CGS 12066B, 1–15 mg kg^{-1} i.p. (a) or 0.2–40 μg i.c.v. (b). Control mice were injected with vehicle (0.25 ml i.p. (a), or 2 μl i.c.v. (b)). Results are plotted as mean ± s.e.mean; $n = 4$ groups of mice.

The effects of 5-HT receptor antagonists on RU 24969-induced hyperlocomotion

Various 5-HT receptor antagonists or the appropriate vehicle were administered 30 min before injection of RU 24969 (7.5 mg kg^{-1}). The resulting locomotor response was attenuated by (±)-pindolol (4 mg kg^{-1}), (±)-propranolol (20 mg kg^{-1}), spiroxatrine (0.1 mg kg^{-1}) and ketanserin (1 mg kg^{-1}) (Table 2). The (+)-enantiomer of WAY 100135 (5 mg kg^{-1}, s.c.), ritanserin (0.1 mg kg^{-1}), ondansetron (1 mg kg^{-1}), metergoline (3 mg kg^{-1}) and methysergide (3 mg kg^{-1}) were without effect (Table 2). At these doses, metergoline was the only compound that inhibited spontaneous locomotor

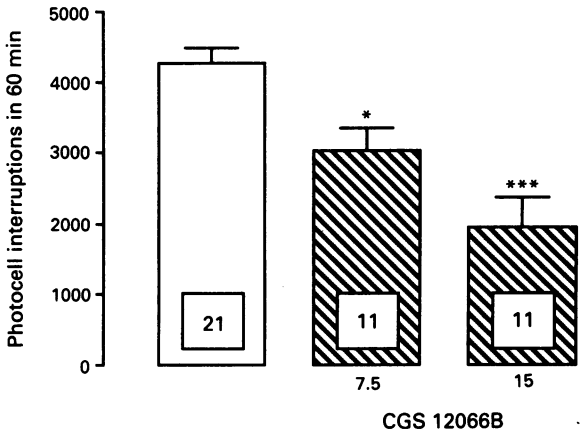


Figure 3 The effects of CGS 12066B on RU 24969-induced hyperlocomotion. Groups of two mice were injected with CGS 12066B (7.5 and 15 mg kg^{-1}, i.p.) 10 min prior to RU 24969 (7.5 mg kg^{-1}, i.p.) shown by the hatched columns. Control mice received vehicle (0.25 ml, i.p.) as shown by the open column. Results are the accumulated number of photocell interruptions in 60 min ± s.e.mean with the number of groups tested shown in each column. Statistical evaluations were performed with Student's t test. Although control data have been pooled for clarity, statistical comparisons were made against the appropriate subgroup of control values. Significantly different from appropriate control. $^*P<0.05$; $^{***}P<0.001$.

Table 2 Effect of various 5-HT receptor antagonists on RU 24969-induced hyperlocomotion in mice

Antagonist	*5-HT receptor selectivity*	*Dose* (mg kg^{-1})	*RU 24969 activity*		*Saline activity*	
			Control	*Antagonist-treated*	*Control*	*Antagonist-treated*
(±)-Pindolol	5-HT$_1$	4	5070 ± 220	3217 ± 272***	231 ± 46	130 ± 47
(±)-Propranolol	5-HT$_1$	20	5567 ± 212	2346 ± 306***	211 ± 76	304 ± 64
Spiroxatrine	5-HT$_{1A}$	0.1	4918 ± 358	2265 ± 134***	421 ± 151	307 ± 81
(+)-WAY 100135	5-HT$_{1A}$	5	4246 ± 378	4652 ± 251	16 ± 9	68 ± 25
Ketanserin	5-HT$_{2A}$	1	4947 ± 266	2991 ± 123***	134 ± 48	70 ± 23
Ondansetron	5-HT$_3$	1	4961 ± 263	5257 ± 276	175 ± 39	107 ± 24
Ritanserin	5-HT$_{2A/2C}$	0.1	5191 ± 278	5123 ± 301	83 ± 39	76 ± 35
Methysergide	5-HT$_{2A/2C}$	3	5026 ± 239	5370 ± 243	145 ± 46	183 ± 42
Metergoline	5-HT$_{1A/1D/2A/2C}$	3	4713 ± 310	4325 ± 372	198 ± 56	67 ± 18*

Pairs of mice were given an intraperitoneal or in the case of the (+)-enantiomer of WAY 100135 subcutaneous injection of antagonist or vehicle. Thirty minutes later these mice received either RU 24969 (7.5 mg kg^{-1}, i.p.) or saline (0.25 ml, i.p.) to determine the effects on hyperlocomotion and control activity, respectively. Results are mean ± s.e.mean of the photocell interruptions in 60 min; $n = 7–12$ groups. Results were analysed by Student's t test. Significantly different from appropriate control: $^*P<0.05$; $^{***}P<0.001$.

activity in control mice (Table 2). The stereoselectivity of the inhibition produced by pindolol and propranolol was also determined. The locomotor response to RU 24969 (7.5 mg kg^{-1}) was markedly inhibited by the (−)-isomers of pindolol (4 mg kg^{-1}) and propranolol (20 mg kg^{-1}), but at identical doses, the (+)-isomers were without effect (Figure 4). The saline responses of control mice were not altered by administration of the isomers of pindolol or propranolol given at 4 mg kg^{-1} and 20 mg kg^{-1} respectively (data not shown).

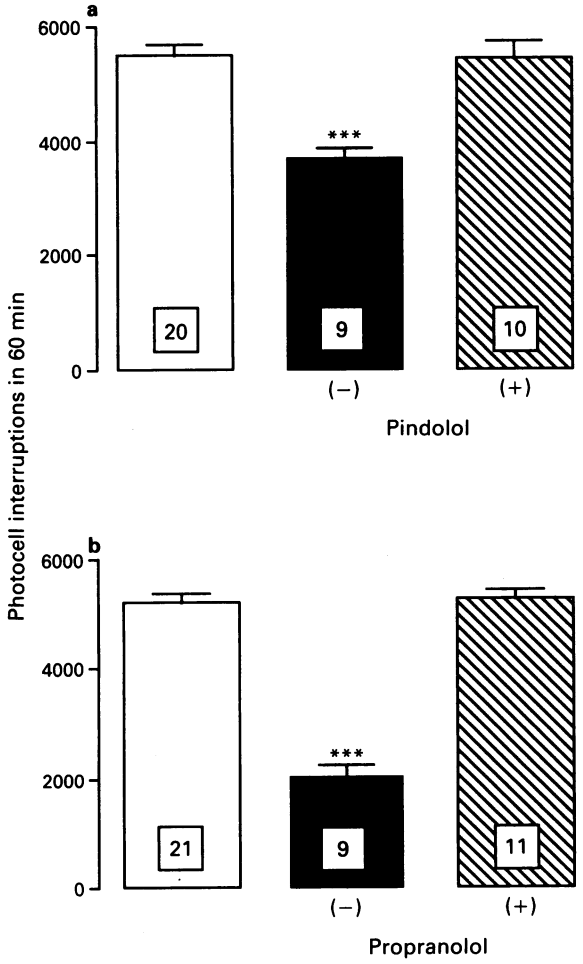


Figure 4 The effects of the isomers of pindolol and propranolol on RU 24969-induced hyperlocomotion. Groups of two mice were injected with the (−)- and (+)-isomers of (a) pindolol (4 mg kg^{-1}, i.p.) or (b) propranolol (20 mg kg^{-1}, i.p.) shown by the solid and hatched columns, respectively. Control mice received vehicle (0.25 ml, i.p.) as shown by the open columns. Results are the accumulated number of photocell interruptions in 60 min ± s.e.mean with the number of groups tested shown in each column. Statistical evaluations were performed with Student's t test. Although control data have been pooled for clarity, statistical comparisons were made against the appropriate subgroup of control values. Significantly different from appropriate control. ***$P<0.001$.

The effects of catecholamine receptor antagonists on RU 24969-induced hyperlocomotion

The locomotor activity of pairs of mice induced by RU 24969 (7.5 mg kg^{-1}) was measured 30 min after i.p. injection of various catecholamine receptor antagonists or appropriate vehicle. Prazosin (1 mg kg^{-1}), SCH 23390 (0.05 mg kg^{-1}) and BRL 34778 (0.03 mg kg^{-1}) inhibited the hyperlocomotion without impairing the saline control response (Table 3). Idazoxan (1 mg kg^{-1}), metoprolol (10 mg kg^{-1}) and ICI 118, 551 (5 mg kg^{-1}) had no effect on RU 24969-induced hyperlocomotion (Table 3).

Effects of selective neuronal lesions and monoamine depletion on RU 24969-induced hyperlocomotion and 5-MeODMT-induced head-twitches

5-HT-containing neurones were selectively lesioned by administration of 5,7-DHT (75 μg, i.c.v.). This procedure reduced the 5-HT content of whole brain by 73% with only minor effects on noradrenaline and dopamine levels (Table 4). Head-twitch and locomotor responses to 5-MeODMT and RU 24969 were measured on two consecutive days commencing 15 days after 5,7-DHT lesioning. Although this procedure increased the head-twitch response to 5-MeODMT (2 mg kg^{-1}) by 139%, it did not modify the hyperlocomotion produced by RU 24969 at doses of 3.75 or 7.5 mg kg^{-1} (Figure 5). In a second study, the 5-HT content of the brain was reduced by 14 days of administration of the synthesis inhibitor, PCPA (200 mg kg^{-1}). This treatment, which produced a pronounced and selective 75% reduction of 5-HT concentrations (Table 4), also markedly increased 5-MeODMT (2 mg kg^{-1})-induced head-twitches by 155%, but had no effect on RU 24969 (3.75 or 7.5 mg kg^{-1})-induced locomotor activity (Figure 5).

Noradrenergic neurones were lesioned by administration of DSP-4 (100 mg kg^{-1}) which reduced noradrenaline concentrations in whole brain by 72% with no effect on dopamine or 5-HT levels (Table 4). This noradrenergic lesion attenuated RU 24969-induced hyperlocomotion by 39% at 3.75 mg kg^{-1} and 45% at 7.5 mg kg^{-1} when these responses were tested 8 days after DSP-4 administration (Figure 6).

Reserpine (0.25–2 mg kg^{-1}) dose-dependently reduced brain 5-HT, noradrenaline and dopamine concentrations (Table 4). Although 5-HT and noradrenaline concentrations were significantly reduced at all doses of reserpine and dopamine concentrations were significantly reduced at 0.5, 1 and 2 mg kg^{-1}, marked reductions (>60%) were only obtained at doses of 1 and 2 mg kg^{-1}. Reserpine did not significantly modify the hyperlocomotion induced by RU

Table 3 Effects of various catecholamine receptor antagonists on RU 24969-induced hyperlocomotion

Antagonist	*Catecholamine receptor selectivity*	*Dose* (mg kg^{-1}, i.p.)	*RU 24969 activity*		*Saline activity*	
			Control	*Antagonist-treated*	*Control*	*Antagonist-treated*
Prazosin	α_1	1	5044 ± 211	2496 ± 290***	67 ± 27	42 ± 16
Idazoxan	α_2	1	5331 ± 437	4426 ± 213	210 ± 37	264 ± 75
Metoprolol	β_1	10	4379 ± 222	4003 ± 208	192 ± 65	160 ± 54
ICI 118,551	β_2	5	4353 ± 444	3852 ± 343	65 ± 23	170 ± 77
SCH 23390	D_1	0.05	4734 ± 158	1100 ± 232***	141 ± 49	141 ± 70
BRL 34778	D_2	0.03	5162 ± 266	1133 ± 170***	145 ± 58	125 ± 31

Pairs of mice were given an intraperitoneal injection of antagonist or vehicle. Thirty minutes later these mice received either RU 24969 (7.5 mg kg^{-1}, i.p.) or saline (0.25 ml, i.p.) to determine the effects on hyperlocomotion and control activity, respectively. Results are mean ± s.e.mean of the locomotor counts accumulated in 60 min; $n = 7-11$ groups. Results were analysed by Student's t test. Significantly different from appropriate control: ***$P<0.001$.

Table 4 Effects of lesioning and monoamine depleting agents on 5-HT, noradrenaline and dopamine concentrations in mouse brain

Treatment	n	*Dose*	*5-HT*	*Noradrenaline*	*Dopamine*
Vehicle	36	4 μl i.c.v.	890 ± 11	678 ± 7	1563 ± 16
5,7-DHT	28	75μg i.c.v.	242 ± 18*** (73%)	581 ± 12*** (14%)	1463 ± 20*** (6%)
Saline	36	0.25 ml i.p. × 14	888 ± 14	465 ± 7	1054 ± 17
PCPA	36	200 mg kg^{-1} i.p. × 14	220 ± 7*** (75%)	459 ± 8 (1%)	1062 ± 13 (+1%)
Saline	42	0.25 ml i.p.	950 ± 13	476 ± 13	950 ± 13
DSP-4	38	100 mg kg^{-1} i.p.	924 ± 11 (3%)	134 ± 5*** (72%)	924 ± 11 (3%)
Vehicle	20	0.25 ml i.p.	891 ± 10	551 ± 9	1170 ± 14
Reserpine	8	0.25 mg kg^{-1} i.p.	676 ± 31** (24%)	437 ± 21** (21%)	1099 ± 26 (7%)
	8	0.5 mg kg^{-1} i.p.	618 ± 46** (31%)	363 ± 51** (34%)	984 ± 51** (16%)
	8	1 mg kg^{-1} i.p.	329 ± 28** (63%)	101 ± 14** (82%)	450 ± 50** (62%)
	10	2 mg kg^{-1} i.p.	210 ± 24** (76%)	61 ± 18** (89%)	203 ± 50** (83%)

Groups of mice were injected with the above depleting agents or appropriate vehicle as described in Methods. Monoamine concentrations in mouse whole brain are reported as mean ± s.e.mean in ng g^{-1} wet weight; percentage depletion in parentheses. Statistical comparisons between appropriate vehicle and 5,7-DHT, PCPA or DSP-4 treated groups were made with Student's t test, ***$P<0.001$. Comparisons between vehicle- and reserpine-treated groups were made by analysis of variance followed by Williams' test, **$P<0.01$. For abbreviations, see text.

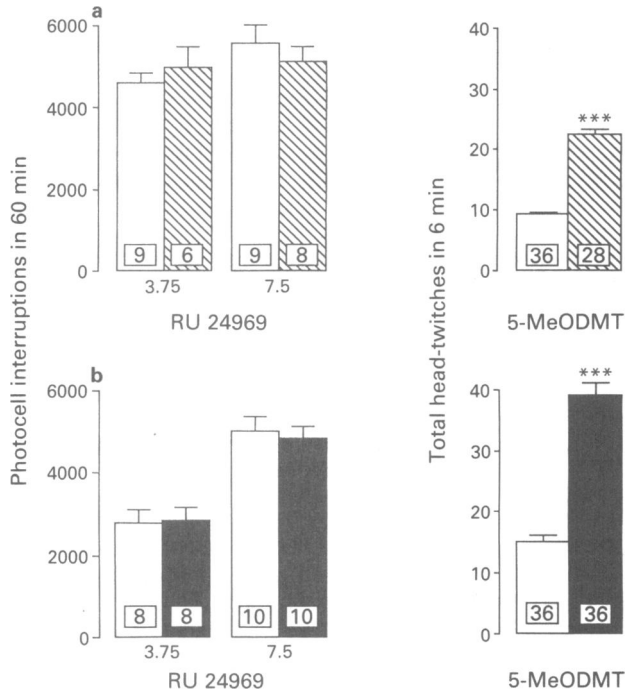


Figure 5 The effects of (a) 5,7-dihydroxytryptamine (5,7-DHT) lesioning and (b) repeated *p*-chlorophenylalanine (PCPA) administration on RU 24969-induced hyperlocomotion. Groups of mice were injected with: (a) 5,7-DHT (75 μg, i.c.v.; hatched columns) or saline-ascorbate vehicle (4 μl, i.c.v.; open columns); (b) PCPA (200 mg kg^{-1}, i.p.; solid columns) or saline (0.25 ml, i.p.; open columns) once daily for 14 days. The head-twitch response of all mice were determined using 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeODMT) (2 mg kg^{-1}, i.p.) 15 days after 5,7-DHT lesioning or on the day of the final PCPA injection. Twenty-four h later pairs of control and 5-HT-depleted mice were tested for their locomotor response to RU 24969 (3.75 or 7.5 mg kg^{-1}, i.p.). Results are shown as mean ± s.e.mean with the number of observations shown in each column. Statistical comparisons were made with Student's t test. Significantly different from appropriate control. ***$P<0.001$.

24969 (7.5 mg kg^{-1}) at the lower doses of 0.25 and 0.5 mg kg^{-1}, but, virtually abolished the response at 1 and 2 mg kg^{-1} by 95% and 98%, respectively (Figure 6). In both the DSP-4-treated and reserpine-treated mice hyperlocomotion was not replaced by any other behavioural effect.

Effects of RU 24969 administration on brain concentrations of monoamines and metabolites

Mice were injected with 3.75–10 mg kg^{-1} of RU 24969 60 min before determination of the concentrations of various monoamine neurotransmitters and their metabolites. RU 24969 increased the concentration of 5-HT in whole brain and correspondingly decreased the levels of the major metabolite, 5-HIAA (Table 5).

RU 24969 had no effect on the concentrations of noradrenaline in whole brain, but this 5-HT$_1$ agonist increased levels of MHPG (Table 5). Since the primary pharmacological action of RU 24969 is to produce locomotor activity, its effects on dopamine and metabolites were determined in nucleus accumbens rather than whole brain. However, RU 24969 did not alter the concentrations of dopamine, DOPAC or HVA at any of the doses tested (Table 5).

Discussion

In this study, we observed that RU 24969 produces pronounced and sustained locomotor activity in mice and this finding is totally consistent with earlier reports (Green *et al.*, 1984; Goodwin & Green, 1985; De Souza *et al.*, 1986). However, CGS 12066B which is a selective, 5-HT$_{1B}$ ligand (Neale *et al.*, 1987) produced only a very transient increase in locomotor activity whether given by the intraperitoneal or intracerebroventricular route. Moreover, CGS 12066B inhibited RU 24969-induced hyperlocomotion indicating that this compound is almost certainly a partial, rather than full, agonist at 5-HT$_{1B}$ receptors. These results agree with the findings of Neale *et al.* (1987) which showed that CGS 12066B had relatively weak agonist efficacy.

Goodwin & Green (1985) have previously reported that the locomotor response to RU 24969 is inhibited by (−)-propranolol, but not (+)-propranolol or ritanserin suggesting that the hyperlocomotion is specifically mediated by 5-HT$_1$ receptors. We have now confirmed and extended these findings. Thus, RU 24969-induced locomotor activity was unaltered by the (+)-enantiomer of WAY 100135, a potent and selective 5-HT$_{1A}$ antagonist (Bill *et al.*, 1993), ritanserin, a potent 5-HT$_{2A/2C}$ antagonist (Hoyer, 1988), ondansetron, a potent 5-HT$_3$ antagonist (Kilpatrick *et al.*, 1987) and the (+)-isomers of pindolol and propranolol which have poor 5-HT$_1$ affinity. However, this response was markedly inhibited by (−)-pindolol and (−)-propranolol which are potent 5-HT$_1$ ligands (Middlemiss *et al.*, 1977; Hoyer *et al.*,

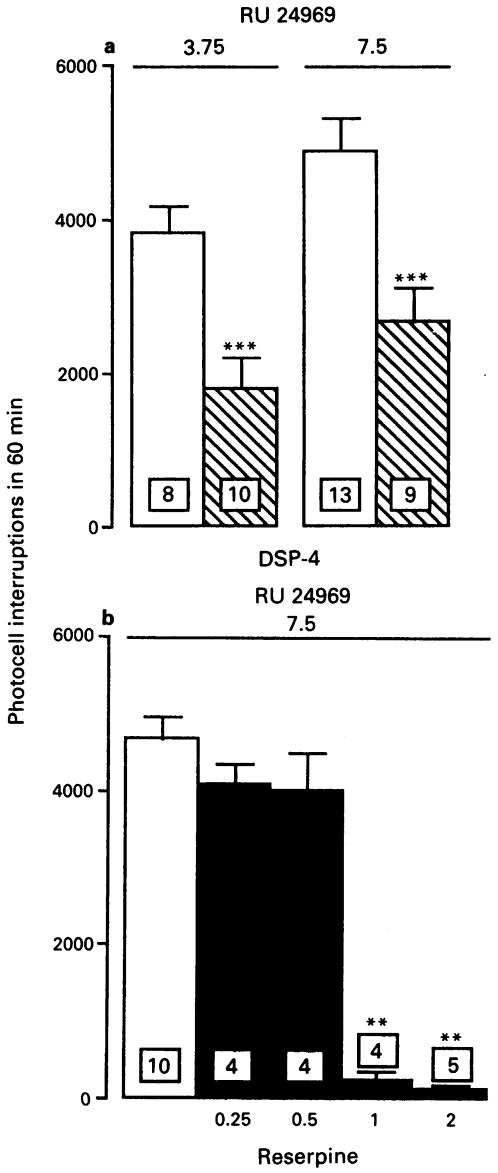


Figure 6 The effects of (a) N-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) lesioning and (b) reserpine administration on RU 24969-induced hyperlocomotion. Groups of mice were injected with: (a) DSP-4 (100 mg kg^{-1}, i.p.; hatched columns) or saline (0.25 ml, i.p.; open columns); (b) Reserpine (0.25–2 mg kg^{-1}, i.p.; solid columns) or vehicle (0.25 ml, i.p. open columns). The locomotor responses of pairs of mice to RU 24969 (3.75 or 7.5 mg kg^{-1}, i.p., DSP-4 or saline; 7.5 mg kg^{-1}, i.p., reserpine or vehicle) were determined 7 days and 18 h after DSP-4 and reserpine, respectively. Results are shown as mean ± s.e.mean with the number of groups shown on each column. Statistical comparisons between saline and DSP-4 treated groups were made with Student's *t* test; ***$P<0.001$. Comparisons between vehicle- and reserpine-treated groups were made by analysis of variance followed by Williams' test; **$P<0.01$.

