1P MULTIPLE PATHWAYS UNDERLIE NO-INDEPENDENT RELAXATION TO THE CALCIUM IONOPHORE A23187 IN THE RABBIT ISOLATED FEMORAL ARTERY

D.T.D. Rowe, C.J. Garland & F. Plane, Dept. of Pharmacology, University of Bristol, Bristol, BS8 1TD

The identity of the mediator of nitric oxide (NO)-independent relaxation of arterial smooth muscle is unclear. In the rabbit isolated femoral artery, endothelium-dependent relaxation to the calcium ionophore A23187 is largely mediated by the release of EDHF (Plane *et al.*, 1995). Thus, in the present study, we have investigated the role of heme oxygenase (HO) and cannabinoid receptor activation in relaxation to A23187 in this vessel.

New Zealand White rabbits of either sex were anaesthetised (sodium pentobarbitone, 60 mg kg^{-1}, i.v.) and killed by rapid exsanguination. Segments of femoral artery (2 mm in length) were mounted in a myograph under a previously determined optimal preload of 1g for isometric recording of tension changes. The tissues were maintained at 37°C in oxygenated Krebs buffer containing indomethacin (1 μM). All data are expressed as mean ± s.e. mean. Differences between mean values were calculated using Students t-test.

A23187 (0.01-10 μM) caused endothelium-dependent relaxation (pD_2 6.2 ± 0.4; maximal relaxation 98.6 ± 1.5 %; n=15) of arterial segments pre-constricted with phenylephrine (1-3 μM) which was not significantly altered by pre-incubation with the NO synthase inhibitors (NOSI) L-N^G-nitroarginine methyl ester and L-N^G-nitroarginine (both 100 μM; 30 mins; n=12; P>0.05). In contrast, the inhibitors of HO, tin protoporphyrin IX (TPP; 10 μM; 20 mins) and zinc protoporphyrin IX (ZPP; 10 μM; 20 mins), each significantly attenuated relaxation to A23187 reducing the maximum relaxation by around 40 % to 60.0 ± 3.1 % (n=7; P <0.01) and 63.1 ± 8.8% (n=8; P<0.01), respectively.

In the presence of the NOSI, TPP and ZPP each caused a greater depression of relaxation to that observed with each inhibitor alone, reducing the maximum response by around 70 % to 29.5 ± 3.8 % and 34.8 ± 4.7 % (n=4; P<0.01), respectively. Similarly, exposure to either TPP or ZPP together with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM; 10 mins), an inhibitor of soluble guanylyl cyclase, depressed the maximum response by around 65 % to 35.4 ± 2.2 % and 37.0 ± 0.5 % (n=4; P<0.01), respectively. The combination of either of the HO inhibitors with the NOSI and 30 mM KCl abolished A23187-evoked responses (n=4; P<0.01).

Pre-incubation with the cannabinoid receptor antagonist SR141716A (1 μM; 20 mins) alone did not alter relaxation to A23187 (n=4; P>0.05). However, in the presence of the NOSI, exposure to SR141716A did cause a significant attenuation of relaxation to A23187, reducing the maximum response by around 20 % to 76.4 ± 2.3 % (n=5; P<0.01). Similarly, exposure to the combination of ODQ and SR141716A caused a further reduction in A23187-evoked relaxation than that seen with ODQ alone, reducing the maximum response to 45.9 ± 4.8 % (n=4; P<0.01). Relaxation to A23187 was abolished in the presence of a combination of SR141716A, the NOSI and either TPP or ZPP (n=5; P<0.01), and ODQ, SR141716A and either TPP or ZPP (n=3; P<0.01).

The HO inhibitors and SR141716A had no effect on relaxation to either ACh, the NO donor 3-morpholino-sydnonimine (SIN-1) or the potassium channel opener levcromakalim (n=4-6 in each case; P>0.05) or on contraction to phenylephrine (n=4; P>0.05).

These data indicate that in the rabbit isolated femoral artery, NO-independent relaxation to A23187, may involve the activation of both HO- and SR141716A-sensitive mechanisms. As this response has previously been attributed to EDHF, these data may indicate that EDHF-mediated responses may be due to the activation of a number of pathways.

D.T.D.R. was supported by a Wellcome Trust Vacation Scholarship.

Plane, F., Pearson, T. & Garland, C.J. (1995). *Br. J. Pharmacol.*, **115**, 31-38.

2P FURTHER CHARACTERISATION OF THE MEDIATOR OF NO-INDEPENDENT DILATATION TO ACETYLCHOLINE IN THE RAT ISOLATED PERFUSED MESENTERIC BED

J. Sehdev, C.J. Garland & F. Plane, Department of Pharmacology, University of Bristol, Bristol, BS8 1TD.

Endothelium-dependent relaxation of the rat isolated perfused mesenteric bed is mediated by both nitric oxide (NO) and an NO-independent endothelium-derived hyperpolarising factor (EDHF; Parsons *et al.*, 1994). The identity of EDHF is as yet unclear although it has recently been suggested to be a product of cytochrome P450 metabolism of arachidonic acid (Bauersachs *et al.*, 1994) or a cannabinoid (Randall *et al.*, 1996). In the present study, the identity of the mediator of NO-independent relaxation was investigated further.