1985) and by CGS 12066B which is 5-HT$_{1B}$ receptor-selective (Neale *et al.*, 1987). This hypothesis is also supported by the observation that RU 24969 did not produce head-twitching in the mice which is a 5-HT$_{2A}$-mediated behavioural effect. Taken together, our results show that the locomotor effects of RU 24969 are mediated via 5-HT$_{1B}$ receptors. The only results which would detract from this conclusion are the inhibition of RU 24969-induced hyperlocomotion by spiroxatrine and ketanserin, which have high affinity for 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors, respectively (Janssen, 1983; Nelson & Taylor, 1986; Alexander & Wood, 1987). Since ritanserin had no effect on this response, the inhibition by ketanserin probably resulted from its known α_1-adrenoceptor antagonist activity (Janssen, 1983). It is likely that the inhibition by spiroxatrine was due to potent antagonism of dopamine D_2 receptors (IC_{50} = 10.8 nM; Alexander & Wood, 1987).

Although the results argue that this locomotor response derives specifically from activation of central 5-HT$_{1B}$ receptors, they do not preclude an action of RU 24969 on 5-HT$_{1A}$ receptors. In fact, the observation that RU 24969 produced marked hypothermia in mice suggests that this agonist may simultaneously activate presynaptic 5-HT$_{1A}$ receptors and it is, therefore, fortunate that this latter effect does not interfere with the locomotor response. The finding that RU 24969-induced hyperlocomotion in mice results solely from 5-HT$_{1B}$ receptor activation contrasts with the contradictory findings which have been derived from studies using rats. For example, the response in rats was not inhibited by either racemic propranolol or its active (−)-isomer and it was potentiated by ritanserin (Green *et al.*, 1984; Goodwin & Green, 1985). In contrast, Tricklebank *et al.* (1986) found inhibition with propranolol and pindolol, but this inhibition was not stereoselective. It is obvious from these reports that there is no evidence to support the argument that RU 24969-induced hyperlocomotion in rats is a response specifically initiated by 5-HT$_{1B}$ receptor activation.

Although 5-HT$_{1B}$ receptors function as nerve terminal autoreceptors in rodent brain (Middlemiss, 1984a,b), the receptors responsible for the locomotor effects of RU 24969 are almost certainly postsynaptic as lesioning of 5-HT-containing neurones or depletion of 5-HT did not inhibit this behavioural response. This 5-HT$_{1B}$-mediated effect did not, however, show supersensitivity after these procedures, which contrasts with the marked potentiation of 5-HT$_{2A}$-mediated head-twitches observed in mice. Studies previously performed in rats have also found that the 5-HT receptors mediating the locomotor effects of RU 24969 are postsynaptic (Green *et al.*, 1984; Nisbet & Marsden, 1984; Tricklebank *et al.*, 1986; Oberlander *et al.*, 1986; 1987). However, there is disagreement over the induction of 5-HT$_{1B}$ receptor supersensitivity in rats; Tricklebank *et al.* (1986) and Oberlander *et al.* (1987) reported its occurrence after 5,7-DHT lesioning, whilst Nisbet & Marden (1984) failed to observe this effect.

The role of other monoaminergic neurones in the expression of RU 24969-induced hyperlocomotion has also been determined. Destruction of noradrenergic neurones with the selective neurotoxin, DSP-4 (Jaim-Etcheverry & Zieher, 1980; Jonsson *et al.*, 1981) and depletion of 5-HT, noradrenaline

Table 5 Effects of administration of RU 24969 on the concentrations of monoamines and their major metabolites in mouse brain

		Whole brain		*Whole brain minus cerebellum*	*Nucleus accumbens*		
Treatment	*5-HT*	*5-HIAA*	*Noradrenaline*	*MHPG*	*Dopamine*	*DOPAC*	*HVA*
Saline	1209 ± 28	388 ± 28	514 ± 7	89 ± 2	5899 ± 184	767 ± 88	785 ± 40
RU 24969 (3.75)	1436 ± 30**	243 ± 11**	508 ± 4	102 ± 3*	5855 ± 208	783 ± 78	809 ± 19
RU 24969 (7.5)	1602 ± 53**	195 ± 17**	515 ± 12	104 ± 4**	5819 ± 143	770 ± 59	861 ± 33
RU 24969 (10)	1499 ± 34**	190 ± 9**	492 ± 10	114 ± 5**	5904 ± 322	877 ± 50	866 ± 33

Groups of mice were injected with RU 24969 (3.75–10 mg kg^{-1}, i.p.) or saline (0.25 ml, i.p.). Sixty minutes later brains were removed and the concentrations of monoamines and their metabolites were measured by h.p.l.c. with electrochemical detection as described in Methods. Results are reported as mean ± s.e.mean in ng g^{-1} wet weight; $n = 6–8$. Statistical comparisons between saline and RU 24969 treated groups were made using analysis of variance followed by Williams' test. Significantly different from control. *$P<0.05$, **$P<0.01$. For abbreviations, see text.

and dopamine concentrations by reserpine markedly inhibited this locomotor response demonstrating the involvement of catecholaminergic neurones. Receptor antagonist studies revealed mediation of RU 24969-induced hyperlocomotion via α_1-adrenoceptors, and dopamine D_1 and D_2 receptors. These findings are consistent with and, in addition, complement earlier investigations performed on C57/Bl/6 mice which have shown that activation of α_1-adrenoceptors with phenylephrine or methoxamine produces intense locomotor activity and this response is in turn expressed via dopaminergic neurones (Heal, 1984). Other research workers have similarly reported that catecholaminergic neurones were involved in the expression of RU 24969-induced locomotor activity in the rat because the response was also inhibited by α_1-adrenoceptor and dopamine receptor antagonists in this species (Tricklebank *et al.*, 1986; Oberlander *et al.*, 1986). In the rat, the influence of other monoamine neurotransmitter systems on RU 24969-induced hyperlocomotion may be more complex than in the mouse. For example, Tricklebank *et al.* (1986) found that monoamine depletion with reserpine resulted in the progressive release of behavioural components of the 5-HT syndrome, an effect not seen in the mouse, whilst Oberlander *et al.* (1986) reported that lesioning the globus pallidus to disrupt motor outputs of the dopamine system enhanced the RU 24969 locomotor response and suppressed the inhibitory effect of dopamine receptor antagonists.

The effects of RU 24969 administration on the concentrations of monoamine neurotransmitters and their metabolites in the brain were almost entirely consistent with the observed behavioural effects of this 5-HT_1 agonist. In keeping with its potent actions as a 5-$HT_{1A/1B}$ receptor agonist, RU 24969 markedly decreased the concentration of the 5-HT metabolite, 5-HIAA, while simultaneously increasing the concentration of the parent monoamine. These effects are almost certainly due to a reduction of 5-HT neuronal activity as a result of the concurrent activation of inhibitory 5-HT_{1B} terminal autoreceptors and inhibitory 5-HT_{1A} somatodendritic autoreceptors, and this has been reported by numerous earlier workers (Euvrard & Boissier, 1980; Green *et al.*, 1984; Goodwin & Green, 1985; Carli *et al.*, 1988). Although noradrenaline concentrations were unaltered by RU 24969, levels of MHPG were increased. In mice, this metabolite is a good index of noradrenaline turnover and functional utilization (Heal *et al.*, 1989). By contrast, RU 24969 had no effect on the concentrations of dopamine or its metabolites in the nucleus accumbens which is involved in the generation of locomotor activity (Pijnenburg & Van Rossum, 1973; Costall & Naylor, 1976). Taken together, these results suggest that RU 24969-induced locomotor activity is mediated via noradrenergic neurones acting on α_1-adrenoceptors and, subsequently, via dopaminergic neurones acting on dopamine D_1 and D_2 receptors. This sequence of events has previously been demonstrated for phenylephrine-induced activity in mice (Heal, 1984).

In conclusion, the results presented demonstrate that RU 24969 produces a syndrome of pronounced locomotor activity in mice which is specifically initiated via 5-HT_{1B} receptor activation and, consequently, this response may serve as a valuable model for monitoring 5-HT_{1B} receptor function *in vivo*. The 5-HT_{1B} receptors involved are postsynaptic and do not show supersensitivity following prolonged and extensive 5-HT depletion or neuronal lesioning. Catecholaminergic neurones are involved in the expression of this locomotor response.

Note added in proof

5-HT receptors have recently been reclassified (Humphrey *et al.*, 1993). Of particular relevance to this paper is that the increasingly recognised similarity between the 5-HT_{1C} and 5-HT_2 receptor has led to the former being reclassified as the 5-HT_{2C} receptor. The receptor previously referred to as the 5-HT_2 receptor is now termed the 5-HT_{2A} receptor. These names have been used in the text.

The authors wish to thank Mike Prow and Sarah Butler for performing the h.p.l.c. analyses on the brain samples, and Jo Hilliard and Jean Smith for preparing the manuscript. We also gratefully acknowledge the donation of drugs by the following pharmaceutical companies: Farmitalia, ICI, Janssen, Roussel-Uclaf, Sandoz and SmithKline Beecham.

References

ALEXANDER, B.S. & WOOD, M.D. (1987). Monoamine receptor binding profile of the 5-HT_{1A} antagonist spiroxatrine. *Br. J. Pharmacol.*, **91**, 449P.

ARMITAGE, P. & BERRY, G. (1987). Comparison of several groups. In *Statistical Methods in Medical Research*, 2nd edition. pp. 186–196. Oxford: Blackwell.

BILL, D.J., BROWN, G. & FLETCHER, A. (1993). Stereospecific blockade of 8-OH-DPAT-induced hypothermia in rodents by the selective 5-HT_{1A} receptor antagonist, WAY 100135. *Br. J. Pharmacol.*, **108**, 94P.

CARLI, M., INVERNIZZI, R., CERVO, L. & SAMANIN, R. (1988). Neurochemical and behavioural studies with RU-24969 in the rat. *Psychopharmacology*, **94**, 359–364.

COSTALL, B. & NAYLOR, R.J. (1976). Antagonism of the hyperactivity induced by dopamine applied intracerebrally to the nucleus accumbens septi by typical neuroleptics and by clozapine, sulpiride and thioridazine. *Eur. J. Pharmacol.*, **35**, 161–168.

DE SOUZA, R.J., GOODWIN, G.M., GREEN, A.R. & HEAL, D.J. (1986). Effect of chronic treatment with 5-HT_1 agonist (8-OH-DPAT and RU 24969) and antagonist (isapirone) drugs on the behavioural responses of mice to 5-HT_1 and 5-HT_2 agonists. *Br. J. Pharmacol.*, **89**, 377–384.

EUVRARD, C. & BOISSIER, J.R. (1980). Biochemical assessment of the central 5-HT agonist activity of RU 24969 (a piperidinyl indole). *Eur. J. Pharmacol.*, **63**, 65–72.

GOODWIN, G.M. & GREEN, A.R. (1985). A behavioural and biochemical study in mice and rats of putative selective agonists and antagonists for 5-HT_1 and 5-HT_2 receptors. *Br. J. Pharmacol.*, **84**, 743–753.

GREEN, A.R., GUY, A.P. & GARDNER, C.R. (1984). The behavioural effects of RU 24969, a suggested 5-HT_1 receptor agonist in rodents and the effect on the behaviour of treatment with antidepressants. *Neuropharmacology*, **23**, 655–661.

HEAL, D.J. (1984). Phenylephrine-induced activity in mice as a model of central α_1-adrenoceptor function. Effects of acute and repeated administration of antidepressant drugs and electroconvulsive shock. *Neuropharmacology*, **23**, 1241–1251.

HEAL, D.J., PHILPOT, J., MOLYNEUX, S.G. & METZ, A. (1985). Intracerebroventricular administration of 5,7-dihydroxytryptamine to mice increases both head-twitch response and the number of cortical 5-HT_2 receptors. *Neuropharmacology*, **24**, 1201–1205.

HEAL, D.J., PROW, M.R. & BUCKETT, W.R. (1989). Measurement of 3-methoxy-4-hydroxyphenylglycol (MHPG) in mouse brain by h.p.l.c. with electrochemical detection, as an index of noradrenaline utilisation and presynaptic α_2-adrenoceptor function. *Br. J. Pharmacol.*, **96**, 547–556.

HEAL, D.J., PROW, M.R. & BUCKETT, W.R. (1991). Determination of the role of noradrenergic and 5-hydroxytryptaminergic neurones in postsynaptic α_2-adrenoceptor desensitization by desipramine and ECS. *Br. J. Pharmacol.*, **103**, 1865–1870.

HOYER, D. (1988). Molecular pharmacology and biology of 5-HT_{1C} receptors. *Trends Pharmacol. Sci.*, **9**, 89–94.

HOYER, D., ENGEL, G. & KALKMAN, H.O. (1985). Molecular pharmacology of 5-HT_1 and 5-HT_2 recognition sites in rat and pig brain membranes: radioligand binding studies with [^{3}H]5-HT, [^{3}H]8-OH-DPAT, (−)[^{125}I]iodocyanopindolol, [^{3}H]mesulergine and [^{3}H]ketanserin. *Eur. J. Pharmacol.*, **118**, 13–23.

HUMPHREY, P.P.A., HARTIG, P. & HOYER, D. (1993). A proposed new nomenclature for 5-HT receptors. *Trends Pharmacol. Sci.*, **14**, 233–236.

HUNT, P. & OBERLANDER, C. (1981). The interaction of indole derivatives with the serotonin receptor and non-dopaminergic circling behaviour. *Adv. Exp. Med. Biol.*, **133**, 547–561.

JAIM-ETCHEVERRY, G. & ZIEHER, L.M. (1980). DSP-4: a novel compound with neurotoxic effects on noradrenergic neurons of adult and developing rats. *Brain Res.*, **188**, 513–523.

JANSSEN, P.A.J. (1983). 5-HT$_2$ receptor blockade to study serotonin-induced pathology. *Trends Pharmacol. Sci.*, **4**, 198–206.

JONSSON, G., HALLMAN, H., PONZIO, F. & ROSS, S. (1981). DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine)- a useful denervation tool for central and peripheral noradrenaline neurons. *Eur. J. Pharmacol.*, **72**, 173–188.

KILPATRICK, G.J., JONES, B.J. & TYERS, M.B. (1987). Identification and distribution of 5-HT$_3$ receptors in rat brain using radioligand binding. *Nature*, **330**, 746–748.

MIDDLEMISS, D.N. (1984a). Stereoselective blockade at [^{3}H]5-HT binding sites and at the 5-HT autoreceptor by propranolol. *Eur. J. Pharmacol.*, **101**, 289–293.

MIDDLEMISS, D.N. (1984b). 8-Hydroxy-2-(di-n-propylamino) tetralin is devoid of activity at the 5-HT autoreceptor in the rat frontal cortex. Implications for the proposed link between the autoreceptor and the [^{3}H]5-HT recognition site. *Naunyn-Schmied. Arch. Pharmacol.*, **327**, 18–22.

MIDDLEMISS, D.N., BLAKEBOROUGH, L. & LEATHER, S.R. (1977). Direct evidence for an interaction of β-adrenergic blockers with the 5-HT receptor. *Nature*, **267**, 289–290.

NEALE, R.F., FALLON, S.L., BOYAR, W.C., WASLEY, J.W.F., MARTIN, L.L., STONE, G.A., GLAESER, B.S., SINTON, C.M. & WILLIAMS, M. (1987). Biochemical and pharmacological characterisation of CGS 12066B, a selective serotonin-1B agonist. *Eur. J. Pharmacol.*, **136**, 1–9.

NELSON, D.L. & TAYLOR, E.W. (1986). Spiroxatrine: a selective serotinin$_{1A}$ receptor antagonist. *Eur. J. Pharmacol.*, **124**, 207–208.

NISBET, A.P. & MARSDEN, C.A. (1984). Increased behavioural response to 5-methoxy-N, N-dimethyltryptamine but not to RU-24969 after intraventricular 5,7-dihydroxytryptamine administration. *Eur. J. Pharmacol.*, **104**, 177–180.

OBERLANDER, C., BLAQUIÈRE, B. & PUJOL, J.-F. (1986). Distinct functions for dopamine and serotonin in locomotor behaviour: evidence using the 5-HT$_1$ agonist RU 24969 in globus pallidus-lesioned rats. *Neurosi. Letts.*, **67**, 113–118.

OBERLANDER, C., DEMASSEY, Y., VERDU, A., VAN DE VELDE, D. & BARDELAY, C. (1987). Tolerance to the serotonin 5-HT$_1$ agonist RU 24969 and effects on dopaminergic behaviour. *Eur. J. Pharmacol.*, **139**, 205–214.

PEROUTKA, S.J., LEBOVITZ, R.M. & SYNDER, S.H. (1981). Two distinct central serotonin receptors with differential physiological functions. *Science*, **212**, 827–829.

PIJNENBURG, A.J.J. & VAN ROSSUM, J.M. (1973). Stimulation of locomotor activity following injection of dopamine into the nucleus accumbens. *J. Pharm. Pharmacol.*, **25**, 1003–1005.

TRICKLEBANK, M.D., MIDDLEMISS, D.N. & NEILL, J. (1986). Pharmacological analysis of the behavioural and thermoregulatory effects of the putative 5-HT$_1$ receptor agonist, RU 24969, in the rat. *Neuropharmacology*, **25**, 877–886.

WILLIAMS, D.A. (1972). The comparison of several dose levels with a zero dose control. *Biometrics*, **28**, 519–531.

(Received July 16, 1993
Revised August 18, 1993
Accepted September 1, 1993)

Br. J. Pharmacol. (1993), **110**, 1630–1634 © Macmillan Press Ltd, 1993

Effect of activation on adhesion of flowing neutrophils to cultured endothelium: time course and inhibition by a calcium channel blocker (nitrendipine)

I. Perry, S.M. Buttrum & [1]G.B. Nash

Department of Haematology, The Medical School, University of Birmingham, Birmingham B15 2TT

1 Adhesion of neutrophils to vascular endothelium plays an important role in inflammation and thrombosis. Modulation of adhesion may be therapeutic in these conditions.

2 A flow model was used to quantify adhesion of neutrophils to human cultured umbilical vein endothelial cells. The time course of the neutrophil response to activation by N-formyl-methionyl-leucyl-phenylalanine (fMLP, 10^{-7} M) was studied and the inhibitory effects of the calcium-channel blockers, nitrendipine and nifedipine, were investigated.

3 Neutrophils adhered firmly to the endothelial cells without rolling, but initial attachment was highly dependent on shear stress; doubling the stress from 0.05 to 0.1Pa decreased the number of neutrophils adhering by over 80%.

4 Adhesion rapidly increased after activation of neutrophils by fMLP, peaking at 1–3 min post-treatment, and then decreased over the next 10–12 min. A monoclonal antibody to the β2-integrin component CD18 inhibited adhesion by over 80% for activated or unactivated cells.

5 The Ca-channel blocker, nitrendipine, but not nifedipine, significantly inhibited the fMLP-induced increase of adhesion in a dose-dependent manner (10^{-8} to 10^{-6} M). Dihydropyridines may be useful agents for modifying neutrophil function.

Keywords: Neutrophils; endothelium; adhesion; rheology; calcium-channel blockers; nitrendipine

Introduction

The adhesion of neutrophilic granulocytes to vascular endothelium and their subsequent migration from the circulation, is essential for the development of a normal inflammatory response. However, this process has also been implicated in the pathogenesis of diverse conditons in which vascular damage is a key event, including reperfusion injury after ischaemia, vasculitis, peripheral vascular disease, and shock (Hernandez *et al.*, 1987; Vedder *et al.*, 1988; Fein, 1991). Damage results because adhesion may be followed by the release of lytic enzymes and oxygen free radicals from immobilised, activated neutrophils, and also because the neutrophils themselves may be obstructive to the microcirculation (Harlan, 1985; Worthen *et al.*, 1989). Adherent neutrophils may also promote thrombosis by interaction with platelets at sites of vascular damage (De-Gaetano *et al.*, 1990; Bazzoni *et al.*, 1991). The processes which control neutrophil adhesion are therefore of great pathophysiological interest, while pharmacological modification of these processes has immense therapeutic potential.

Neutrophil adhesion can occur as a result of stimulation of either the endothelium or neutrophils by cytokines, chemoattractants and elements of the complement and clotting cascades (Zimmerman & McIntyre, 1988; Smith *et al.*, 1989; Luscinkas *et al.*, 1991). Experimental evidence suggests that the adhesion of flowing neutrophils *in vivo* is a multistep process (Osborn, 1990; Zimmerman *et al.*, 1992 for review). The first step, margination, is dependent on rheological mechanisms which exclude neutrophils from the central region of flow (Goldsmith, 1984). In the presence of endothelial stimulation, adhesive interactions between endothelial selectins and their ligands on unstimulated neutrophils act to slow the fact moving marginated cells, and create a rolling motion of the neutrophils over the endothelium (Ley *et al.*, 1991; Smith *et al.*, 1991). Rolling slows the neutrophils, and if neutrophil activation occurs, a second type of interaction, mediated by the β_2-integrins on the neutrophil and the endothelial receptor ICAM-1 (and possibly ICAM-2) can arrest the moving cells (Lawrence *et al.*, 1990; Lawrence & Springer, 1991; Smith *et al.*, 1991). It is generally believed that rolling is a necessary pre-requisite for immobilisation, because the integrin-mediated adhesion does not act efficiently for free-flowing neutrophils. The rate of response of neutrophils to activation will control whether they become successfully immobilised in the target tissue. Thus, modification of adhesion might be achieved at any of the steps, i.e., endothelial stimulation, rolling adhesion, neutrophil activation and integrin-mediated binding.

Most reports to date have described the *in vitro* adhesion of neutrophils to endothelium under static conditions. In this study we have used a new assay (Cooke *et al.*, 1993) to study neutrophil adhesion to unstimulated, cultured endothelial cells under conditions of flow. Others previously reported that adhesion to unstimulated endothelium was critically dependent on the shear stress in the flow, was increased after activation of neutrophils by the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP) and was mediated predominantly by the β_2-integrin class of adhesion molecules (Lawrence *et al.*, 1990). Here we give details of the time-course of the adhesive response of neutrophils after activation by fMLP, and describe the effects of two structurally similar, dihydropyridine, calcium channel blockers (nitrendipine and nifedipine) on this response.

Methods

Neutrophil preparation

Venous blood was collected from healthy, adult volunteers into buffered citrate anticoagulant (CPDA-1, 8:1, Blood Transfusion Service, Birmingham, UK) or into preservative-free sodium heparin (5 u ml^{-1}, Leo Laboratories Ltd., Princes Risborough, UK). All subsequent procedures were at

[1] Author for correspondence.

room temperature. A portion of the heparinised blood was centrifuged at 1500 *g* for 10 min and the plasma was harvested and filtered through a 0.2 μm pore, sterile filter.

Whole citrated blood was layered over a two-step gradient of equal volumes of Histopaque-1077 and Histopaque-1119 (Sigma Chemical Co. Ltd., Poole, UK) and centrifuged at 700 *g* for 25 min. The middle, neutrophil fraction was harvested and washed in 5% autologous heparinized plasma in RPMI-1640 (ICN Flow Laboratories Ltd., Irvine, Scotland) and centrifuged for a further 7 min at 600 *g*. Contaminating red blood cells were removed by 30 s hypotonic lysis with water, followed by return of the osmolarity with 4 × concentrated phosphate buffered saline. The neutrophils were washed twice in 5% plasma in RPMI and a sample was counted in a Coulter counter (model ZF, Coulter Electronics Ltd, Luton, UK). The cell suspension was diluted to a final concentration of 1×10^6 neutrophils ml^{-1} in 5% autologous plasma in RPMI. Review of the morphology of fixed neutrophils prepared in this manner showed consistently low levels of activation (mean 7% deviation from spherical shape) and a high level of viability; 98% of neutrophils developed pseudopodia in response to stimulation with 10^{-7} M fMLP (Sigma Chemical Co. Ltd.). In early experiments 5% autologous serum was used in place of plasma. However, this was found to give higher levels of activation and was substituted by 5% autologous plasma.

Endothelial culture

Culture of human umbilical vein endothelial cells (HUVEC) in flattened glass capillary tubes (microslides) was carried out as recently described (Cooke *et al.*, 1993). Briefly, endothelial cells were harvested by collagenase digestion of human umbilical veins and cultured in tissue culture flasks in Medium 199 (ICN Flow Ltd.) supplemented with gentamicin sulphate, glutamine, heparin, and 20% human serum. Confluent monolayers were dispersed using EDTA/trypsin, washed and seeded in microslides (internal dimensions 50 mm × 3.0 mm × 0.03 mm; Camlab Ltd., Cambridge, U.K.) which had been pre-coated with 3-aminopropyltriethoxysilane. The cells were cultured for 24 h until confluent, with medium being allowed to flow through the microslides at two-hourly intervals by an automated flow/culture system. All media and reagents were tested and found to be free of endotoxin (<0.25 endotoxin units ml^{-1} by *Limulus* amoebocyte lysate assay; E.Toxate, Sigma Chemical Co. Ltd.).

Adhesion assay

Endothelialised microslides were removed from the culture dish, glued to a glass microscope slide and incorporated into a flow system as previously described (Cooke *et al.*, 1993). Either neutrophil suspension (1×10^6 cells ml^{-1}) or cell-free wash medium (5% autologous plasma in RPMI, pH 7.4) was perfused over the endothelium at a controlled flow rate, and hence known wall shear stress, generated by a syringe pump (model 906, Harvard Apparatus, South Natick, U.S.A.). The flow rate required to generate a desired shear stress (τ) was calculated from the equation: $6 \times \tau \times Q/a^2 \times b$, where, η = viscosity of fluid, Q = volumetric flow rate, a = microslide internal height, b = microslide internal width (Lawrence *et al.*, 1990).

The endothelium was viewed by phase contrast microscopy and observations made either directly by eye or recorded on video tape for retrospective analysis. The number of adherent neutrophils was counted in a whole number of microscope or video fields, the dimensions of which were calibrated, so that adhesion could be expressed as the number of neutrophils mm^{-2} endothelium. The velocity of moving cells was determined by using the video digitising system recently described (Cooke *et al.*, 1993), which measured the distance moved by cells in a known period of time.

Two types of assay were performed:

(i) Boluses of neutrophils were perfused through separate endothelialised microslides for 2 to 5 min at a desired wall shear stress. Adhesion was quantitated after an equal period of washout with cell-free medium;

(ii) Neutrophils were perfused through microslides at a desired shear stress for up to 30 min, with adherent cells being counted every 2 min.

In order to study the effects of activation, neutrophils were stimulated with 10^{-7} M fMLP prior to testing. In a first series of experiments, 2 min boluses of neutrophils were perfused through separate microslides, either 1, 3 or 8 min after stimulation. Because of the dead-space within the flow system, earlier time points could not be tested. In a second series, the treated neutrophils were immediately and continuously perfused through microslides for up to 30 min after stimulation. In other experiments, both unstimulated and fMLP-stimulated neutrophils were treated with a monoclonal antibody to CD-18, the common β subunit of the neutrophil β_2-integrins (M783, Dako Ltd., High Wycombe, U.K.). An isotype-matched control antibody was mouse IgG1 against mycobacterial purified protein derivative (gift of Dr S.K. Parida).

Drugs

The dihydropyridine calcium-channel antagonists nitrendipine and nifendipine (gift of Bayer AG, Wuppertal, Germany) were dissolved in ethanol. Neutrophils were preincubated for 30 min with varying concentrations of these agents, prior to stimulation with 10^{-7} M fMLP and perfusion through microslides for 16 min. Control samples were treated with ethanol (0.1% v/v) alone. Comparisons between the adhesion of different samples were made by paired *t* test after logarithmic transformation of data.

Results

Response of neutrophils to activation

Table 1 shows the effects of fMLP treatment on neutrophil adhesion at two wall shear stresses (0.05 Pa and 0.1 Pa). Activation with fMLP caused an increase in the level of adhesion at both wall shear stresses, but adhesion was much reduced at 0.1 Pa compared to 0.05 Pa. Preliminary experiments showed that adhesion was not detectable at a wall shear stress of 0.2 Pa, while at a low shear stress of 0.02 Pa, adhesion was relatively high even for unstimulated neutrophils (data not shown). Others have found that a relatively high percentage of unstimulated neutrophils can bind to HUVEC in the absence of flow, and that adhesion decreases monotonically with increasing wall shear stress (Lawrence *et al.*, 1990; Smith *et al.*, 1991). In this study, all subsequent experiments were performed at 0.05 Pa so that statistically adequate counts of adherent cells were obtained at a shear stress close to that acting *in vivo* (see Discussion).

Table 1 Effect of shear stress and activation on adhesion of neutrophils to human cultured umbilical vein endothelial cells

Wall shear stress	Number of adherent neutrophils* ($/mm^2/10^6$ cells perfused)	
	Untreated	*fMLP-treated*
0.05 Pa	26 ± 16	372 ± 48
0.1 Pa	5 ± 2	23 ± 2

*Data are mean ± s.e.mean for 3 experiments. In each experiment boluses were perfused through separate microslides for 5 min; since the flow rate for 0.1 Pa was double that for 0.05 Pa, the adhesion has been normalised per 10^6 cells perfused.

The time course of the changes in neutrophil adhesion induced by fMLP was studied in two ways. When separate 2 min boluses of neutrophils were allowed to flow over the endothelium after addition of fMLP, it was evident that activation caused a rapid but short-lived increase in the number of adherent cells (Figure 1). The increase in adhesion was maximal at the first time point following stimulation (i.e., after 1–3 min) and declined thereafter. Adhesion was also quantitated during continuous perfusion of neutrophils (Figure 2). In experiments performed with unstimulated neutrophils, the number of adherent cells increased steadily with time, reaching 250 cells mm^{-2} after 30 min. However, after treatment with fMLP, adhesion increased rapidly over the first few minutes (attaining 266 cells mm^{-2} after 4 min), increased less rapidly from about 4 to 14 min, and then remained essentially constant at about 650 cells mm^{-2}.

When unstimulated or fMLP-stimulated neutrophils were treated with a monoclonal antibody to CD18 before and during perfusion over the endothelium, the levels of adhesion were greatly reduced (Figure 3). The number of cells adherent after 16 min perfusion was reduced by over 80% for either stimulated or unstimulated neutrophils (3 and 5 experiments respectively). A non-specific, isotype-matched, antibody had no consistent effect on adhesion (data not shown).

Effects of nitrendipine and nifedipine

Pretreatment with nitrendipine caused a consistent reduction in the level of adhesion of fMLP-stimulated neutrophils perfused over endothelium (Figure 4). Judged by the number of adherent cells after 16 min of perfusion, both 10^{-6} M and 10^{-7} M nitrendipine significantly reduced adhesion (Table 2). Inhibition occurred in all experiments with 10^{-8} M nitrendpine, but despite this trend, the effect was not statistically significant. Identical experiments were performed with nifedipine, but no inhibition of attachment was observed at concentrations of 10^{-8} M to 10^{-6} M (Table 2).

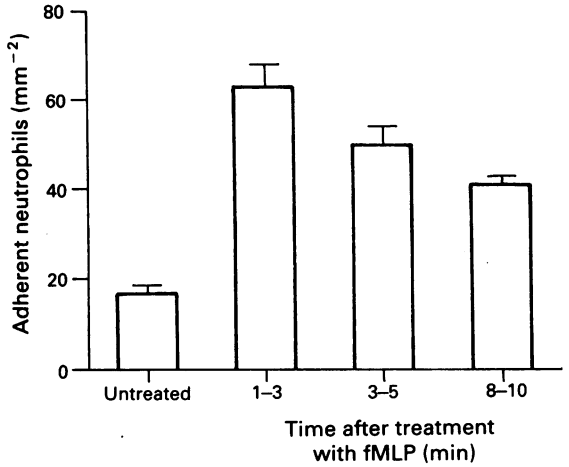


Figure 1 Time course of changes in the adhesion of neutrophils after activation by fMLP (10^{-7} M). Data are mean ± s.e.mean for 3 experiments in which 2 min boluses of neutrophils were perfused through separate HUVEC-coated microslides at a wall shear stress of 0.05 Pa.

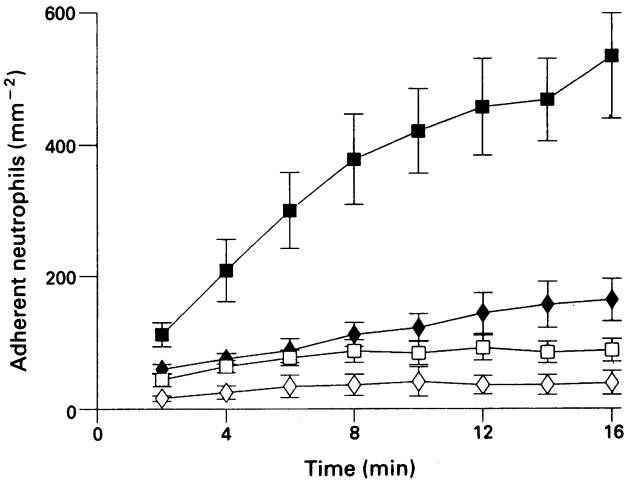


Figure 3 Effect of a monoclonal antibody to CD18 on the time course of the adhesion of neutrophils, with (■, □) and without (◆, ◇) activation by fMLP (10^{-7} M); open symbols refer to samples treated with the antibody. Data are mean ± s.e.mean for 5 or 3 experiments (fMLP-activated or unactivated cells respectively) in which neutrophils were continuously perfused through separate HUVEC-coated microslides at a wall shear stress of 0.05 Pa.

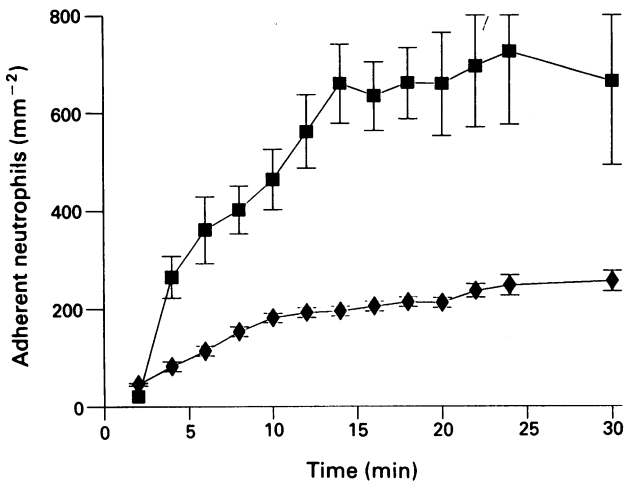


Figure 2 Time course of the adhesion of neutrophils, with (■) and without (◆) activation by fMLP (10^{-7} M). Data are mean ± s.e.mean for 3 experiments in which neutrophils were continuously perfused through separate HUVEC-coated microslides at a wall shear stress of 0.05 Pa.

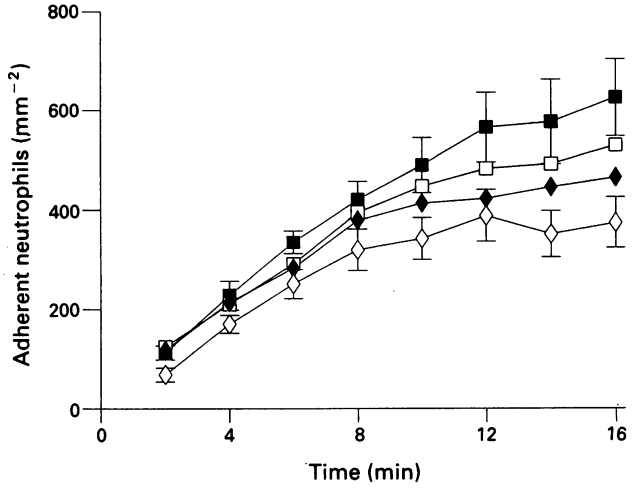


Figure 4 Effect of nitrendipine on the time course of the adhesion of fMLP-activated neutrophils: (■) 0, (□) 10^{-8}, (◆) 10^{-7}, (◇) 10^{-6} M nitrendipine. Data are mean ± s.e.mean for 6 experiments in which neutrophils were continuously perfused through separate HUVEC-coated microslides at a wall shear stress of 0.05 Pa; for clarity s.e.means are shown only for 0 and 10^{-6} M nitrendipine.

Table 2 Effect of treatment with nitrendipine or nifedipine on adhesion of fMLP-treated neutrophils to human cultured umbilical vein endothelial cells

	Number of adherent neutrophils* (% relative to control)	
Concentration (M)	*Nitrendipine* ($n = 6$)	*Nifedipine* ($n = 5$)
0	100	100
10^{-8}	87 ± 5	97 ± 3
10^{-7}	70 ± 5**	94 ± 8
10^{-6}	63 ± 6**	94 ± 4

*Data are mean ± s.e.mean for n experiments.
**$P < 0.01$ compared to control by paired two-tailed t test on logarithmically transformed data.

Microscopic observations of adhesive behaviour

Retrospective analysis of video recordings permitted analysis of cell velocities. The mean velocity of non-adherent neutrophils moving over endothelium was 370 $\mu m\ s^{-1}$ with no neutrophil observed to move at a velocity of below 130 $\mu m\ s^{-1}$. Adhesion occurred essentially instantaneously; the arrest of moving cells occurred so rapidly that the deceleration phase could not be measured. No rolling adhesion was seen in this study (defined as an adherent cell moving over the endothelium at a velocity $< 50\ \mu m\ s^{-1}$; Lawrence & Springer, 1991; Smith *et al.*, 1991). Once arrested, cells were not all completely immobile. If video recordings of several minutes duration were viewed, then some adherent neutrophils could be seen to extend pseudopodia and more over the endothelium at a mean velocity of 12 ± 2.5 $\mu m\ min^{-1}$ (mean ± s.e.mean for 25 cells). Stationary or motile cells were firmly attached to the endothelium, in that cells were rarely washed away by the flow, even after the fMLP-induced increase in initial adhesiveness had subsided. Adherent cells sometimes acted as an obstruction to flow and could be seen to deflect the otherwise linear stream of flowing cells. Neutrophils colliding with an obstructing cell often adhered to endothelium immediately behind or adjacent to it.

Discussion

Neutrophil adhesion to endothelium is critically dependent on the local flow forces. In agreement with previous reports (Lawrence *et al.*, 1990), we found that increasing the wall shear stress from 0.05 Pa to 0.1 Pa caused the number of cells adhering to unstimulated, cultured endothelial cells to decrease markedly, regardless of neutrophil activation, while adhesion was not detectable at higher stress. Others have reported that rolling adhesion on interleukin-1-stimulated endothelial cells expressing E-selectin (Smith *et al.*, 1991), or on the purified endothelial receptor P-selectin (Lawrence & Springer, 1991) can be established at higher stress. These reports have suggested that selectin-mediated rolling adhesion is a prerequisite for stationary, integrin-mediated attachment of flowing neutrophils. Although *in vivo* studies suggest that 0.1 Pa represents the lowest wall shear stress in venules (Lipowsky *et al.*, 1978; Klitzman & Duling, 1979), it is likely that shear stress falls below this level in situations of compromised flow, e.g. in ischaemic tissues. Thus, our observation of adhesion without rolling, indicates that activated neutrophils might become immobilised in low flow states without endothelial stimulation or expression of selectins.

We found that adhesion of activated neutrophils was maximal at the first measurable time (1–3 min) after stimulation with fMLP, and then declined, so that there was little additional adhesion after the initial 15 min. Adherent neutrophils did not spontaneously detach from the endothelial surface, and some at least, actively moved over it. Thus, although the stimulus to adhere subsided, adherent cells did not completely reverse their adhesive bonds after an extended period of activation.

Treatment with a monoclonal antibody to the CD18 molecule greatly reduced adhesion of unstimulated and fMLP-stimulated neutrophils, demonstrating that the attachment of flowing neutrophils to endothelium was mediated by the β2 class of integrin molecules. Neutrophils constitutively express β2 integrins on their surface; upon activation there is a rapid change in the conformation of CD18 which alters binding to ICAM-1, and a more gradual increase in the quantity of CD11b/CD18 derived from intracellular stores (see Arnaout, 1990, for review). The rapid time course of the increase in integrin-mediated adhesion following fMLP stimulation is consistent with the conformational change in CD18, and with the previous demonstration that recruitment of CD11b/CD18 is not necessary for increased adhesion to endothelium in a static assay (Vedder & Harlan, 1988).

The activation-dependent increase in neutrophil adhesion was significantly inhibited by nitrendipine, but not by the structurally similar compound nifedipine. Nifedipine, and several other calcium channel antagonists, have been shown to inhibit neutrophil activation, as judged by production of oxidants (Zimmerman *et al.*, 1989; Nalini *et al.*, 1991). A previous, preliminary report suggested that nitrendipine inhibited neutrophil adhesion by disregulating G-proteins in a manner independent of its calcium channel blocking activity, and thus interrupted the signal transduction process of chemoattractant stimulation (Rosen & Wheyell, 1990). Recently, we have quantified the activation-dependent increase in expression of neutrophil integrins in whole blood, by immunofluorescence (Bateman *et al.*, 1993). Subsequently, we have found that increased expression of the β2-linked integrin CD11b after fMLP treatment is not inhibited by nitrendipine (unpublished observations). Thus, nitrendipine does not inhibit all pathways of activation, but appears to block selectively the activation-dependent alteration of the affinity of integrin adhesion molecules.

The above-mentioned results suggest that structurally similar calcium-channel blockers, such as nitrendipine and nifedipine, may affect neutrophil function via different mechanisms, or be selective, in that their efficiency for inhibiting different functions varies. Inhibition of neutrophil adhesion to endothelium may reduce neutrophil-mediated tissue damage *in vivo* (Vedder *et al.*, 1988). We have shown that nitrendipine can inhibit up-regulation of integrin-mediated adhesion, at concentrations close to those likely to be achieved *in vivo* ($\cong 5 \times 10^{-8}$ M) (Goa & Sorkin, 1987). Thus, further studies of this and related compounds would be useful to evaluate their potentials as inhibitors of neutrophil-related tissue injury in ischaemic and thrombotic disorders.

We gratefully acknowledge the advice and assistance of Dr Brian Cooke with culture procedures, the helpful discussions with Dr Bruno Rosen, and the financial support of Bayer AG.

References

ARNAOUT, M.A. (1990). Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood*, **75**, 1037–1050.

BATEMAN, J., PARIDA, S.K. & NASH, G.B. (1993). Neutrophil integrin assay for clinical studies. *Cell Biochem. Funct.*, **11**, 87–91.

BAZZONI, G., DEJANA, E. & DEL MASCHIO, A. (1991). Platelet-neutrophil interactions: possible relevance in the pathogenesis of thrombosis and inflammation. *Haematologica*, **76**, 491–499.

COOKE, B.M., PERRY, I., USAMI, S. & NASH, G.B. (1993). A simplified method for the culture of endothelial cells and analysis of adhesion of blood cells under conditions of flow. *Microvasc. Res.*, **45**, 35–45.

DE-GAETANO, G., EVANGELISTA, V., RAJTAR, G., DEL MASCHIO, A. & CERLETTI, C. (1990). Activated polymorphonuclear leukocytes stimulate platelet function. *Thrombos. Res.*, Suppl. XI, 25–32.

FEIN, A.M. (1991). Neutrophil endothelial interaction in critical illness. *Chest*, **99**, 1456–1462.

GOA, K. & SORKIN, E. (1987). Nitrendipine: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the treatment of hypertension. *Drugs*, **33**, 123–155.

GOLDSMITH, H.L. (1984). Radial distribution of white cells in tube flow. In *White Cell Mechanics: Basic Science and Clinical Aspects.* ed. Meiselman, H.J., Lichtman, M. & LaCelle, P.L. pp. 131–146. New York: Alan R. Liss.