Male Wistar rats (250-350 g) were killed by cervical dislocation. Isolated mesenteric preparations were perfused with oxygenated Krebs buffer (37 °C; 5-7 ml min^{-1}) containing indomethacin (1 μM) and the NO synthase inhibitors L-N^G-nitroarginine methyl ester and L-N^G-nitroarginine (both 100 μM). Perfusion pressure was measured via a pressure transducer. In a parallel study, the NO synthase inhibitors abolished acetylcholine (ACh)-induced increases in cyclic GMP as detected by radio-immunoassay (n=6; P<0.01). All data are expressed as mean ± se mean % relaxation of PE-induced constriction. Differences between mean values were calculated using the Student's t-test or ANOVA.

Injection of bolus doses of acetylcholine (ACh; 0.2-2000 nmol), sodium nitroprusside (SNP; 0.02-20 nmol) and the potassium channel opener levcromakalim (0.2-200 nmol) each evoked dose-dependent dilation of preparations pre-constricted with phenylephrine (PE; 30 μM). The maximum reversal of PE-induced tone by each dilator was 70.0 ± 3.7 % (n=20), 84.6 ± 5.2 % (n=12) and 89.3 ± 4.1 % (n=14), respectively.

The cytochrome P450 inhibitors miconazole (10 μM) clotrimazole (1 μM), 7-ethoxyresorufin (1 μM), and proadifen (10 μM) each attenuated dilatation to ACh, reducing the maximum response to 51.7 ± 5.2 %, 18.0 ±3.4 %, 23.4 ± 3.6 % and 37.3 ± 5.9 %, respectively (n=4 in each case; P<0.01). However, these inhibitors also inhibited dilatation to the potassium channel opener levcromakalim. Miconazole and proadifen abolished dilatation to levcromakalim (n=4 in each case; P<0.01) whereas clotrimazole and 7-ethoxyresorufin each reduced the maximum dilatation to levcromakalim to 15.9 ± 7.8 % and 21.5 ± 8.1 %, respectively (n=3; P<0.01). In contrast, 17-octadecynoic acid (17-ODYA; 5 μM) inhibited ACh-evoked dilatation of PE-induced tone, reducing the maximum response to 31.5 ± 5.4 % (n=4; P<0.01), but did not alter dilatation to either SNP or to levcromakalim (n=4 in each case; P>0.05).

The cannabinoid receptor antagonist SR141716A (10 μM) and the heme oxygenase inhibitor zinc protoporphyrin (10 μM), each attenuated dilatation to ACh but did not significantly reduce the maximum response (63.8 ± 10.9 % and 58.3 ± 10.2 %, respectively; n=4 in each case; P>0.05). In contrast, the superoxide anion generating agents methylene blue (10 μM), hypoxanthine (0.1 mM)/xanthine oxidase (16 $mUml^{-1}$) and pyrogallol (10 μM), each significantly inhibited relaxation to ACh, reducing the maximum response to 42.7 ± 3.2 %, 19.4 ± 8.6 % and 15 ± 6.4 %, respectively (n=4 in each case; P<0.01).

These data indicate that, in contrast to other inhibitors of this enzyme, 17-ODYA may selectively inhibit NO-independent dilatation to ACh in the rat isolated perfused mesenteric bed. In addition, the observed inhibition of NO-independent dilatation by methylene blue and other generators of superoxide anions suggests that the mediator of NO-independent dilatation, like NO, is sensitive to destruction by superoxide anions.

J. Sehdev was supported by a Nuffield Foundation Vacation Scholarship.

Bauersachs, J., Hecker, M. & Busse, R. (1994) *Br. J. Pharmacol.*, **113**, 1548-1553.
Parsons, S.J.W., Hill, A., Waldron, G.J. *et al.*, (1994) *Br. J. Pharmacol.*, **113**,1275-1280.
Randall, M.D., Alexander, S.P.H., Bennett *et al.*, (1996) *Biochem. Biophys. Res. Commun.*, **229**, 114-120.

3P THE ROLE OF CYCLIC GMP AND POTASSIUM CHANNELS IN NO-INDEPENDENT RELAXATION OF THE RABBIT ISOLATED FEMORAL ARTERY

F. Plane & C.J. Garland, Dept. of Pharmacology, University of Bristol, Bristol, BS8 1TD

Endothelium-dependent relaxation of the rabbit isolated femoral artery to the calcium ionophore A23187 is resistant to inhibitors of nitric oxide (NO) synthase and appears to be largely mediated by the release endothelium-derived hyperpolarising factor (EDHF; Plane *et al.*, 1995). In the present study, we have further investigated the mechanisms underlying the NO-independent relaxation to A23187 in this artery.