HARLAN, J.M. (1985). Leukocyte-endothelial interactions. *Blood*, **65**, 513–525.

HERNANDEZ, L.A., GRISHAM, M.B., TWOHIG, B., ARFORS, K.-E., HARLAN, J.M. & GRANGER, D.N. (1987). Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am. J. Physiol.*, **253**, H699–H703.

KLITZMAN, B. & DULING, B.R. (1979). Microvascular haematocrit and red cell flow in resting and contracting striated muscle. *Am. J. Physiol.*, **237**, H481–H490.

LAWRENCE, M.B., SMITH, C.W., ESKIN, S. & MCINTIRE, L.V. (1990). Effect of venous shear stress on CD-18 mediated neutrophil adhesion to cultured endothelium. *Blood*, **75**, 227–237.

LAWRENCE, M.B. & SPRINGER, T.A. (1991). Leukocytes roll on a selectin at physiological flow rates: distinction from and prerequisite for adhesion through integrins. *Cell*, **65**, 859–873.

LEY, K., GAEHTGENS, P., FENNIE, C., SINGER, M.S., LASKY, L.A. & ROSEN, S.D. (1991). Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood*, **77**, 2553–2555.

LIPOWSKY, H.H., KOVALCHEK, S. & ZWEIFACH, B.W. (1978). The distribution of blood rheological parameters in the microcirculation of cat mesentery. *Circ. Res.*, **43**, 738–749.

LUSCINKAS, F., CYBULSKY, M., KELY, J., PERKINS, C., DAVIS, V. & GIMBRONE, M. (1991). Cytokine activated human neutrophil transmigration via mechanism involving both ELAM-1 and ICAM-1. *J. Immunol.*, **146**, 1617–1625.

NALINI, K., ANDRABI, K., GANGULAY, N. & WAKI, P. (1991). Nifedipine affects neutrophil functions by a non-calcium mediated mechanism. *Eur. Heart J.*, **12**, (suppl. D), 105–107.

OSBORN, L. (1990). Leukocyte adhesion to endothelium in inflammation. *Cell*, **62**, 3–6.

ROSEN, B. & WHEYWELL, S. (1990). fMLP induced neutrophil adhesion on endothelial cells can be inhibited by nitrendipine. *Int. J. Microcir. Clin. Exp.*, **9**, (suppl.1), 80.

SMITH, C.W., KISHOMOTO, T., ABBAS, O., HUGHES, B., ROTHLIEN, R. & MCINTIRE, L.V. (1991). Chemotactic factors regulate lectin adhesion molecule 1 (LECAM-1) dependent neutrophil adhesion to cytokine stimulated endothelial cells in vitro. *J. Clin. Invest.*, **87**, 609–618.

SMITH, C.W., MARLIN, A., ROTHLEIN, R., TOMA, C. & ANDERSON, D. (1989). Cooperative interaction of LFA-1 and Mac-1 with ICAM-1 in facilitating adherence and transendothelial migration of human neutrophils *in vivo*. *J. Clin. Invest.*, **83**, 2008–2017.

VEDDER, N.B. & HARLAN, J.M. (1988). Increased surface expression of CD11b/CD18 (Mac-1) is not required for stimulated neutrophil adherence to cultured endothelium. *Blood*, **81**, 676–682.

VEDDER, N.B., WINN, R.K., RICE, L.C., CHI, E.Y., ARFORS, K.-E. & HARLAN, J.M. (1988). A monoclonal antibody to the adherence-promoting leukocyte glycoprotein, CD18, reduces organ injury and improves survival from hemorhrhagic shock and resuscitation in rabbits. *J. Clin. Invest.*, **81**, 939–944.

WORTHEN, G.S., SCHWAB, B., ELSON, E.L. & DOWNEY, G.P. (1989). Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries. *Science*, **245**, 183–186.

ZIMMERMAN, G.A. & MCINTYRE, T. (1988). Neutrophil adherence to human endothelium in vitro occurs by CD18 glycoprotein dependent and independent mechanisms. *J. Clin. Invest.*, **81**, 531–537.

ZIMMERMAN, G.A., PRESCOTT, S.M. & MCINTYRE, T.M. (1992). Endothelial cell interactions with granulocytes: tethering and signalling molecules. *Immunol. Today*, **13**, 93–100.

ZIMMERMAN, J.J., ZUK, S.M. & MILLARD, J.R. (1989). In vitro modulation of human neutrophil superoxide anion generation by various calcium channel antagonists used in ischaemia-reperfusion resuscitation. *Biochem. Pharmacol.*, **38**, 3601–3610.

(Received April 27, 1993
Revised July 14, 1993
Accepted September 1, 1993)

Br. J. Pharmacol. (1993), **110**, 1635–1639 © Macmillan Press Ltd, 1993

Mediation of the hypotensive action of systemic clonidine in the rat by α_2-adrenoceptors

[1]J. Paul Hieble & David C. Kolpak

Department of Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406, U.S.A.

1 During the past few years it has been shown that the sympatholytic effect resulting from localized microinjection of clonidine and other imidazolines into the rostral ventrolateral medulla (RVL) results from activation of 'imidazoline' receptors (I_1 receptors) rather than from an α_2-adrenoceptor-mediated effect.

2 The relative contributions of these two receptor systems to the hypotensive action of systemically administered clonidine have not been studied. Clonidine has afffinity for both I_1 and α_2-adrenoceptors; guanabenz represents a useful pharmacological tool, since it activates only the α_2-adrenoceptor.

3 Antagonists acting at both I_1 and α_2-adrenoceptors (idazoxan) and at only α_2-adrenoceptors (SK&F 86466; 6-chloro-3-methyl-2,3,4,5-tetrahydro-3-benzazepine) are available. Idazoxan (1 mg kg^{-1}, i.v.) and SK&F 86466 (3 mg kg^{-1}, i.v.) produced an equivalent degree of blockade of the pressor response to guanabenz or clonidine in the pithed rat, a response mediated by the α_2-adrenoceptor.

4 Guanabenz (30 μg kg^{-1}, i.v.) and clonidine (10 μg kg^{-1}, i.v.) lowered blood pressure in the chloralose-anaesthetized spontaneously hypertensive rat by 48 ± 4.6 mmHg and 44 ± 5.4 mmHg, respectively; this response, for either agonist, was blocked by both idazoxan and SK&F 86466.

5 These data show that the hypotensive effect of intravenously administered clonidine results from activation of central α_2-adrenoceptors, with no significant contribution from an I_1-mediated effect. Thus clonidine can lower blood pressure by different receptor mechanisms, dependent on the route of administration.

Keywords: α_2-Adrenoceptors; imidazoline receptors; idazoxan; guanabenz; SK&F 86466; centrally acting antihypertensives; rostral ventrolateral medulla

Introduction

It is now recognized that there are specific 'receptors' which recognize compounds containing imidazole, imidazoline and guanidinum moities, although the endogenous ligand(s) for these receptors has not yet been identified (Ernsberger *et al.*, 1987; Parini *et al.*, 1989; Lehmann *et al.*, 1989; Bricca *et al.*, 1989a; Michel *et al.*, 1989; Wikberg & Uhlen, 1990). Most of the characterization of these receptors has been performed via radioligand binding assays, and two major subtypes of imidazoline receptor have been identified, and designated I_1 and I_2 (Michel & Ernsberger, 1992). The I_1 receptor is preferentially labelled by [^{3}H]-clonidine or [^{3}H]-*para*-aminoclonidine, and the I_2 by [^{3}H]-idazoxan. In both man and experimental animals, a high density of I_1 receptors is found in a specific area of the brainstem, the rostral ventrolateral medullar (RVL; also referred to as the nucleus reticularis lateralis (NRL)) (Ernsberger *et al.*, 1987; Bricca *et al.*, 1989b), and, in the rat, the hypotensive and bradycardiac action of clonidine and other imidazolines, administered via localized microinjection to this region, has been shown to result from activation of I_1 receptors (Ernsberger *et al.*, 1990; 1992; Gomez *et al.*, 1991). Thus, the hypotensive action of clonidine could be antagonized by local injection of idazoxan, an antagonist having affinity for both I_1 receptors and α_2-adrenoceptors, but not by SK&F 86466 (6-chloro-3-methyl-2,3,4,5-tetrahydro-3-benzazepine) which blocks only the α_2-adrenoceptor. Guanabenz, an agonist at α_2-adrenoceptors but not at I_1 receptors, had little effect on blood pressure and heart rate when administered to the RVL (Ernsberger *et al.*, 1990).

It has long been assumed that the ability of clonidine and related compounds to lower blood pressure results from stimulation of central α_2-adrenoceptors. The selective α_2-adrenoceptor antagonists, yohimbine and rauwolscine, will produce dose-related blockade of the hypotensive effect of clonidine, administered via the vertebral artery to the anaesthetized cat (Timmermans *et al.*, 1981). Similar results are observed in the dog (Schmitt *et al.*, 1973). Intravenous yohimbine has been shown to block the hypotensive action of intravenous clonidine in the anaesthetized normotensive rat (Gutkind *et al.*, 1986). In the anaesthetized cat (Hamilton *et al.*, 1980) or rat (Borkowski & Finch, 1979), centrally administered yohimbine can block the hypotensive response to intracerebroventricular clonidine. Since yohimbine has essentially no affinity for the I_1 receptor (Ernsberger *et al.*, 1987; Bricca *et al.*, 1989a), it is likely that clonidine is acting via α_2-adrenoceptors in the above models. However, a recent study (Tibirica *et al.*, 1991a) found a low dose of intracisternal yohimbine to be ineffective against the hypotensive effect of intravenous clonidine, although the clonidine-induced metabolic effects in the locus coeruleus were attenuated.

In this report, we examine the relative role of α_2-adrenoceptors and I_1-receptors in the blood pressure lowering effect of intravenous clonidine in the anaesthetized spontaneously hypertensive rat.

Methods

Measurement of α_2-adrenoceptor-mediated pressor activity in the pithed rat

Male Sprague-Dawley rats (Charles River Labs, Wilmington, Mass., U.S.A.) weighing 250–350 g were anaesthetized with methohexitone (10 mg kg^{-1}, i.v.). The trachea was cannulated and polyethylene cannulae containing heparinized (20 units ml^{-1}) saline were introduced into the femoral artery and vein for monitoring blood pressure and intravenous drug administration, respectively. One orbit was perforated and the spinal cord destroyed by pushing a stainless steel rod (2 mm dia-

[1] Author for correspondence.

meter) forward through the spinal canal. Immediately after pithing, the rats were connectd to a rodent respirator (Harvard Model No. 683) via the tracheal cannula, and artificially respired at 60 strokes min^{-1}, with a tidal volume of 1 ml $100\,g^{-1}$ of body weight. Animals were gently secured to a water-circulating heating pad maintained at 37°C.

Clonidine ($0.1-100\,\mu g\,kg^{-1}$) or guanabenz ($1.0-300\,\mu g\,kg^{-1}$) were administered serially via i.v. bolus. Changes in blood pressure were monitored via the arterial cannula with a pressure transducer and physiological recorder. Doses were administered when the blood pressure response to the previous dose had stabilized. Only one dose-response curve was determined in each animal. Test antagonists were administered via slow i.v. bolus (1–2 min) 5 min prior to determination of the agonist dose-response curve.

Measurement of blood pressure effects in anaesthetized rats

Male spontaneously hypertensive rats (Taconic Farms, Germantown, NY, U.S.A.), age 16–20 weeks, were anaesthetized with α-chloralose ($80\,mg\,kg^{-1}$, i.v.) and placed on a water-circulating heating pad maintained at 37°C. Polyethylene cannulae containing heparinized (20 units ml^{-1}) saline were inserted into the femoral artery and vein.

Animals were given a single i.v. bolus dose of either clonidine ($10\,\mu g\,kg^{-1}$) or guanabenz ($30\,\mu g\,kg^{-1}$) and blood pressure was monitored for 70 min. Test antagonists (SK&F 86466 or idazoxan) were administered via slow (1–2 min) i.v. bolus injetion 5 min before agonist challenge.

Drugs used

Clonidine, guanabenz and idazoxan were obtained from Research Biochemicals Inc (Natick, Mass, U.S.A.). α-Chloralose was obtained from Fisher Scientific Company. SK&F 86466 (6-chloro-3-methyl-2,3,4,5-tetrahydro-3-benzazepine) was synthesized at SmithKline Beecham Pharmaceuticals.

Results

Both clonidine and guanabenz produced dose-related increases in blood pressure in the pithed normotensive rat (Figures 1 and 2). Clonidine was somewhat more potent ($ED_{50} = 3\,\mu g\,kg^{-1}$) and produced a slightly greater maximum increase in diastolic blood pressure (108 mmHg) than guanazbenz ($ED_{50} = 20\,\mu g\,kg^{-1}$; maximum response = 95 mmHg). SK&F 86466, at a dose of $3\,mg\,kg^{-1}$, i.v., produced a 5.2 fold shift to the right in the clonidine dose-response curve, idazoxan, at a dose of $1\,mg\,kg^{-1}$, i.v., produced a 3.6 fold shift (Figure 1). These antagonists produced a slightly greater shift in the dose-response curve for guanabenz (8.7 and 10 fold shifts for idazoxan and SK&F 86466, respectively at the same doses tested against clonidine) (Figure 2).

In chloralose-anaesthetized spontaneously hypertensive rats, clonidine ($10\,\mu g\,kg^{-1}$, i.v.) produced its well known biphasic effect on blood pressure, with an initial increase, followed by a long lasting fall in pressure. The hypotensive phase was attenuated by SK&F 86466 at $1\,mg\,kg^{-1}$, i.v., administered 5 min prior to clonidine. Increasing the SK&F 86466 dose to $3\,mg\,kg^{-1}$ resulted in a nearly complete abolition of the hypotensive phase, and a substantial attenuation of the initial pressor response (Figure 3). Similar results were obtained with guanabenz ($30\,\mu g\,kg^{-1}$; Figure 4). Figure 5 compares the ability of idazoxan and SK&F 86466 to block the antihypertensive action of clonidine and guanabenz. It is clear that the two agonists are equally sensitive to both idazoxan and SK&F 86466. Furthermore treatment with idazoxan ($1\,mg\,kg^{-1}$) or SK&F 86466 ($3\,mg\,kg^{-1}$) produces an equivalent degree of blockade, as measured by attenuation of the peak antihypertensive effect.

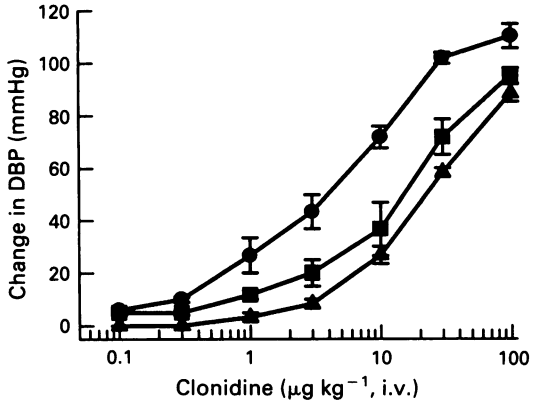


Figure 1 Effect of idazoxan ($1\,mg\,kg^{-1}$, i.v., ■) and SK&F 86466 ($3\,mg\,kg^{-1}$, i.v., ▲) on the pressor response to clonidine in the pithed rat; control (●). Response to clonidine expressed as increase in diastolic blood pressure (DBP). Basal DBP (mean of treatment groups) = 42 ± 3 mmHg. Each curve represents the mean ± s.e.mean of six animals.

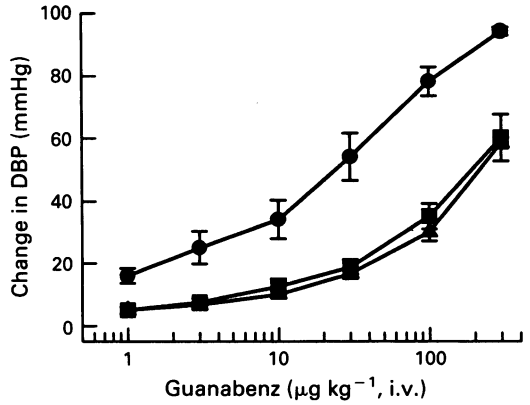


Figure 2 Effect of idazoxan ($1\,mg\,kg^{-1}$, i.v., ■) and SK&F 86466 ($3\,mg\,kg^{-1}$, i.v., ▲) on the pressor response to guanabenz in the pithed rat; control (●). Response to guanabenz expressed as increase in diastolic blood pressure (DBP). Basal DBP (mean of treatment groups) = 41 ± 2 mmHg. Each curve represents the mean ± s.e.mean of six animals.

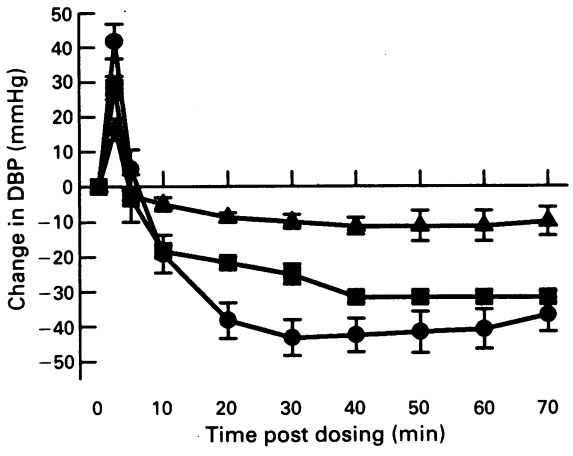


Figure 3 Blockade by SK&F 86466 of the antihypertensive action of clonidine ($10\,\mu g\,kg^{-1}$) in the chloralose-anaesthetized spontaneously hypertensive rat. Clonidine administered in time = 0, in SK&F 86466-treated animals, the antagonist was administered 5 min prior to clonidine challenge. All drugs were given via i.v. bolus administration: (●) control; (■) $1\,mg\,kg^{-1}$ SK&F 86466; (▲) $3\,mg\,kg^{-1}$ SK&F 86466. Each curve represents the mean ± s.e.mean of six animals.

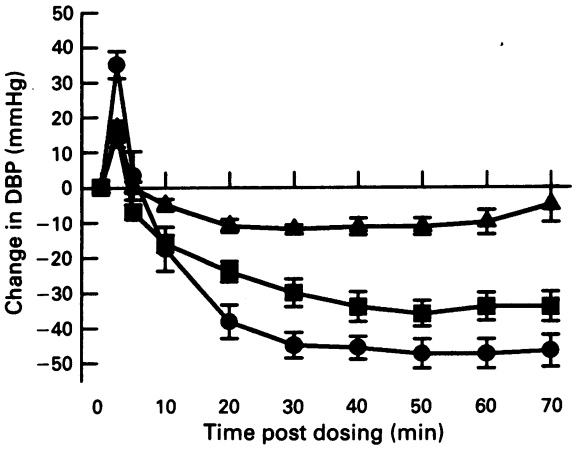


Figure 4 Blockade by SK&F 86466 of the antihypertensive action of guanabenz (30 μg kg^{-1}) in the chloralose-anaesthetized spontaneously hypertensive rat. Guanabenz administered at time = 0; in SK&F 86466-treated animals, the antagonist was administered 5 min prior to clonidine challenge. All drugs were given via i.v. bolus administration. Symbols as in Figure 3. Each curve represents the mean ± s.e.mean of six animals.

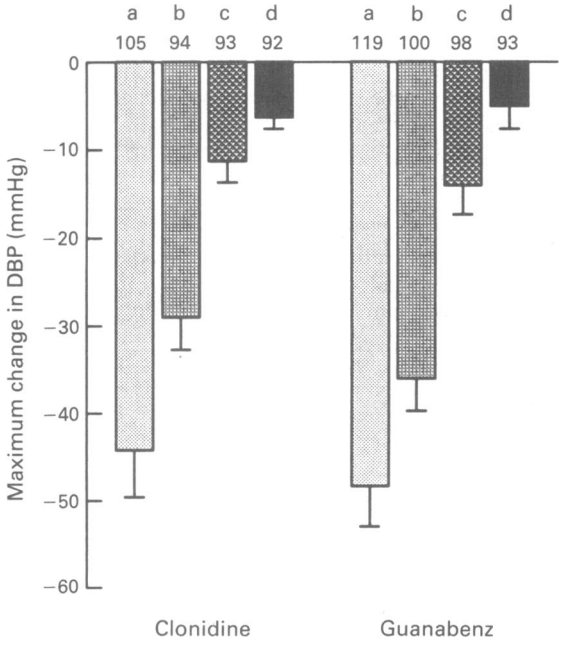


Figure 5 Effect of SK&F 86466 and idazoxan on the antihypertensive action of clonidine (10 μg kg^{-1}) and guanabenz (30 μg kg^{-1}) in the chloralose-anaesthetized rat. All drugs were administered via i.v. bolus. Each column represents the maximum decrease in diastolic blood pressure (DBP) observed during the 70 min interval following agonist administration (see Figures 3 and 4 for the time course of the response to clonidine and guanabenz, respectively). Basal DBP prior to antagonist administration was 108.5 ± 2.5 mmHg (mean of all groups). DBP subsequent to antagonist dosing, but prior to agonist challenge is shown above each column. (a) Control; (b) SK&F 86466 1 mg kg^{-1}; (c) SK&F 86466 3 mg kg^{-1}; (d) idazoxan 1 mg kg^{-1}. Each column represents the mean with s.e.mean of six animals.

Discussion

The evidence for involvement of I_1 receptors in the sympatholytic action of clonidine, administered by localized injection to the RVL region of the rat, is quite convincing. Indeed, since the hypotensive effect of clonidine is insensitive to SK&F 86466, and the ability of a structurally diverse series of compounds to lower blood pressure and heart rate correlates well with I_1 receptor, but not with α_2-adrenoceptor affinity (Ernsberger *et al.*, 1990) it appears that the α_2-adrenoceptor may not be involved to any significant degree when agonists are administered via this route.

On the other hand, the early studies showing blockade by yohimbine or rauwolscine of the effects of clonidine, administered via the intravertebral or intra-cerebroventricular route, support an α_2-adrenoceptor-mediated effect. At least in some cases (Borkowski & Finch, 1979) the yohimbine doses are high, and it is known that high doses of yohimbine can block α_1-adrenoceptors as well as other neurotransmitter receptors (e.g. 5-hydroxytryptamine (5-HT) and dopamine). It is unlikely that clonidine would lower blood pressure via activation of 5-HT or dopamine receptors. Although a hypotensive action mediated via central α_1-adrenoceptors has been suggested (Gutkind *et al.*, 1986), the studies of Timmermans *et al.* (1981) using yohimbine, rauwolscine and corynanthine suggest that clonidine does not act via this mechanism, at least in the cat.

Clonidine, which crosses the blood brain barrier readily, will have access to many sites within the central nervous system upon intravenous or intra-vertebral arterial administration. It is known that blood pressure can be reduced by α-adrenoceptor stimulation in locations other than the RVL, including the nucleus tractus solitarius (NTS) (Kubo & Misu, 1981; Brody *et al.*, 1984). It also appears that different agonists, each presumed to be acting at α_2-adrenoceptors, can lower blood pressure by acting at different sites within the central nervous system (Scholtysik *et al.*, 1975; Gutkind *et al.*, 1986; van den Buuse *et al.*, 1993). Gutkind *et al.* (1986) found the hypotensive action of clonidine, but not guanabenz to be sensitive to yohimbine blockade. These results differ from our data, obtained with different antagonists, which suggest that these agonists act via a similar mechanism.

Considering the multiple pathways controlling sympathetic outflow, it is likely that different receptor mechanisms could be involved in the generalized central application of clonidine from when the drug is locally applied to a specific nucleus. Intracerebroventricular administration may represent an intermediate situation, since access may depend on the distance of individual nuclei from the ventricular surface. It is even possible that different receptor mechanisms are involved in conscious and anaesthetized animals, since intracerebroventricular yohimbine failed to block the hypotensive effect of intracerebroventricular clonidine in conscious normotensive or hypertensive rats (Kawasaki *et al.*, 1992), contrasting to similar experiments in anaesthetized animals where the same intracerebroventricular dose of yohimbine was effective (Borkowski & Finch, 1979).

Functional and radioligand binding studies suggest that the agonists and antagonists used in this study should be suitable tools for evaluating the relative contributions of α_2-adrenoceptors and I_1 receptors. Table 1 shows representative values showing clonidine and idazoxan to have affinity for both receptors, and guanabenz and SK&F 86466 to be highly selective for the α_2-adrenoceptor. Guanabenz was found to be slightly more potent than clonidine at α_2-adrenoceptors in the RVL. Radioligand binding studies at this receptor in rat, canine and bovine cortex have shown either agent to be more potent, although the potency ratio is generally two or less (Table 1). Consistent with our data (Figures 1 and 2), guanabenz has been reported to be slightly less potent than clonidine as a pressor agent in the pithed rat, with a lower maximum response (Gutkind *et al.*, 1986). Guanabenz is also less potent than clonidine in producing hypotension and bradycardia in the anaesthetized rat (Gutkind *et al.*, 1986) and as an antihypertensive drug in man (Walker *et al.*, 1982). SK&F 86466 has about ten fold lower α_2-adrenoceptor affinity than idazoxan in the bovine RVL (Ernsberger *et al.*, 1990); however, in other α_2-adrenoceptor binding assays, the two compounds have essentially equal

Table 1 Receptor affinities of pharmacological tools for differentiation of α_2-adrenoceptors and I_1 receptors

	Affinity (nM)[1]				
Tissue	*Clon*	*GBZ*	*SKF*	*IDZ*	*Reference*
α_2-Adrenoceptors					
Human adipocyte	32	39	26	19	Langin *et al.*, 1990
Bovine RVL[2]	28	7.2	35	3.6	Ernsberger *et al.*, 1990
Bovine cortex	5.3	11	84	61	Ernsberger *et al.*, 1990
Guinea-pig ileum[3]	8.5	14	300	31	Hieble *et al.*, 1990
Guinea-pig atrium[4]	15	ND	17	13	Hieble *et al.*, 1986a,b
I_1-receptors					
Bovine RVL[5]	1	$>10^6$	93,000	186	Ernsberger *et al.*, 1990

[1]Affinity of clonidine (Clon), guanabenz (GBZ), SK&F 86466 (SKF) and idazoxan (IDZ) expressed as K_i for inhibition of radioligand binding, K_B (functional potency of antagonists) or IC_{50} (functional potency of agonists). Unless otherwise noted, affinity determined by radioligand binding assay.
[2]α_2-Adrenoceptor component of [^{3}H]-*p*-amino clonidine binding.
[3]Functional potency for inhibition of short circuit current, or blockade of the inhibitory effect of clonidine.
[4]Functional potency for inhibition of the inotropic response to field stimulation, or blockade of the inhibitory effect of B-HT 920.
[5]I_1 receptor component of [^{3}H]-*p*-amino clonidine binding.

affinity (Ernsberger *et al.*, 1990; Blaxall *et al.*, 1991), and functional *in vitro* assays show the two antagonists to be essentially equipotent (Hieble *et al.*, 1986a,b).

Our *in vivo* assay in the pithed rat shows idazoxan to be about three fold more potent as an α_2-adrenoceptor antagonist, as reflected by blockade of guanabenz-induced increases in blood pressure (Figure 2). The lower absolute potency of both SK&F 86466 and idazoxan against the response to clonidine in this model may reflect the greater contribution of the α_1-adrenoceptor to the clonidine-induced pressor response, consistent with previous data in the pithed rat (Timmermans & Van Zweiten, 1980; Gutkind *et al.*, 1986). Clonidine is less selective than guanabenz as an α_2-adrenoceptor agonist, based on ability to inhibit binding of antagonist ligands to central α_1- and α_2-adrenoceptors (Megens *et al.*, 1986).

In contrast to α-adrenoceptor-mediated pressor responses in the pithed rat, the hypotensive response to clonidine could be mediated either by α_2-adrenoceptors, I_1-receptors, or by a combination of the two mechanisms. Since SK&F 86466 was essentially equipotent against clonidine and guanabenz, it is unlikely that an I_1 receptor-mediated effect makes a significant contribution to the hypotensive response to intravenous clonidine.

It has been suggested that clonidine and guanabenz may lower blood pressure in the rat by an action at different centres within the central nervous system (Gutkind *et al.*, 1986). Based on the different *in vitro* affinities of these two agonists for I_1 receptors, this certainly remains a possibility. Studies with local administration of a variety of centrally acting sympatholytic agents supports differences in their mechanism of action. Nevertheless the current results would suggest that both clonidine and guanabenz act by stimulation of α_2-adrenoceptors when administered intravenously, although it cannot be established that identical neuronal populations are activated.

Several agents, e.g. rilmenidine and moxonidine, have been postulated to have greater selectivity than clonidine for I_1 receptors versus α_2-adrenoceptors (Bricca *et al.*, 1989b; Gomez *et al.*, 1992; Ernsberger *et al.*, 1992; 1993). It has been proposed that the relative lack of side effects of these drugs, *vis-à-vis* clonidine and other clinically used α_2-adrenoceptor agonists, results from this selectivity (King *et al.*, 1992; Tibirica *et al.*, 1991b). Alternatively, some other difference in the overall pharmacological profile could contribute to the favourable properties of the newer agents. For example, rilmenidine lacks the histamine (H_2), receptor agonist activity of clonidine (Li *et al.*, 1989).

Most of the evidence supporting an I_1 receptor contribution to the antihypertensive action of these drugs has come from studies using local administration to the RVL. The results presented here with clonidine and guanabenz would suggest that studies examining the mechanism by which these drugs lower blood pressure when systemically administered be performed before assuming that I_1 receptor selectivity is the only factor contributing to their novel clinical profile.

References

BLAXALL, H.S., MURPHY, T.J., BAKER, J.C., RAY, C. & BYLUND, D.B. (1991). Characterization of the alpha-2C adrenergic receptor subtype in the opossum kidney and in the OK cell line. *J. Pharmacol. Exp. Ther.*, **259**, 323–329.

BRICCA, G., DONTENWILL, M., MOLINES, A., FELDMAN, J., BELCOURT, A. & BOUSQUET, P. (1989a). The imidazoline preferring receptor: binding studies in bovine, rat and human brainstem. *Eur. J. Pharmacol.*, **162**, 1–9.

BRICCA, G., DONTENWILL, M., MOLINES, A., FELDMAN, J., BELCOURT, A. & BOUSQUET, P. (1989b). Rilmenidine selectivity for imidazoline receptors in human brain. *Eur. J. Pharmacol.*, **163**, 373–377.

BRODY, M.J., O'NEILL, T.P. & PORTER, J.P. (1984). Role of central catecholaminergic systems in pathogenesis and treatment of hypertension. *J. Cardiovasc. Pharmacol.*, **6** (Suppl 5), S727–S741.

BORKOWSKI, K.R. & FINCH, L. (1979). A comparison of the cardiovascular effects of centrally administered clonidine and adrenaline in the anesthetized rat. *J. Pharm. Pharmacol.*, **31**, 16–19.

ERNSBERGER, P., DAMON, T.H., GRAFF, L.M., SCHAFER, S.G. & CHRISTEN, M.O. (1993). Moxonidine, a centrally acting antihypertensive agent, is a selective ligand for I_1-imidazoline sites. *J. Pharmacol. Exp. Ther.*, **264**, 172–182.

ERNSBERGER, P., GIULIANO, R., WILLETTE, R.N. & REIS, D.J. (1990). Role of imidazole receptors in the vasodepressor response to clonidine analogs in the rostral ventrolateral medulla. *J. Pharmacol. Exp. Ther.*, **253**, 408–418.

ERNSBERGER, P., HAXHIU, M.A., DAMON, T.H. & WENDORFF, L.A. (1992). Imidazoline I_1 receptors and brainstem autonomic control: membrane binding, autoradiographic and functional studies. *FASEB J.*, **6**, A1874.

ERNSBERGER, P., MEELEY, M.P., MANN, J.J. & REIS, D.J. (1987). Clonidine binds to imidazole binding sites as well as α_2-adrenoceptors in the ventrolateral medulla. *Eur. J. Pharmacol.*, **134**, 1–13.

GOMEZ, R.E., ERNSBERGER, P., FEINLAND, G. & REIS, D.J. (1991). Rilmenidine lowers arterial pressure via imidazole receptors in brainstem C1 area. *Eur. J. Pharmacol.*, **195**, 181–191.

GUTKIND, J.S., KAZANETZ, M. & ENERO, M.A. (1986). Cardiovascular effects of alpha-adrenergic drugs: differences between clonidine and guanfacine. *Naunyn-Schmied Arch. Pharmacol.*, **332**, 370–375.

HAMILTON, T.C., HUNT, A.A.E. & POYSER, R.H. (1980). Involvement of central α_2-adrenoceptors in the mediation of clonidine-induced hypotension in the cat. *J. Pharm. Pharmacol.*, **32**, 788–789.

HIEBLE, J.P., DEMARINIS, R.M., FOWLER, P.J. & MATTHEWS, W.D. (1986a). Selective alpha-2 adrenoceptor blockade by SK&F 86466: in vitro characterization of receptor selectivity. *J. Pharmacol. Exp. Ther.*, **236**, 90–96.

HIEBLE, J.P., MCCAFFERTY, J.P., NASELSKY, D. & BONDINELL, W.E. (1990). In vitro characterization of the α_2-adrenoceptor of the rat enterocyte. *Eur. J. Pharmacol.*, **183**, 1204–1205.

HIEBLE, J.P., SULPIZIO, A.C., NICHOLS, A.J., DEMARINIS, R.M., PFEIFFER, F.R., LAVANCHY, P.G. & RUFFOLO, R.R. Jr. (1986b). Pharmacological differentiation of pre- and postjunctional alpha$_2$-adrenoceptors. *J. Hyperten.*, **4** (Suppl 6), S189–S192.

KAWASAKI, H., NAKAMURA, S. & TAKASAKI, K. (1992). Central α_2-adrenoceptor mediated pressor response to clonidine in conscious, spontaneously hypertensive rats. *Jpn. J. Pharmacol.*, **59**, 321–331.

KING, P.R., GUNDLACH, A.L., JAROTT, B. & LOUIS, W.J. (1992). α_2-Adrenoceptor and catecholamine-insensitive binding sites for [^{3}H] rilmenidine in membranes from rat cerebral cortex. *Eur. J. Pharmacol.*, **218**, 101–108.

KUBO, T. & MISU, Y. (1981). Pharmacological characterization of the alpha-adrenoceptors responsible for a decrease of blood pressure in the nucleus tractus solitarii of the rat. *Naunyn-Schmied. Arch. Pharmacol.*, **317**, 120–125.

LANGIN, D., PARIS, H. & LAFONTAN, M. (1990). Binding of [^{3}H] idazoxan and of its methoxy derivative [^{3}H] RX 821002 in human fat cells: [^{3}H] Idazoxan but not [^{3}H] RX821002 labels additional non α_2-adrenergic binding sites. *Mol. Pharmacol.*, **37**, 876–885.

LEHMANN, J., KOENIG-BERARD, E. & VITOU, P. (1989). The imidazoline-preferring receptor. *Life Sci.*, **45**, 1609–1615.

LI, C.G. & RAND, M.J. (1989). Rilmenidine differs from clonidine in that it lacks histamine-like activity. *J. Pharm. Pharmacol.*, **41**, 464–468.

MEGENS, A.A.H.P., LEYSEN, J.FE., AWOUTERS, F.H.L. & NIEMEGEERS, C.J.E. (1986). Further validation of in vivo and in vitro pharmacological procedures for assessing the α_2/α_1-selectivity of test compounds: α-adrenoceptor agonists. *Eur. J. Pharmacol.*, **129**, 57–64.

MICHEL, M.C., BRODDE, O.-E., SCHNEPEL, B., BEHRENDT, J., TSCHADA, R., MOTULSKY, H.J. & INSEL, P.A. (1989). [^{3}H] Idazoxan and some other α_2-adrenergic drugs also bind with high affinity to a nonadrenergic site. *Mol. Pharmacol.*, **35**, 324–330.

MICHEL, M.C. & ERNSBERGER, P. (1992). Keeping an eye on the I site: imidazoline-preferring receptors. *Trends Pharmacol. Sci.*, **13**, 369–370.

PARINI, A., COUPRY, I., GRAHAM, R.M., UZIELLI, I., ATLAS, D. & LANIER, S. (1989). Characterization of an imidazoline/guanidinium receptive site distinct from the α_2-adrenergic receptor. *J. Biol. Chem.*, **264**, 11874–11878.

SCHMITT, H., SCHMITT, H. & FENARD, S. (1973). Action of α-blocking drugs on the sympathetic centres and their interactions with the central sympatho-inhibitory effect of clonidine. *Arzneim-Forschung*, **23**, 40–45.

SCHOLTYSIK, G., EICHENBERGER, E., BURKI, H., SALZMANN, R., MULLER-SCHWEINITZER, E. & WAITE, R. (1975). Pharmacological actions of the antihypertensive agent N-amidino-2(2,6-dichlorophenyl) acetamide hydrochloride (BS 100–141). *Arzneim. Forsch.*, **25**, 1483–1491.

TIBIRICA, E., FELDMAN, J., MERMET, C., GONON, F. & BOUSQUET, P. (1991a). An imidazoline-specific mechanism for the hypotensive effect of clonidine: a study with yohimbine and idazoxan. *J. Pharmacol. Exp. Ther.*, **256**, 606–613.

TIBIRICA, E., FELDMAN, J., MERMET, C., MONASSIER, L., GONON, F. & BOUSQUET, P. (1991b). Selectivity of rilmenidine for the nucleus reticularis lateralis, a ventrolateral medullary structure containing imidazoline-preferring receptors. *Eur. J. Pharmacol.*, **209**, 213–221.

TIMMERMANS, P.B.M.W.M., SCHOOP, A.M.C., KWA, H.Y. & VAN ZWIETEN, P.A. (1981). Characterization of α-adrenoceptors participating in the central hypotensive and sedative effects of clonidine using yohimbine, rauwolscine and corynanthine. *Eur. J. Pharmacol.*, **70**, 7–15.

TIMMERMANS, P.B.M.W.M. & VAN ZWIETEN, P.A. (1980). Postsynaptic α_1- and α_2-adrenoceptors in the circulatory system of the pithed rat: selective stimulation of the α_2-type by B-HT 933. *Eur. J. Pharmacol.*, **63**, 199–202.

VAN DEN BUUSE, M., HEAD, G.A. & KORNER, P.I. (1993). Differential role of brain ascending noradrenergic bundles in the circulatory effects of α-methyldopa and clonidine. *J. Cardiovasc. Pharmacol.*, **21**, 112–119.

WALKER, B.R., HARE, L.E. & DEITCH, M.W. (1982). Comparative antihypertensive effects of guanabenz and clonidine. *J. Int. Med. Res.*, **10**, 6–14.

WIKBERG, J.E.S. & UHLEN, S. (1990). Further characterization of the guinea pig cerebral cortex idazoxan receptor: solubilization, distinction from the imidazole site, and demonstration of cirazoline as an idazoxan receptor-selective drug. *J. Neurochem.*, **55**, 192–203.

(Received April 8, 1993
Revised September 1, 1993
Accepted September 2, 1993)

Br. J. Pharmacol. (1993), **110**, 1640–1644 © Macmillan Press Ltd, 1993

Insulin-mediated sensitization of adenylyl cyclase activation

[1]Ross D. Feldman

Departments of Medicine and Pharmacology and Toxicology, University of Western Ontario, Canada

1 Insulin may be an important regulator of vascular function. We have previously studied lymphocyte β-adrenoceptors as a model for the human vascular β-adrenoceptor. To examine the effects of insulin on human β-adrenoceptor responsiveness, adenylyl cyclase activity, cyclic AMP-dependent protein kinase activity and β-adrenoceptor radioligand binding assays were performed on permeabilized mononuclear leukocytes.

2 With acute exposure to insulin *in vitro*, followed by washing and permeabilization there was a dose-dependent increase in both lymphocyte NaF-stimulated activity and β-adrenoceptor-stimulated adenylyl cyclase activity paralleling an increase in β-adrenoceptor-stimulated protein kinase A activity. Manganese-, forskolin- and forskolin plus guanylimidodiphosphate-stimulated adenylyl cyclase activities were not altered by insulin pretreatment. Additionally, mononulcear leukocyte β-adrenoceptor density, proportion of externalized receptors and receptor affinity for agonist were not altered.

3 The data indicate that acute exposure to insulin sensitizes G-protein-stimulated adenylyl cyclase activity. These findings suggest a potential role for insulin in the regulation of β-adrenoceptor responsiveness in man.

Keywords: Insulin; β-adrenoceptors; adenylyl cyclase; mononuclear leukocytes

Introduction

Insulin may have an important role in physiological and pathophysiological cardiovascular regulation. Defects in insulin secretion/insulin receptor responsiveness, namely hyperinsulinemia and insulin resistance have been implicated as potential mechanisms in the pathogenesis and/or maintenance of hypertension (Reaven, 1991; Ferrannini *et al.*, 1987). Effects on sympathetic activity and catecholamine-mediated responses may be important mechanisms by which insulin regulates vascular function. Recent studies have focused on the effects of insulin on sympathetic activity. High insulin concentrations increase sympathetic activity (Anderson *et al.*, 1991). However, even with euglycaemic clamping, insulin infusion increases forearm blood flow (Creager *et al.*, 1985; Anderson *et al.*, 1991) (as opposed to the reduction in forearm blood flow that might be expected under other circumstances in the setting of increased sympathetic activity under conditions where systemic blood pressure was maintained).

The effect of insulin on vascular adrenoceptor responsiveness is unclear. High insulin levels have been associated with both increased (Gans *et al.*, 1991; Townsend *et al.*, 1992) and decreased (Wamback & Liu, 1991) α-adrenoceptor responsiveness. The effect of insulin on vascular β-adrenoceptor responsiveness is unknown. However, a possible interaction between insulin and the β-adrenoceptor complex has been inferred from the observations of Creager *et al.* (1985). They demonstrated that the effect of insulin to increase forearm blood flow was blocked by β-adrenoceptor antagonists.

To determine whether insulin might have a role in regulating human β-adrenoceptor cardiovascular responsiveness we examined the effects of insulin *in vitro* on human lymphocytes. We have previously utilized human lymphocytes as a model for the vascular human β-adrenoceptor complex. We and others have demonstrated that under certain conditions (i.e. aging and hypertension) alterations in lymphocyte β-adrenoceptor properties parallel alterations in vascular β-adrenoceptor responsiveness (Feldman *et al.*, 1987; Feldman, 1990; 1992; Naslund *et al.*, 1990). The data to be presented show that insulin sensitizes lymphocyte β-adrenoceptor-mediated adenylyl cyclase and adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase activity probably at the level of guanine-nucleotide regulatory protein (G-protein) function.

[1] Address for correspondence: P.O. Box 5339, University Hospital, London, Ontario, Canada N6A 5A5.

Methods

Lymphocytes were isolated from whole blood samples drawn from normotensive healthy subjects between the age of twenty and forty years. Samples were drawn in the morning following an overnight fast. Mononuclear leukocytes were isolated from Ficoll-Hypaque gradients by the methods of Boyum (1968) as previously described (Feldman, 1989). Isolated mononuclear leukocytes were resuspended in RPMI 1640 (GIBCO, Grand Island, NY, U.S.A.) and incubated with various concentrations of human insulin (Humulin, Eli Lilly & Company, Indianapolis, Indiana, U.S.A.). Mononuclear leukocytes were permeabilized (Digitonin 10 μg ml^{-1} for 15 min at 4°C) in Hanks' Basic Salt Solution supplemented with magnesium sulphate 1 mM, EDTA 0.5 mM, HEPES 20 mM, (pH 7.4 at 25°C). Preparations were centrifuged at 400 *g* for 10 min at 4°C, resuspended in Hanks' supplemented solution as above, recentrifuged at 400 *g* for 10 min at 4°C and resuspended in Hanks' supplemented buffer for assay.

Assays of adenylyl cyclase activity

Assays of adenylyl cyclase activity were performed in digitonin-permeabilized mononuclear leukocyte preparations according to previously published methods (Feldman, 1989). Adenylyl cyclase activity was assessed by the conversion of [α^{32}P]-adenosine triphosphate (ATP) to [^{32}P]-cyclic adenosine 3':5'-monophosphate ([^{32}P]-cyclic AMP) by modifications of the methods of Salomon *et al.* (1974). Cells, suspended in supplemented Hanks' balanced salt solution (pH 7.4 at 4°C), were added at a final concentration of 4 to 6 × 10^6 cells ml^{-1}. Assays were performed with an incubation volume of 200 μl with ~1 μCi [α^{32}P]-ATP (New England Nuclear, Boston, U.S.A.), 0.3 mM ATP, 0.1 mM cyclic AMP, 5 mM phosphoenolpyruvate, 40 μg ml^{-1} pyruvate kinase and 20 μg ml^{-1} myokinase for 10 min at 37°C. Assays were performed at a

free magnesium concentration of 1 mM. The ^{32}P-labelled nucleotides were separated by sequential Dowex and alumina chromatography and assessed by scintillation spectroscopy (Beckman LS6000). (−)-Isoprenaline-stimulated activity (with guanosine triphosphate, GTP, 100 μM) was expressed as the increase in activity above that seen with GTP alone. Forskolin-, manganese- and sodium fluoride-(NaF)-stimulated activities was expressed as the increase in activity above basal levels.

Assessment of cyclic AMP-dependent protein kinase activity

Cyclic AMP-dependent protein kinase (PKA) activity was determined in permeabilized mononuclear leukocytes preparations as previously described (Feldman, 1989). PKA activity was assessed by ^{32}P-phosphorylation of synthetic substrate (Keptide-Sigma Chemical Company) using [γ^{32}P]-ATP. Briefly, cells were incubated for 12 min at 30°C with 1 mM Kemptide, 1 μg ml^{-1} bovine serum albumin, 0.5 mM ascorbic acid, 0.8 mM ATP and ~1 μCi [α^{32}P]-ATP. Phosphorylated substrate was separated by adherence to phosphocellulose (Whatman P81 chromatography paper). Following six washes in phosphoric acid (75 mM), radioactivity on the phosphocellulose strips was assessed by scintillation spectroscopy. (−)-Isoprenaline-stimulated activity was assessed at six concentrations ranging from 1 nM to 10 μM. The EC_{50} for (−)-isoprenaline and maximal (−)-isoprenaline-stimulated activity was determined by computerized non-linear curve-fitting (GraphPad, Inplot 4.0, Sigmoid Plot Subroutine, GraphPad Software, San Diego, CA, U.S.A.).

Assessment of β-adrenoceptor specific radioligand binding

Radioligand binding studies were performed in permeabilized mononuclear leukocytes according to previously described methods (Feldman, 1989). The high affinity β-adrenoceptor-specific radioligand [^{125}I]-iodopindolol (2.2 Ci μM^{-1}; New England Nuclear, Boston, MA, U.S.A.) was used. Assays were performed at 25°C for 90 min by which time steady state was achieved at all radioligand concentrations used.

Saturation binding curve techniques were used to determine receptor density (B_{max}) and receptor affinity for the radioligand (K_D). [^{125}I]-iodopindolol labelling in the presence of propranolol (1 μM) was defined as non-specific binding. The proportion of specific sites blocked by the hydrophilic β-adrenoceptor antagonist CGP-12177 (30 nM) was used as an index of 'non-sequestered', externalized β-receptor sites, as previously described (Feldman, 1989). Receptor affinity for the agonist (−)-isoprenaline in the presence and absence of guanylylimidodiphosphate, a hydrolysis-resistant analogue of GTP (Gpp(NH)p 100 μM), was determined from 12 point competition curves using permeabilized cells. Radioligand binding data were analysed by computerized curve-fitting techniques. Saturation binding curves were analysed for receptor density (B_{max}) and receptor affinity (K_D) for [^{125}I]-iodopindolol (Rectangular Hyperbola Subroutine, Inplot 4.0, GraphPad Software Inc., San Diego, CA, U.S.A.). Agonist competition curves in the presence of Gpp(NH)p were analysed according to a one-site model (Competition Curve Subroutine, Inplot 4.0). Agonist competition curves in the absence of guanine nucleotides were analysed according to a two-site model where the K_D for the low affinity state was that determined experimentally as the K_D for (−)-isoprenaline in the presence of Gpp(NH)p. The overall IC_{50} for (−)-isoprenaline (derived from agonist competition curves in the absence of Gpp(NH)p) was determined by computerized non-linear curve-fitting of the data (Sigmoid Curve Subroutine, Inplot 4).

Statistical analysis

Measures of potency (i.e. EC_{50}, K_D) were expressed as geometric means ± standard errors of the mean (s.e.mean). Other parameters were expressed as arithmetic means ± s.e.mean. Paired Student's t tests were used for normally distributed data and Wilcoxon signed ranks tets were used for non-normally distributed data. Dose-response curves were initially analysed by ANOVA, followed by Student's t test. A value of $P<0.05$ on a two-tailed test was used as a minimum level of significance.

Results

Insulin-mediated alterations in adenylyl cyclase activity

To assess the effect of pre-exposure to insulin on the subsequent assessment of adenylyl cyclase responsiveness, human mononuclear leukocytes were incubated with insulin at a concentration of 1 pM to 1 nM for 10 min at 37°C. Cells were washed and permeabilized. β-Adrenoceptor-stimulated adenylyl cyclase activity was assessed by the response to (−)-isoprenaline (100 μM). G-protein stimulated adenylyl cyclase activity was assessed by the response to NaF (3 mM). Insulin exposure was associated with a dose-dependent increase in both NaF- and (−)-isoprenaline-stimulated adenylyl cyclase activities (Figure 1). Interestingly, the insulin effect on NaF-stimulated activity appeared to be biphasic with a peak effect at an insulin concentration of 100 pM. In contrast, a significant effect of insulin on (−)-isoprenaline-stimulated adenylyl cyclase activity was apparent only at a concentration of 1 nM. Basal activity was not affected by insulin pretreatment (Table 1). Proinsulin (1 nM for 10 min) had no significant effect on (−)-isoprenaline-stimulated activity (98 ± 15% of control, $n = 3$). Subsequent studies utilized an insulin concentration of 1 nM for assessment of alterations in (−)-isoprenaline-mediated response and 100 pM for NaF and other stimulators of adenylyl cyclase.

The effect of insulin pretreatment was also evident at more prolonged preincubation times. Following 30 min of preincubation with insulin (1 nM) (−)-isoprenaline-stimulated activity was increased 25 ± 10% vs. control (−)-isoprenaline-stimulated activity ($n = 4$) and following 60 min was increased 29 ± 15% ($n = 4$).

To assess whether the effect of insulin pretreatment on β-adrenoceptor responsiveness might represent an apparent alteration in the K_{act} for magnesium (and hence might be minimized at higher magnesium concentrations), assays were

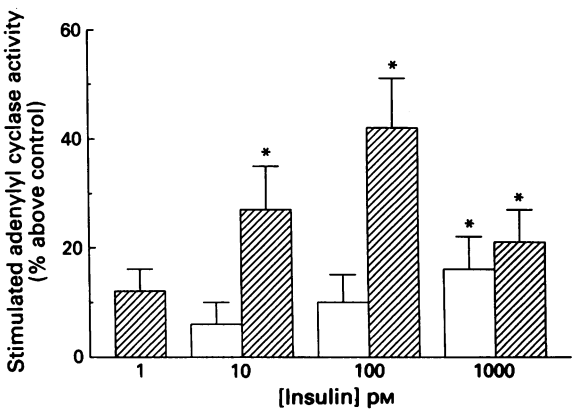


Figure 1 Insulin-mediated alterations in (−)-isoprenaline- and NaF-stimulated adenylyl cyclase activation. The effect of varying concentrations of insulin is expressed as the increase in activity vs. control (−)-isoprenaline- (open columns) and NaF-(hatched columns) stimulated activities. Data were analysed by ANOVA followed by paired comparisons vs. control. *$P<0.05$ vs. control.

Table 1 Effects of insulin exposure (1 nM) on (−)-isoprenaline-stimulated adenylyl cyclase activity

	Control	*Insulin-treated*
	(pmol $min^{-1}/10^6$ cells)	
GTP	0.80 ± 0.10	0.85 ± 0.12
Iso	1.19 ± 0.13	1.34 ± 0.18‡
Iso − GTP	0.39 ± 0.04	0.49 ± 0.07*

GTP denotes adenylyl cyclase activity with guanosine triphosphate (GTP, 100 μM) only. Iso denotes activity with (−)-isoprenaline (100 μM) with GTP. Iso − GTP represents the difference between (−)-isoprenaline- and GTP-stimulated activity. Data represent the mean ± s.e.mean from 12 experiments performed under identical conditions.
*$P<0.05$ vs. corresponding control activity. ‡$P=0.05$ vs. corresponding control activity.

Table 2 Effects of insulin on cyclic AMP-dependent protein kinase activity (PKA)

	Control	*Insulin-treated*
	(pmol $min^{-1}/10^6$ cells)	
Basal	124 ± 6	129 ± 4
Iso − Basal	51 ± 8	63 ± 12*
Cyclic AMP	346 ± 35	390 ± 55

Iso − basal denotes (−)-isoprenaline-stimulated PKA activity above basal levels. Cyclic AMP denotes PKA activity stimulated by cyclic AMP (1 mM). *$P<0.05$. Data represent the mean ± s.e.mean from 6 experiments performed under identical conditions.

Table 3 Effect of insulin on total β-adrenoceptor density and externalized β-adrenoceptor density

	Control	*Insulin-treated*
K_D IPIN (pM) [log K_D ± s.e.mean]	4.2 [−11.28 ± 0.10]	3.9 [−11.41 ± 0.10]
$B_{max\text{-}tot}$ (sites/cell)	663 ± 60	656 ± 79
$B_{max\text{-}ext}$ (sites/cell)	615 ± 46	632 ± 76

Total receptor density ($B_{max\text{-}tot}$) was determined from [^{125}I]-iodopindolol (IPIN) saturation binding curves where propranolol (1 μM) was used to assess non-specific binding. Externalized receptor density ($B_{max\text{-}ext}$) was determined from [^{125}I]-IPIN saturation binding curves where CGP-12177 (30 nM) was used to assess non-specific and sequestered receptor binding. Data represent the mean ± s.e.mean from 6 studies performed under identical conditions.

Table 4 Effect of insulin on receptor affinity for agonists

	Control	*Insulin-treated*
K_H (nM)	4.5	4.0
[log K_H ± s.e.mean]	[−8.35 ± 0.36]	[−8.40 ± 0.52]
K_L (nM)	392	367
[log K_L ± s.e.mean]	[−6.41 ± 0.07]	[−6.43 ± 0.15]
R_H (%)	50 ± 3	52 ± 6

K_H and K_L are the K_D for the high and low affinity states of the receptor for agonist (respectively). R_H is the % of receptors in the high affinity state. K_H, K_L and R_H estimates were determined from (−)-isoprenaline competition curves for [^{125}I]-iodopindolol binding in the absence and presence of guanine nucleotides. Data are the mean ± s.e.mean from 5 experiments performed under identical conditions.

performed at a free magnesium concentration of 2.5 mM (as opposed to the 1 mM free magnesium concentration used in the previous assays). At a 2.5 mM free magnesium concentration, insulin pretreatment (1 nM) was still associated with an increase in (−)-isoprenaline-stimulated adenylyl cyclase activity comparable to that seen at lower free magnesium concentrations (25 ± 6% greater than control, $n=11$, $P<0.05$).

To determine whether sensitization of adenylyl cyclase responsiveness reflected a membrane-delimited effect of insulin on adenylyl cyclase activity (versus an effect which is dependent on an intact cell system), NaF-stimulated adenylyl cyclase activity was assessed in permeabilized mononuclear leukocyte samples in which insulin was added concurrently with sodium fluoride. Under these conditions, insulin (100 pM) was not associated with any significant sensitization of response (95 ± 16% of control, $n=5$).

To attempt to determine whether insulin was acting at the level of the G-protein complex or directly on the catalytic subunit, manganese chloride (5 mM)-, forskolin (10 μM)- and forskolin + Gpp(NH)p (100 μM)-stimulated activities were assessed. Manganese chloride stimulated adenylyl cyclase activity in permeabilized lymphocytes to a level of 0.76 ± 0.16 pmol $min^{-1}/10^6$ cells over basal activity. Insulin pretreatment (100 pM) was not associated with any significant increase in manganese chloride-stimulated activity (104 ± 6% of control, $n=6$). Forskolin stimulated adenylyl cyclase activity to a level of 32.1 ± 4.1 pmol $min^{-1}/10^6$ cells. Insulin pretreatment did not significantly alter the extent of forskolin stimulation (102 ± 2% of control, $n=4$). The extent of inhibition of forskolin-stimulated activity by co-incubation with guanine nucleotides has been used previously as an index of the activity of the inhibitory G-protein (G_i) (Pyne *et al.*, 1989). Co-incubation of lymphocytes with forskolin and Gpp(NH)p decreased adenylyl cyclase activity to 17.1 ± 4.1 pmol $min^{-1}/10^6$ cells – consistent with a selective effect of G_i. Insulin pretreatment did not significantly alter the extent of Gpp(NH)p inhibition of forskolin-stimulated activity (101 ± 4% of control, $n=4$).

Insulin-mediated alterations in cyclic AMP-dependent protein kinase activity

Previous studies have reported that in some cell models insulin activates cyclic AMP phosphodiesterase (Weber *et al.*, 1987; Weiss & Wheeler, 1988; Smoake & Solomon, 1989). Thus the physiological effect of insulin-sensitization of adenylyl cyclase activation could be attenuated further 'downstream' in the signal transduction pathway. Therefore, we next determined whether insulin-mediated sensitization of β-adrenoceptor responsiveness was still evident at the level of cyclic AMP-dependent protein kinase activity (PKA). It should be noted that these studies were performed without the addition of a phosphodiesterase inhibitor. (−)-Isoprenaline-stimulated PKA was assessed in control and insulin-pretreated mononuclear leukocytes (1 nM for 10 min). Insulin pretreatment increased maximal (−)-isoprenaline-stimulated PKA activation 20 ± 6% ($n=6$, $P<0.05$, Table 2). The EC_{50} for (−)-isoprenaline was not significantly changed with insulin pretreatment (control: 35 nM [+12/−10], insulin: 39 nM [+32/−17], $P>0.3$). Additionally, basal activity and cyclic AMP-stimulated activity were not altered (Table 2).

Effects of insulin on β-adrenoceptor radioligand binding assays

To determine whether the sensitization of β-adrenoceptor responsiveness reflected a change in either total receptor density or in the density of externalized (nonsequestered) receptors, saturation binding curve studies were performed. Total adrenoceptor density was assessed with propranolol (1 μM) as the measure of non-specific binding. Externalized

Table 5 Effects of insulin on adenylyl cyclase-linked systems

Cell type	*Author, year*	*Assays*	*Effect of insulin*
Fat cells	Engfeldt *et al.*, 1988	Binding studies adenylyl cyclase activity	Reduced isoprenaline-mediated effect and β-adrenoceptor density
Hepatocytes	Irvine & Housley, 1988	Adenylyl cyclase activity	Reduced cholera-toxin-stimulated activity
Adrenal cells	Begeot *et al.*, 1989	Adenylyl cyclase activity	Increased cholera-toxin-stimulated activity
Platelets	Kahn & Sinha, 1990	Binding studies and adenylyl cyclase activity	Increased PGE-stimulated activity and receptor density
UMR-106-01 cells	Hickman & McElduff, 1990	Adenylyl cyclase activity	Increased PTH-, isoprenaline- and cholera toxin-stimulated activities
HT29 cells	Devedjian *et al.*, 1991	Binding studies and adenylyl cyclase activity	Reduced α_2-adrenoceptor mediated effect and density
UMR-106-01 cells	Iida-Klein & Hahn, 1991	Adenylyl cyclase activity	No change within physiological range
DDT, MF-2 cells	Hadcock *et al.*, 1992	Adenylyl cyclase activity, β-adrenoceptor-phosphorylation	Reduced isoprenaline-stimulated activity. Increased β-adrenoceptor phosphorylation
Lung explants	Davis *et al.*, 1992	Cyclic AMP accumulation	No change within physiological range
SK-N-MC cells	Bahouth & Lopez, 1992	Adenylyl cyclase activity	Reduced isoprenaline-stimulated activity

receptor sites were assessed with CGP-12177 ((−)-(3-t-butylamino-2-hydroxypropoxy)-benzimidazole-2-one; 30 nM). (Previous studies have demonstrated that agonist-mediated 'internalization' of receptor sites is still detectable in the digitonin-permeabilized leukocyte preparation; Feldman, 1989.) Insulin exposure (1 nM for 10 min) was not associated with any alterations in either total receptor density or in the proportion of externalization sites (Table 3).

To determine whether insulin-mediated sensitization of β-adrenoceptor responsiveness might be associated with a more subtle alteration in adrenoceptor function reflected by altered 'coupling', agonist competition curves were studied comparing samples from control and insulin-pretreated preparations (1 nM for 10 min). Insulin exposure was associated with no significant alterations in the IC_{50} for (−)-isoprenaline inhibition of [^{125}I]-iodopindolol binding, in the proportion of receptors in the high affinity state for agonist (R_H), or in the K_D for the high or low affinity states of the receptor for agonist (Table 4).

Discussion

The present studies indicate that insulin sensitizes β-adrenoceptor responsiveness probably due to effects on G-protein stimulated adenylyl cyclase activity. An effect of insulin at the level of the G-protein complex is suggested by the parallel insulin-mediated increases in both (−)-isoprenaline- and sodium fluoride-stimulated activities. The lack of any apparent sensitization of forskolin- or manganese chloride-stimulated activities suggest that the effect of insulin is not directly on the catalytic subunit, adenylyl cyclase. Further, the radioligand binding studies indicate that acute insulin exposure does not alter β-adrenoceptor density, the proportion of externalized receptor sites nor receptor affinity for agonists.

The effects of insulin on adenylyl cyclase-linked systems are variable. Most studies have used either cell culture models or systems in which insulin is important as a metabolic regulator (hepatocytes and fat cells). In these systems insulin has been reported to impair (Engfeld *et al.*, 1988; Irvine & Houslay, 1988; Bahouth & Lopez, 1992; Davis *et al.*, 1992) or facilitate (Begeot *et al.*, 1989; Kahn & Sinha, 1990; Hickman & McElduff, 1990; Devedjian *et al.*, 1991) receptor-stimulated adenylyl cyclase activity (Table 5). Interpretation of cell culture studies, however, are complicated by the potential confounding effects of insulin as a growth factor, especially in those studies which have examined the longer term effects of insulin in serum-starved cell cultures (Hickman & McElduff, 1990; Iida-Klein & Hahn, 1991; Begeot *et al.*, 1992; Davis *et al.*, 1992), or used higher concentrations of insulin which may interact with other growth factor receptors (Begeot *et al.*, 1989; Iida-Klein & Hahn, 1991; Bahouth & Lopez, 1992; Davis *et al.*, 1992). In a single report examining the effects of insulin in a circulating blood cell system, platelet prostaglandin E (PGE)-stimulated adenylyl cyclase activity was increased by insulin and this paralleled an increase in PGE-receptor density (Kahn & Sinha, 1990). These findings contrast with the lymphocyte model where the increase in β-adrenoceptor-stimulated adenylyl cyclase activity was clearly not related to any alteration in the level of the receptor. Additionally, whereas in the platelet model NaF-stimulated activity was unaltered with insulin exposure, in the present studies insulin comparably sensitized both β-adrenoceptor and NaF-stimulated activities.

In the lymphocyte model the predominant effect of insulin appears to be at the level of G-protein function. A role for insulin in regulating G-protein function has been suggested by Houslay and colleagues (Heyworth *et al.*, 1986; Pyne *et al.*, 1989). They demonstrated that the ability of G_i to be ADP-ribosylated is inhibited by insulin (Pyne *et al.*, 1989) leading to attenuated Gpp(NH)p inhibition of forskolin-stimulated adenylyl cyclase activity. Whether the effect of insulin on the lymphocyte adenylyl cyclase is via sensitization of G_s or inhibition of G_i is unclear. However, Gpp(NH)p inhibition of forskolin-stimulated activity was unchanged with insulin pretreatment. These findings suggest that in the lymphocyte the insulin-mediated sensitization of adenylyl cyclase activity is not due to a reduction in G_i activity. However, the effects of insulin on guanine nucleotide-mediated inhibition of forskolin-stimulated activity was assessed at maximal concentrations of Gpp(NH)p. More subtle alterations in G_i function (e.g. alterations in potency of guanine nucleotides on G_i function) could not be ruled out by the present studies.

The intermediary mechanism by which insulin modulates adenylyl cyclase-linked systems remains unknown. However, the lack of any effect of insulin on adenylyl cyclase activity, when added to permeabilized preparations suggests that the mechanism requires an intact cell system and is probably not a 'membrane delimited' effect. It is notable that insulin may stimulate both phospholipase C (Kellerer *et al.*, 1991) and protein kinase C (Farese *et al.*, 1992). However, the link between PKC activation and the observed alterations in G-protein-stimulated adenylyl cyclase activity is unclear.

Regardless of the mechanism involved, insulin-mediated sensitization of β-adrenoceptor responsiveness could represent an important mechanism by which insulin modulates cardiovascular response to sympathetic activation. Previous *in vivo* studies have demonstrated that insulin infusion both increased sympathetic activity but unexpectedly increased forearm blood flow (Creager *et al.*, 1985; Anderson *et al.*, 1991) and that this effect is mediated through the β-adrenoceptor (Creager *et al.*, 1985) (based on its attenuation in the presence of a β-blocker). Sensitization of β-adrenoceptor responsiveness concurrent with increased sympathetic activity could be a plausible explanation for the observed direct effects of insulin in mediating vasodilatation even in the face of increased sympathetic activity. If so, insulin could be important in regulating the vascular response to sympathetic-activation. In support of this concept we have recently demonstrated that insulin mediates a sensitization of vascular β-adrenoceptor mediated relaxation to an extent which is comparable to its sensitization of lymphocyte adenylyl cyclase activity (R. Gros, K. Borkowski and R. Feldman, manuscript in preparation). In insulin-resistant states, attenuation of this regulatory effect could be important in the development of increased peripheral resistance and ultimately hypertension.

The author is grateful to Mr Jozef Chorazyczewski for his excellent technical assistance and to Mrs Joanne Bianco for her expert preparation of this manuscript. This work was supported by a grant from the Heart and Stroke Foundation of Ontario. R.D.F. is supported by a Career Investigator Award from the Heart and Stroke Foundation of Ontario.

References

ANDERSON, E.A., HOFFMAN, R.P., BALON, T.C., SINKEY, C.A. & MARK, A.L. (1991). Hyperinsulinemia produces both sympathetic neural activation and vasodilation in normal humans. *J. Clin. Invest.*, **87**, 2246–2252.

BAHOUTH, S.W. & LOPEZ, S. (1992). Insulin desensitizes β_1-adrenergic receptor-mediated stimulation of adenylyl cyclase in SK-N-MC cells. *Life Sci.*, **51**, 271–276.

BEGEOT, M., LANGLOIS, D. & SAEZ, J.M. (1989). Insulin-like growth factor-I and insulin increase the stimulatory guanine nucleotide binding protein (Gs) in cultured bovine adrenal cells. *Mol. Cell. Endocrinol.*, **66**, 53–57.

BOYUM, A. (1968). Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.*, **21** (Suppl. 97), 77–89.

CREAGER, M.A., LIANG, C.-S. & COFFMAN, J.D. (1985). Beta adrenergic-mediated vasodilator response to insulin in the human forearm. *J. Pharmacol. Exp. Ther.*, **235**, 709–714.

DAVIS, D.J., HICKMAN, J.M., LEFEBVRE, C.A. & LYON, M.E. (1992). Insulin inhibits β-adrenergic responses in fetal rabbit lung in explant culture. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)*, **7**, L562–L567.

DEVEDJIAN, J.-C., FARGUES, M., DENIS-PROUXVIEL, C., DAVIAUD, D., PRATS, H. & PARIS, H. (1991). Regulation of α_{2A}-adrenergic receptor in the HT29 cell line. *J. Biol. Chem.*, **266**, 14359–14366.

ENGFELDT, P., HELLMER, J., WAHRENBERG, H. & ARNER, P. (1988). Effects of insulin on adrenoceptor binding and the rate of catecholamine-induced lipolysis in isolated human fat cells. *J. Biol. Chem.*, **263**, 15553–15580.

FARESE, R.V., STANDAERT, M.L., ARNOLD, T., YU, B., ISHIZUKA, T., HOFFMAN, J., VILA, M. & COOPER, D.R. (1992). The role of protein kinase C in insulin action. *Cell. Signalling*, **4**, 133–143.

FELDMAN, R.D. (1989). β-adrenergic desensitization reduces the sensitivity of adenylate cyclase for magnesium in permeabilized lymphocytes. *Mol. Pharmacol.*, **35**, 304–310.

FELDMAN, R.D. (1990). Defective venous beta-adrenergic response in borderline hypertensive subjects is corrected by a low sodium diet. *J. Clin. Invest.*, **85**, 647–652.

FELDMAN, R.D. (1992). A low-sodium diet corrects the defect in β-adrenergic response in older subjects. *Circulation*, **85**, 612–618.

FELDMAN, R.D., LAWTON, W.J. & MCARDLE, W.L. (1987). Low sodium diet corrects the defect in lymphocyte β-adrenergic responsiveness in hypertensive subjects. *J. Clin. Invest.*, **79**, 290–294.

FERRANNINI, E., BUZZIGOLI, G., BONADONNA, R., GIORICO, M.A., OLEGGINI, M., GRAZIADEI, L., PEDRINELLI, R., BRANDI, L. & BEVILACQUA, S. (1987). Insulin resistance in essential hypertension. *N. Engl. J. Med.*, **317**, 350–357.

GANS, R.O.B., BILO, H.J.G., V. MAARSCHALKERWEERD, W.W.A., HEINE, R.J., NAUTA, J.J.P. & DONKER, A.J.M. (1991). Exogenous insulin augments in healthy volunteers the cardiovascular reactivity to noradrenaline but not to angiotensin II. *J. Clin. Invest.*, **88**, 512–518.

HADCOCK, J.R., PORTS, J.D., GELMAN, M.S. & MALBON, C.C. (1992). Cross-talk between tyrosine kinase and G-protein-linked receptors. *J. Biol. Chem.*, **267**, 26017–26022.

HEYWORTH, C.M., GRAY, A.M., WILSON, S.R., HANSKI, E. & HOUSLAY, M.D. (1986). The action of islet activating protein (pertussis toxin) on insulin's ability to inhibit adenylate cyclase and activate cyclic AMP phosphodiesterases in hepatocytes. *Biochem. J.*, **235**, 145–149.

HICKMAN, J. & MCELDUFF, A. (1990). Insulin sensitizes a cultured rat osteogenic sarcoma cell line to hormones which activate adenylate cyclase. *Calcif. Tissue Int.*, **46**, 401–405.

IIDA-KLEIN, A. & HAHN, T.J. (1991). Insulin acutely suppresses parathyroid hormone second messenger generation in UMR-106-01 osteoblast-like cells: differential effects on phospholipase C and adenylate cyclase activation. *Endocrinology*, **129**, 1016–1024.

IRVINE, F.J. & HOUSLEY, M.D. (1988). Insulin and glucagon attenuate the ability of cholera toxin to activate adenylate cyclase in intact hepatocytes. *Biochem. J.*, **251**, 447–452.

KAHN, N.N. & SINHA, A.K. (1990). Stimulation of prostaglandin E_1 binding to human blood platelet membrane by insulin and the activation of adenylate cyclase. *J. Biol. Chem.*, **265**, 4976–4981.

KELLERER, M., MACHICAO, F., SEFFER, E., MUSHACK, J., ULLRICH, A. & HARING, H.U. (1991). Stimulation of phospholipase C activity by insulin is mediated by both isotypes of the human insulin receptor. *Biochem. Biophys. Res. Commun.*, **181**, 566–572.

NASLUND, T., SILBERSTEIN, D.J., MERRELL, J.W., NADEAU, J.H. & WOOD, A.J.J. (1990). Low sodium intake corrects abnormality in β-receptor-mediated arterial vasodilation in patients with hypertension: correlation with β-receptor function *in vitro*. *Clin. Pharmacol. Ther.*, **48**, 87–95.

PYNE, N.J., HEYWORTH, C.M., BALFOUR, N.W. & HOUSLAY, M.D. (1989). Insulin affects the ability of G_i to be ADP-ribosylated but does not elicit its phosphorylation in intact hepatocytes. *Biochem. Biophys. Res. Commun.*, **165**, 251–256.

REAVEN, G.M. (1991). Insulin resistance and compensatory hyperinsulinemia: role in hypertension, dyslipidemia, and coronary heart disease. *Am. Heart J.*, **121**, 1283–1288.

SALOMON, Y., LONDOS, C. & RODBELL, M. (1974). A highly sensitive adenylate cyclase assay. *Analyt. Biochem.*, **58**, 541–548.

SMOAKE, J.A. & SOLOMON, S.S. (1989). Insulin control of cyclic AMP phosphodiesterase. *Life Sci.*, **45**, 2255–2268.

TOWNSEND, R.R., YAMAMOTO, R., NICKOLS, M., DIPETTE, D.J. & NICKOLS, G.A. (1992). Insulin enhances pressor responses to norepinephrine in rat mesenteric vasculature. *Hypertension*, **19** (Suppl II), II-105–II-110.

WAMBACK, G.K. & LIU, D. (1991). Insulin attenuates vasoconstriction induced by noradrenaline, serotonin and potassium chloride in rat mesenteric resistance arteries. *J. Hypertension*, **9** (Suppl 6), S198–S199.

WEBER, H.W., CHUNG, C.-Z., DAY, K. & APPLEMAN, M.M. (1987). Insulin stimulation of cyclic AMP phosphodiesterase is independent from the G-protein pathways involved in adenylate cyclase regulation. *J. Cyclic Nucleotide Res.*, **11**, 345–354.

WEISS, R.M. & WHEELER, M.A. (1988). Insulin activation of cyclic AMP phosphodiesterase in intact ureteral segments. *J. Pharmacol. Exp. Ther.*, **247**, 630–634.

(Received March 10, 1993
Revised August 15, 1993
Accepted September 3, 1993)

British Journal of Pharmacology

VOLUME 110 (4) DECEMBER 1993

OBITUARY

Hugh Blaschko, FRS 1275

SPECIAL REPORTS

H. Matsuno, T. Uematsu & M. Nakashima. Thrombolytic effects of a plasminogen-plasminogen activator chimera in a photochemically induced thrombosis (PIT) model 1278

G. Edwards & A.H. Weston. Induction of a glibenclamide-sensitive K-current by modification of a delayed rectifier channel in rat portal vein and insulinoma cells 1280

R.S. Berman, J.D. Frew & W. Martin. Endotoxin-induced arterial endothelial barrier dysfunction assessed by an *in vitro* model 1282

PAPERS

P.M. Zygmunt, P.K.E. Zygmunt, E.D. Högestätt & K.-E. Andersson. Effects of ω-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the rabbit urethra and detrusor 1285

M. Facklam & N.G. Bowery. Solubilization and characterization of $GABA_B$ receptor binding sites from porcine brain synaptic membranes 1291

G.P. Connolly, P.J. Harrison & T.W. Stone. Action of purine and pyrimidine nucleotides on the rat superior cervical ganglion 1297

P.A. Iredale & S.J. Hill. Increases in intracellular calcium via activation of an endogenous P_2-purinoceptor in cultured CHO-K1 cells 1305

C.A.E. Martin, E. Naline, L. Manara & C. Advenier. Effects of two β_3-adrenoceptor agonists, SR 58611A and BRL 37344, and of salbutamol on cholinergic and NANC neural contraction in guinea-pig main bronchi *in vitro* 1311

J.B. Louttit & R.A. Coleman. The action of Hoe 140 on the bradykinin-induced splenic pressor reflex of the anaesthetized cat 1317

R.G. Hempelmann & A. Ziegler. Endothelium-dependent noradrenaline-induced relaxation of rat isolated cerebral arteries: pharmacological characterization of receptor subtypes involved 1321

G.E. Boeckxstaens, J.G. De Man, P.A. Pelckmans, K.M. Cromheeke, A.G. Herman & Y.M. Van Maercke. Ca^{2+} dependency of the release of nitric oxide from non-adrenergic non-cholinergic nerves 1329

N.G.M. Schipper, J.C. Verhoef, L.M. De Lannoy, S.G. Romeijn, J.H. Brakkee, V.M. Wiegant, W.H. Gispen & F.W.H.M. Merkus. Nasal administration of an ACTH(4–9) peptide analogue with dimethyl-β-cyclodextrin as an absorption enhancer: pharmacokinetics and dynamics 1335

S.-C.G. Hui, E.L. Sevilla & C.W. Ogle. 5-HT_3 antagonists reduce morphine self-administration in rats 1341

J.G. Filep & E. Földes-Filep. Modulation by nitric oxide of platelet-activating factor-induced albumin extravasation in the conscious rat 1347

M.A. Khoyi, H.H. Dalziel, L. Zhang, R.A. Bjur, W.T. Gerthoffer, I.L.O. Buxton & D.P. Westfall. $[Ca^{2+}]_i$-sensitive, IP_3-independent Ca^{2+} influx in smooth muscle of rat vas deferens revealed by procaine 1353

P.-E. Sandström. Inhibition by hydrochlorothiazide of insulin release and calcium influx in mouse pancreatic β-cells 1359

J.C. Wanstall, J.S. Thompson & A.H. Morice. Effects of atrial natriuretic peptide and nitroprusside on isolated pulmonary resistance and conduit arteries from rats with pulmonary hypertension 1363

P. Lopes, D. Regoli & R. Couture. Cardiovascular effects of intrathecally administered bradykinin in the rat: characterization of receptors with antagonists 1369

J.W. Growcott, B. Holloway, M. Green & C. Wilson. Zeneca ZD7114 acts as an antagonist at β_3-adrenoceptors in rat isolated ileum 1375

J. Nogueira de Francischi, D.M. Conroy, K. Maghni & P. Sirois. Inhibition by rapamycin of leukocyte migration and bronchial hyperreactivity induced by injection of Sephadex beads to guinea-pigs 1381

E.J. Kelso, B.J. McDermott & B. Silke. Cardiotonic actions of selective phosphodiesterase inhibitors in rat isolated ventricular cardiomyocytes 1387

H. Okumura, N. Ashizawa, F. Kobayashi, K. Arai, R. Asakura, N. Ashikawa & A. Matsuura. Comparison of haemoconcentration induced by big endothelin-1 and endothelin-1 in mice 1395

A.R. Baydoun, R.G. Bogle, J.D. Pearson & G.E. Mann. Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells 1401

G. Lombardi, M. Alesiani, P. Leonardi, G. Cherici, R. Pellicciari & F. Moroni. Pharmacological characterization of the metabotropic glutamate receptor inhibiting D-[^{3}H]-aspartate output in rat striatum 1407

M. Hernández, U. Simonsen, D. Prieto, L. Rivera, P. García, E. Ordaz & A. García-Sacristán. Different muscarinic receptor subtypes mediating the phasic activity and basal tone of pig isolated intravesical ureter 1413

J.-X. Bao & L. Stjärne. Dual contractile effects of ATP released by field stimulation revealed by effects of α,β-methylene ATP and suramin in rat tail artery 1421

M. Freissmuth, W. Beindl & M. Kratzel. Binding and structure-activity-relation of benzo[*f*]isoquinoline- and norcodeinone-derivatives at μ-opioid receptors in the rat cerebral cortex 1429

H. Tsunoda, T. Miyauchi, K. Fujita, T. Kubo & K. Goto. Mechanism of rat uterine smooth muscle contraction induced by endothelin-1 1437

M.N. Perkins & D. Kelly. Induction of bradykinin B_1 receptors *in vivo* in a model of ultra-violet irradiation-induced thermal hyperalgesia in the rat 1441

M. Kobari, Y. Fukuuchi, M. Tomita, N. Tanahashi, S. Konno & H. Takeda. Effects of sumatriptan on the cerebral intraparenchymal microcirculation in the cat 1445

J.M. Dickenson & S.J. Hill. Homologous and heterologous desensitization of histamine H_1- and ATP-receptors in the smooth muscle cell line, DDT_1MF-2: the role of protein kinase C 1449

S.M. Gardiner, P.A. Kemp, T. Bennett, R.M.J. Palmer & S. Moncada. Regional and cardiac haemodynamic effects of N^G, N^G, dimethyl-L-arginine and their reversibility by vasodilators in conscious rats 1457

K. Kurz, I. von Kügelgen & K. Starke. Prejunctional modulation of noradrenaline release in mouse and rat vas deferens: contribution of P_1- and P_2-purinoceptors **1465**

J.F. Kleha, P. Devesly & A. Johns. EDRF release from canine coronary artery by lectins **1473**

M.J. Rand & C.G. Li. Modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves **1479**

R.G. Pertwee, L.A. Stevenson & G. Griffin. Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabimimetic agents, CP 55,940, WIN 55,212-2 and anandamide **1483**

A.U. Ziganshin, C.H.V. Hoyle, X. Bo, G. Lambrecht, E. Mutschler, H.G. Bäumert & G. Burnstock. PPADS selectively antagonizes P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder **1491**

M.M. Teixeira & P.G. Hellewell. Suppression by intradermal administration of heparin of eosinophil accumulation but not oedema formation in inflammatory reactions in guinea-pig skin **1496**

S. Yamanaka, H. Iwao, T. Yukimura, S. Kim & K. Miura. Effect of the platelet-activating factor antagonist, TCV-309, and the cyclo-oxygenase inhibitor, ibuprofen, on the haemodynamic changes in canine experimental endotoxic shock **1501**

H.-C. Huang, Y.-I. Weng, C.-R. Lee, T.-R. Jan, Y.-L. Chen & Y.-T. Lee. Protection by scoparone against the alterations of plasma lipoproteins, vascular morphology and vascular reactivity in hyperlipidaemic diabetic rabbit **1508**

M.M. Teixeira, T.J. Williams & P.G. Hellewell. Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin **1515**

C. David, N. Davis, R. Mason & V.G. Wilson. Evidence for functional dissociation of dependence and tolerance in guinea-pig isolated ileal segments following 20 hour exposure to morphine *in vitro* **1522**

M. Hori, K. Sato, S. Miyamoto, H. Ozaki & H. Karaki. Different pathways of calcium sensitization activated by receptor agonists and phorbol esters in vascular smooth muscle **1527**

S.M. Gardiner, P.A. Kemp & T. Bennett. Regional haemodynamic responses to intravenous and intraarterial endothelin-1 and big endothelin-1 in conscious rats **1532**

G. Bricca, J. Zhang, H. Greney, M. Dontenwill, J. Stutzmann, A. Belcourt & P. Bousquet. Relevance of the use of [^{3}H]-clonidine to identify imidazoline receptors in the rabbit brainstem **1537**

I. von Kügelgen, L. Späth & K. Starke. Ionotropic glutamate receptor types leading to adenosine-mediated inhibition of electrically evoked [^{3}H]-noradrenaline release in rabbit brain cortex slices **1544**

A. MacDonald & M. Lamont. Effects of selective antagonism of β-adrenoceptor subtypes on responses to isoprenaline in rat distal colon *in vitro* **1551**

T. Ibbotson, G. Edwards & A.H. Weston. Antagonism of levcromakalim by imidazoline- and guanidine-derivatives in rat portal vein: involvement of the delayed rectifier **1556**

W. Paul, P. Gresele, S. Momi, G. Bianchi & C.P. Page. The effect of defibrotide on thromboembolism in the pulmonary vasculature of mice and rabbits and in the cerebral vasculature of rabbits **1565**

B. Costall, R.J. Naylor & B.R. Tuladhar. 5-HT_4 receptor mediated facilitation of the emptying phase of the peristaltic reflex in the guinea-pig isolated ileum **1572**

M.K. Pugsley, D.A. Saint, M.P. Penz & M.J.A. Walker. Electrophysiological and antiarrhythmic actions of the κ agonist PD 129290 and its **R,R** (+)-enantiomer, PD 129289 **1579**

M.J. Oset-Gasque, M. Parramón & M.P. González. $GABA_B$ receptors modulate catecholamine secretion in chromaffin cells by a mechanism involving cyclic AMP formation **1586**

K.A. Wardle & G.J. Sanger. The guinea-pig distal colon – a sensitive preparation for the investigation of 5-HT_4 receptor-mediated contractions **1593**

R.P. Brownlie, N.J. Brownrigg, H.M. Butcher, R. Garcia, R. Jessup, V.J. Lee, S. Tunstall & M.G. Wayne. ZD1542, a potent thromboxane A_2 synthase inhibitor and receptor antagonist *in vitro* **1600**

N.M.J. Rupniak, S. Boyce, A.R. Williams, G. Cook, J. Longmore, G.R. Seabrook, M. Caesar, S.D. Iversen & R.G. Hill. Antinociceptive activity of NK_1 receptor antagonists: non-specific effects of racemic RP67580 **1607**

H. Inoue, N. Nagata & Y. Koshihara. Profile of capsaicin-induced mouse ear oedema as neurogenic inflammatory model: comparison with arachidonic acid-induced ear oedema **1614**

S.C. Cheetham & D.J. Heal. Evidence that RU 24969-induced locomotor activity in C57/Bl/6 mice is specifically mediated by the 5-HT_{1B} receptor **1621**

I. Perry, S.M. Buttrum & G.B. Nash. Effect of activation on adhesion of flowing neutrophils to cultured endothelium: time course and inhibition by a calcium channel blocker (nitrendipine) **1630**

J.P. Hieble & D.C. Kolpak. Mediation of the hypotensive action of systemic clonidine in the rat by α_2-adrenoceptors **1635**

R.D. Feldman. Insulin-mediated sensitization of adenylyl cyclase activation **1640**

BRITISH JOURNAL OF PHARMACOLOGY

The *British Journal of Pharmacology* welcomes contributions in all fields of experimental pharmacology including neuroscience, biochemical, cellular and molecular pharmacology. The Board of Editors represents a wide range of expertise and ensures that well-presented work is published as promptly as possible, consistent with maintaining the overall quality of the journal.

Edited for the British Pharmacological Society by

A.T. Birmingham
(Chairman)

R.W. Horton **W.A. Large**
(Secretaries)

Editorial Board

J.A. Angus *Victoria, Australia*
M.L.J. Ashford *Cambridge*
G.W. Bennett *Nottingham*
W.C. Bowman *Glasgow*
N.G. Bowery *London*
Alison F. Brading *Oxford*
S.D. Brain *London*
K.T. Bunce *Ware*
K.D. Butler *Horsham*
M. Caulfield *London*
M.K. Church *Southampton*
S.J. Coker *Liverpool*
R.A. Coleman *Ware*
A.J. Cross *London*
V. Crunelli *Cardiff*
T.C. Cunnane *Oxford*
F. Cunningham *London*
A. Dray *London*
J.R. Docherty *Dublin*
J.M. Edwardson *Cambridge*
W. Feniuk *Cambridge*
J.R. Fozard *Basle, Switzerland*
Allison D. Fryer *Baltimore, USA*
Sheila M. Gardiner *Nottingham*
C.J. Garland *Bristol*
L.G. Garland *Beckenham*
A. Gibson *London*
R. Gristwood *Barcelona, Spain*
T. Hedner *Gothenburg, Sweden*
P.E. Hicks *Leuville-sur-Orge, France*
S.J. Hill *Nottingham*
J.C. Hunter *Cambridge*
C.C. Jordan *Ware*
K.A. Kane *Glasgow*
D.A. Kendall *Nottingham*
P. Leff *Loughborough*
H.D. Lux *Planegg, Germany*
R. McMillan *Macclesfield*
J. Maclagan *London*
G. Martin *Beckenham*
W. Martin *Glasgow*
A. Mathie *London*
D.N. Middlemiss *Harlow*
R.C. Miller *Strasbourg, France*
P.K. Moore *London*
R.J. Naylor *Bradford*
C.D. Nicholson *Oss, The Netherlands*
C.P. Page *London*
B.K. Park *Liverpool*
A.N. Payne *Beckenham*
F.L. Pearce *London*
J.D. Pearson *London*
M.H.T. Roberts *Cardiff*
P.J. Roberts *Bristol*
C. Robinson *London*
G.J. Sanger *Harlow*
M.A. Simmonds *London*
J.M. Sneddon *Sunderland*
P. Sneddon *Glasgow*
K. Starke *Freiburg, Germany*
P.V. Taberner *Bristol*
C. Thiemermann *London*
M.D. Tricklebank *Harlow*
M.B. Tyers *Ware*
S.P. Watson *Oxford*
K.J. Watling *Cambridge*
A.H. Weston *Manchester*
B.J.R. Whittle *Beckenham*
Eileen Winslow *Riom, France*

Corresponding Editors

P.R. Adams *Stony Brook, U.S.A.*
C. Bell *Melbourne, Australia*
F.E. Bloom *La Jolla, U.S.A.*
A.L.A. Boura *Clayton, Australia*
N.J. Dun *Toledo, U.S.A.*
R.F. Furchgott *New York, U.S.A.*
T. Godfraind *Brussels, Belgium*
S.Z. Langer *Paris, France*
R.J. Miller *Chicago, U.S.A.*
R.C. Murphy *Denver, U.S.A.*
E. Muscholl *Mainz, Germany*
R.A. North *Portland, U.S.A.*
M. Otsuka *Tokyo, Japan*
M.J. Rand *Melbourne, Australia*
S. Rosell *Södertalje, Sweden*
P. Seeman *Toronto, Canada*
L. Szekeres *Szeged, Hungary*
B. Uvnas *Stockholm, Sweden*
P.A. Van Zwieten *Amsterdam, Netherlands*
V.M. Varagič *Belgrade, Yugoslavia*
G. Velo *Verona, Italy*
Wang Zhen Gang *Beijing, China*
M.B.H. Youdim *Haifa, Israel*

Submission of manuscripts: Manuscripts (two copies) should be sent to The Editorial Office, British Journal of Pharmacology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE.

Authors should consult the Instructions to Authors and the Nomenclature Guidelines for Authors in Vol. **108,** 275–284. These Instructions and Guidelines also appear with the journal Index for Volumes 105–107, 1992. A checklist of the essential requirements is summarised in each issue of the journal on the inside of the back cover.

The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd.

The journal is covered by *Current Contents*, *Excerpta Medica* and *Index Medicus*.

All business correspondence and reprint requests should be addressed to the Scientific & Medical Division, Macmillan Press Ltd., Houndmills, Basingstoke, Hampshire RG21 2XS, UK. Telephone: (0256) 29242; Fax: (0256) 810526.

Enquiries concerning advertising space or rates should be addressed to: Michael Rowley, Advertisement Manager, Macmillan Press Ltd., 4 Little Essex Street, London WC2R 3LF. Telephone: 071 836 6633; Fax: 071 379 0820.

Annual subscription prices for 1993 EC £540, elsewhere £595/US$950 (sterling rate is definitive). Orders must be accompanied by remittance. Cheques should be made payable to Macmillan Press, and sent to: Macmillan Press Ltd., Subscription Department, Brunel Road, Houndmills, Basingstoke, Hampshire RG21 2XS, UK.
Overseas subscribers may make payments into UK Post Office Giro Account No. 5192455. Full details must accompany the payment.

British Journal of Pharmacology (ISSN 0007–1188) is published monthly by Macmillan Publishers Ltd, c/o Mercury Airfreight International Ltd, 2323 Randolph Avenue, Avenel, NJ 07001, USA. Subscription price is $950.00 per annum. 2nd class postage is paid at Rahway NJ. *Postmaster*: send address corrections to Macmillan Publishers, c/o Mercury Airfreight International Ltd, 2323 Randolph Avenue, Avenel NJ 07001.

All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

All material published in this journal is protected by copyright, which covers exclusive rights to reproduce and distribute the material. No material published in this journal may be reproduced or stored on microfilm or in electronic, optical or magnetic form without the written authorisation of the Publisher.

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Macmillan Press Ltd for libraries and other users registered with the Copyright Clearance Center (CCC) Transactional Reporting Service, provided that the base fee of $9.00 per copy is paid directly to CCC, 21 Congress St., Salem, MA 01970, USA.

Apart from any fair dealing for the purposes of research or private study, or criticism or review, as permitted under the Copyright, Designs and Patent Act 1988, this publication may be reproduced, stored or transmitted, in any form or by any means, only with the prior permission in writing of the publishers, or in the case of reprographic reproduction, in accordance with the terms of licences issued by the Copyright Licensing Agency.

© The British Pharmacological Society & Macmillan Press Ltd, 1993.
ISSN 0007–1188
0007–1188/93 $9.00 + $0.00

PREPARATION OF MANUSCRIPTS

Authors are strongly recommended to read the full *Instructions to Authors* and *Nomenclature Guidelines for Authors* (*Br. J. Pharmacol.* 1993, **108,** 275–284) before submitting a manuscript for publication in the *British Journal of Pharmacology*. The manuscript and cover letter should be checked against the following list before mailing.

The original and one copy of the manuscript must be supplied. Manuscripts must be typed in double-line spacing on one side of A4 paper, in type not smaller than 12 characters per inch or 10 point. Both copies to include Tables and a set of labelled Figures. One set of Figures without numbers or letters is also to be included. The text to be arranged in the following subsections:

1. **Title**—To have no more than 150 characters on a separate page, which should also include a Short Title (50 characters maximum) and the name and address of the author for correspondence.
2. **Summary**—To be arranged in numbered paragraphs (Full Papers) or a single paragraph (Special Reports).
   —to include aims, principal results and conclusions.
   —to include Key words (10 maximum) at end of summary.
3. **Introduction**—To contain concise statements of the problem and the aims of the investigation.
4. **Methods**—To have brief but adequate account of the procedures; *full names of drugs* (*including those referred to by manufacturer's code*), sources of drugs and statistical tests to be stated.
5. **Results**—To have no repetition of data in Figures, Tables and text.
6. **Discussion**—Findings and conclusions to be placed in context of other relevant work.
   NB Simple repetition of results and unwarranted speculation are not acceptable.
7. **Acknowledgments**—Sources of support. Sources of drugs not widely available commercially.
8. **References**—All references in the text to be included in the Reference List and *vice versa*. References in alphabetical order with complete citations; Journals publishing 'in press' papers identified.
   References to manuscripts submitted to other journals but not yet accepted are not allowed.
9. **Tables**—Each on a separate page and prepared in accordance with current requirements of the Journal.
10. **Figures**—Both labelled and non-labelled Figures to be prepared in accordance with current requirements of the Journal (see *Instructions to Authors*, 1993, **108,** 275–281) and provided with Figure Number and Authors' names on back (*in pencil*).
    —each legend to be typed on a separate page and carrying keys to symbols.
    —keys to symbols and histograms must not appear on the figures themselves, but in the respective legends.
    —'box style' figures are not in keeping with the Journal style; line drawings etc must have only left-hand and bottom axes.
11. **Manuscripts**—To be accompanied by a declaration signed by each author that
    (a) results are original
    (b) approval of all persons concerned has been given to submit manuscripts for consideration (see also 12b)
    (c) the same material is neither 'in press' (i.e. is in proof or has definitely been accepted for publication) nor under consideration elsewhere. Furthermore it will not be submitted or published elsewhere before a decision has been reached by the Editorial Board of the *British Journal of Pharmacology* and will not be submitted elsewhere if accepted by the *British Journal of Pharmacology*.
    (d) Copyright assignment is included.
12. **Cover letter**—To state clearly
    (a) Corresponding author's full postal address, telephone, telex or Fax number
    (b) where appropriate, that *either* ethical approval has been given for investigation *or* Company or Institutional permission to publish work has been received.
13. **Reminder**—Packaging to be sufficiently robust to protect Figures and to withstand mailing.

Failure to comply with *Instructions to Authors* may lead to substantial delays in processing, review and publication and may even jeopardize acceptance of the manuscript.

NOMENCLATURE

Authors are reminded that accepted receptor and associated terminology is laid out in *Nomenclature Guidelines for Authors,* as published in the *British Journal of Pharmacology, Br. J. Pharmacol.*, 1993, **108,** 282–284.

SPECIAL REPORTS

The purpose of *Special Reports* is to provide rapid publication for **new** and **important** results which the Editorial Board considers are likely to be of special pharmacological significance. *Special Reports* will have publication priority over all other material and so authors are asked to consider carefully the status of their work before submission.

In order to speed publication there is normally no revision allowed beyond very minor typographical or grammatical corrections. If significant revision is required, the Board may either invite rapid re-submission or, more probably, propose that it be re-written as a Full Paper and be re-submitted for consideration. In order to reduce delays, proofs of *Special Reports* will be sent to authors but **essential corrections must reach the Production Office within 48 hours of receipt.** Authors should ensure that their submitted material conforms exactly to the following requirements.

Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain about 400 words. The absolute maximum length of the *Special Report* is 1700 words. For each Figure or Table, please deduct 200 words. The manuscript should comprise a Title page with key words (maximum of 10), a Summary consisting of a single short paragraph, followed by Introduction, Methods, Results, Discussion and References (maximum of 10). In all other respects, the requirements are the same as for Full Papers (see current 'Instructions to Authors').

BRITISH JOURNAL OF PHARMACOLOGY VOLUME 110 (4) 1993

OBITUARY

Hugh Blaschko, FRS 1275

SPECIAL REPORTS

H. Matsuno, T. Uematsu & M. Nakashima. Thrombolytic effects of a plasminogen-plasminogen activator chimera in a photochemically induced thrombosis (PIT) model 1278

G. Edwards & A.H. Weston. Induction of a glibenclamide-sensitive K-current by modification of a delayed rectifier channel in rat portal vein and insulinoma cells 1280

R.S. Berman, J.D. Frew & W. Martin. Endotoxin-induced arterial endothelial barrier dysfunction assessed by an *in vitro* model 1282

PAPERS

P.M. Zygmunt, P.K.E. Zygmunt, E.D. Högestätt & K.-E. Andersson. Effects of ω-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the rabbit urethra and detrusor 1285

M. Facklam & N.G. Bowery. Solubilization and characterization of $GABA_B$ receptor binding sites from porcine brain synaptic membranes 1291

G.P. Connolly, P.J. Harrison & T.W. Stone. Action of purine and pyrimidine nucleotides on the rat superior cervical ganglion 1297

P.A. Iredale & S.J. Hill. Increases in intracellular calcium via activation of an endogenous P_2-purinoceptor in cultured CHO-K1 cells 1305

C.A.E. Martin, E. Naline, L. Manara & C. Advenier. Effects of two β_3-adrenoceptor agonist,s SR 58611A and BRL 37344, and of salbutamol on cholinergic and NANC neural contraction in guinea-pig main bronchi *in vitro* 1311

J.B. Louttit & R.A. Coleman. The action of Hoe 140 on the bradykinin-induced splenic pressor reflex of the anaesthetized cat 1317

R.G. Hempelmann & A. Ziegler. Endothelium-dependent noradrenaline-induced relaxation of rat isolated cerebral arteries: pharmacological characterization of receptor subtypes involved 1321

G.E. Boeckxstaens, J.G. De Man, P.A. Pelckmans, K.M. Cromheeke, A.G. Herman & Y.M. Van Maercke. Ca^{2+} dependency of the release of nitric oxide from non-adrenergic non-cholinergic nerves 1329

N.G.M. Schipper, J.C. Verhoef, L.M. De Lannoy, S.G. Romeijn, J.H. Brakkee, V.M. Wiegant, W.H. Gispen & F.W.H.M. Merkus. Nasal administration of an ACTH(4–9) peptide analogue with dimethyl-β-cyclodextrin as an absorption enhancer: pharmacokinetics and dynamics 1335

S.-C.G. Hui, E.L. Sevilla & C.W. Ogle. 5-HT_3 antagonists reduce morphine self-administration in rats 1341

J.G. Filep & E. Földes-Filep. Modulation by nitric oxide of platelet-activating factor-induced albumin extravasation in the conscious rat 1347

M.A. Khoyi, H.H. Dalziel, L. Zhang, R.A. Bjur, W.T. Gerthoffer, I.L.O. Buxton & D.P. Westfall. $[Ca^{2+}]_i$-sensitive, IP_3-independent Ca^{2+} influx in smooth muscle of rat vas deferens revealed by procaine 1353

P.-E. Sandström. Inhibition by hydrochlorothiazide of insulin release and calcium influx in mouse pancreatic β-cells 1359

J.C. Wanstall, J.S. Thompson & A.H. Morice. Effects of atrial natriuretic peptide and nitroprusside on isolated pulmonary resistance and conduit arteries from rats with pulmonary hypertension 1363

P. Lopes, D. Regoli & R. Couture. Cardiovascular effects of intrathecally administered bradykinin in the rat: characterization of receptors with antagonists 1369

J.W. Growcott, B. Holloway, M. Green & C. Wilson. Zeneca ZD7114 acts as an antagonist at β_3-adrenoceptors in rat isolated ileum 1375

J. Nogueira de Francischi, D.M. Conroy, K. Maghni & P. Sirois. Inhibition by rapamycin of leukocyte migration and bronchial hyperreactivity induced by injection of Sephadex beads to guinea-pigs 1381

E.J. Kelso, B.J. McDermott & B. Silke. Cardiotonic actions of selective phosphodiesterase inhibitors in rat isolated ventricular cardiomyocytes 1387

H. Okumura, N. Ashizawa, F. Kobayashi, K. Arai, R. Asakura, N. Ashikawa & A. Matsuura. Comparison of haemoconcentration induced by big endothelin-1 and endothelin-1 in mice 1395

A.R. Baydoun, R.G. Bogle, J.D. Pearson & G.E. Mann. Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells 1401

G. Lombardi, M. Alesiani, P. Leonardi, G. Cherici, R. Pellicciari & F. Moroni. Pharmacological characterization of the metabotropic glutamate receptor inhibiting D-[^{3}H]-aspartate output in rat striatum 1407

M. Hernández, U. Simonsen, D. Prieto, L. Rivera, P. García, E. Ordaz & A. García-Sacristán. Different muscarinic receptor subtypes mediating the phasic activity and basal tone of pig isolated intravesical ureter 1413

J.-X. Bao & L. Stjärne. Dual contractile effects of ATP released by field stimulation revealed by effects of α,β-methylene ATP and suramin in rat tail artery 1421

M. Freissmuth, W. Beindl & M. Kratzel. Binding and structure-activity-relation of benzo[*f*]isoquinoline- and norcodeinone-derivatives at μ-opioid receptors in the rat cerebral cortex 1429

H. Tsunoda, T. Miyauchi, K. Fujita, T. Kubo & K. Goto. Mechanism of rat uterine smooth muscle contraction induced by endothelin-1 1437

M.N. Perkins & D. Kelly. Induction of bradykinin B_1 receptors *in vivo* in a model of ultra-violet irradiation-induced thermal hyperalgesia in the rat 1441

M. Kobari, Y. Fukuuchi, M. Tomita, N. Tanahashi, S. Konno & H. Takeda. Effects of sumatriptan on the cerebral intraparenchymal microcirculation in the cat 1445

J.M. Dickenson & S.J. Hill. Homologous and heterologous desensitization of histamine H_1- and ATP-receptors in the smooth muscle cell line, DDT_1MF-2: the role of protein kinase C 1449

S.M. Gardiner, P.A. Kemp, T. Bennett, R.M.J. Palmer & S. Moncada. Regional and cardiac haemodynamic effects of N^G, N^G, dimethyl-L-arginine and their reversibility by vasodilators in conscious rats 1457

K. Kurz, I. von Kügelgen & K. Starke. Prejunctional modulation of noradrenaline release in mouse and rat vas deferens: contribution of P_1- and P_2-purinoceptors 1465

J.F. Kleha, P. Devesly & A. Johns. EDRF release from canine coronary artery by lectins 1473

M.J. Rand & C.G. Li. Modulation of acetylcholine-induced contractions of the rat anococcygeus muscle by activation of nitrergic nerves 1479

R.G. Pertwee, L.A. Stevenson & G. Griffin. Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabimimetic agents, CP 55,940, WIN 55,212-2 and anandamide 1483

A.U. Ziganshin, C.H.V. Hoyle, X. Bo, G. Lambrecht, E. Mutschler, H.G. Bäumert & G. Burnstock. PPADS selectively antagonizes P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder 1491

M.M. Teixeira & P.G. Hellewell. Suppression by intradermal administration of heparin of eosinophil accumulation but not oedema formation in inflammatory reactions in guinea-pig skin 1496

S. Yamanaka, H. Iwao, T. Yukimura, S. Kim & K. Miura. Effect of the platelet-activating factor antagonist, TCV-309, and the cyclo-oxygenase inhibitor, ibuprofen, on the haemodynamic changes in canine experimental endotoxic shock 1501

H.-C. Huang, Y.-I. Weng, C.-R. Lee, T.-R. Jan, Y.-L. Chen & Y.-T. Lee. Protection by scoparone against the alterations of plasma lipoproteins, vascular morphology and vascular reactivity in hyperlipidaemic diabetic rabbit 1508

M.M. Teixeira, T.J. Williams & P.G. Hellewell. Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin 1515

C. David, N. Davis, R. Mason & V.G. Wilson. Evidence for functional dissociation of dependence and tolerance in guinea-pig isolated ileal segments following 20 hour exposure to morphine *in vitro* 1522

M. Hori, K. Sato, S. Miyamoto, H. Ozaki & H. Karaki. Different pathways of calcium sensitization activated by receptor agonists and phorbol esters in vascular smooth muscle 1527

S.M. Gardiner, P.A. Kemp & T. Bennett. Regional haemodynamic responses to intravenous and intraarterial endothelin-1 and big endothelin-1 in conscious rats 1532

Contents continue inside back cover

Typeset by Elite Typesetting Techniques, Southampton, Hampshire
and printed in Great Britain by Unwin Brothers Ltd., The Gresham Press, Old Woking, Surrey

G. Bricca, J. Zhang, H. Greney, M. Dontenwill, J. Stutzmann, A. Belcourt & P. Bousquet. Relevance of the use of [^{3}H]-clonidine to identify imidazoline receptors in the rabbit brainstem **1537**

I. von Kügelgen, L. Späth & K. Starke. Ionotropic glutamate receptor types leading to adenosine-mediated inhibition of electrically evoked [^{3}H]-noradrenaline release in rabbit brain cortex slices **1544**

A. MacDonald & M. Lamont. Effects of selective antagonism of β-adrenoceptor subtypes on responses to isoprenaline in rat distal colon *in vitro* **1551**

T. Ibbotson, G. Edwards & A.H. Weston. Antagonism of levcromakalim by imidazoline- and guanidine-derivatives in rat portal vein: involvement of the delayed rectifier **1556**

W. Paul, P. Gresele, S. Momi, G. Bianchi & C.P. Page. The effect of defibrotide on thromboembolism in the pulmonary vasculature of mice and rabbits and in the cerebral vasculature of rabbits **1565**

B. Costall, R.J. Naylor & B.R. Tuladhar. 5-HT$_4$ receptor mediated facilitation of the emptying phase of the peristaltic reflex in the guinea-pig isolated ileum **1572**

M.K. Pugsley, D.A. Saint, M.P. Penz & M.J.A. Walker. Electrophysiological and antiarrhythmic actions of the κ agonist PD 129290 and its R,R (+)-enantiomer, PD 129289 **1579**

M.J. Oset-Gasque, M. Parramón & M.P. González. GABA$_B$ receptors modulate catecholamine secretion in chromaffin cells by a mechanism involving cyclic AMP formation **1586**

K.A. Wardle & G.J. Sanger. The guinea-pig distal colon – a sensitive preparation for the investigation of 5-HT$_4$ receptor-mediated contractions **1593**

R.P. Brownlie, N.J. Brownrigg, H.M. Butcher, R. Garcia, R. Jessup, V.J. Lee, S. Tunstall & M.G. Wayne. ZD1542, a potent thromboxane A$_2$ synthase inhibitor and receptor antagonist *in vitro* **1600**

N.M.J. Rupniak, S. Boyce, A.R. Williams, G. Cook, J. Longmore, G.R. Seabrook, M. Caesar, S.D. Iversen & R.G. Hill. Antinociceptive activity of NK$_1$ receptor antagonists: non-specific effects of racemic RP67580 **1607**

H. Inoue, N. Nagata & Y. Koshihara. Profile of capsaicin-induced mouse ear oedema as neurogenic inflammatory model: comparison with arachidonic acid-induced ear oedema **1614**

S.C. Cheetham & D.J. Heal. Evidence that RU 24969-induced locomotor activity in C57/B1/6 mice is specifically mediated by the 5-HT$_{1B}$ receptor **1621**

I. Perry, S.M. Buttrum & G.B. Nash. Effect of activation on adhesion of flowing neutrophils to cultured endothelium: time course and inhibition by a calcium channel blocker (nitrendipine) **1630**

J.P. Hieble & D.C. Kolpak. Mediation of the hypotensive action of systemic clonidine in the rat by α$_2$-adrenoceptors **1635**

R.D. Feldman. Insulin-mediated sensitization of adenylyl cyclase activation **1640**

See last page of issue for PREPARATION OF MANUSCRIPTS