New Zealand White rabbits of either sex were anaesthetised (sodium pentobarbitone, 60 mg kg^{-1}, i.v.) and killed by rapid exsanguination. Segments of femoral artery (2 mm in length) were mounted in a myograph under a previously determined optimal preload of 1g for isometric recording of tension changes. The tissues were maintained at 37°C in oxygenated Krebs buffer containing indomethacin (1 μM). All data are expressed as mean ± s.e. mean. Differences between mean values were calculated using Students t-test.

A23187 (0.01-10 μM) caused endothelium-dependent relaxation (pD_2 6.3 ± 0.2; maximal relaxation 94.9 ± 0.5 %; n=12) of arterial segments pre-constricted with phenylephrine (1-3 μM). Relaxation was not significantly altered by pre-incubation with the NO synthase inhibitors (NOSI) L-N^G-nitroarginine methyl ester and L-N^G-nitroarginine (both 100 μM; 30 mins; n=10; P>0.05).

In the presence of either 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM; 10 mins), an inhibitor of soluble guanylyl cyclase, or 30 mM KCl, which abolished endothelium-dependent repolarisation, relaxation to A23187 was significantly inhibited by around 35 % in each case (maximum responses 68.1 ± 1.2 % and 62.0 ± 3.1 % (n=4-5; P<0.01), respectively). In the presence of the NOSI and ODQ together, relaxation to A23187 was depressed to a similar extent as in the presence of ODQ alone (maximum relaxation 58.0 ± 2.5 %; n=4). In contrast, the application of the NOSI and 30 mM KCl together caused a further inhibition of relaxation to A23187 than that caused by either treatment alone, reducing the maximum response by around 70 % to 33.8 ± 4.6 % (n=5; P<0.01). Additionally, in the presence of ODQ and 30 mM KCl A23187-evoked relaxation was abolished (n=4; P<0.01).

The potassium channel inhibitors glibenclamide (10 μM), charybdotoxin (ChTX; 50 nM) or apamin (50 nM), alone or in the presence of the NOSI, had no effect on relaxation to A23187 (n=4 in each case; P<0.01). However, in the presence of ChTX and apamin together the maximum relaxation to A23187 was reduced by around 40 % (58.1 ± 6.8 %; n=4; P<0.01) and the combination of the NOSI, ChTX and apamin, depressed the maximum response by around 75 % to 24.5 ± 6.7 % (n=5; P<0.01).

Exposure to a combination of apamin with ODQ, or glibenclamide and ODQ, did not cause a significantly greater depression of relaxation than that observed with ODQ alone (n=4 in each case; P>0.05). However, the application of ChTX with ODQ reduced the maximum relaxation to A23187 to 34.3 ± 6.0 % (n=4: P<0.01) and in the presence of ChTX, apamin and ODQ together A23187-evoked relaxation was abolished (n=5: P<0.01).

These data indicate that both the activation of soluble guanylyl cyclase and potassium channels may contribute to NO-independent relaxation to A23187 in the rabbit isolated femoral artery. NO-independent relaxation in this artery can largely (approximately 70 %) be accounted for by the release of EDHF which appears to cause smooth muscle relaxation via activation of a pathway sensitive to a combination of ChTX and apamin. However, ODQ also reduced the NO-independent relaxation to A23187, and in combination with either 30 mM KCl or, ChTX and apamin, abolished the response. This finding indicates that NO-independent activation of this enzyme may also contribute to NO-independent relaxation in this artery.

This work was supported by the Wellcome Trust.

Plane, F., Pearson, T. & Garland, C.J. (1995). *Br. J. Pharmacol.*, **115**, 31-38.

4P VASOCONSTRICTOR HYPOREACTIVITY IN PERFUSED RAT HINDLIMBS AFTER MYOCARDIAL INFARCTION (MI): ROLE OF BASAL NITRIC OXIDE (NO) PRODUCTION AND EFFECTS OF CAPTOPRIL

D.L. Ceiler, H.J.M.G. Nelissen-Vrancken, J.F.M. Smits & J.G.R. De Mey. Dept. of Pharmacology and Cardiovascular Research Institute Maastricht, Universiteit Maastricht, The Netherlands

Altered basal NO production may be involved in the development and progression of heart failure. The present study addresses the modulatory role of NO in hindlimb vascular contractile ability after MI in the rat and the effects of treatment with the angiotensin converting enzyme inhibitor captopril in this setting.

MI was induced in male Wistar rats (300-320 g) by ligation of the left coronary artery under pentobarbital anesthesia (60 mg/kg i.p.). At 2 (sham n=10; MI n=10) and 5 (sham n=8; MI n=9) weeks post-MI, vascular contractile function was assessed in ex vivo perfused hindlimbs from resistance changes induced by 35 mmol/L and 125 mmol/L potassium (K^+) and from the maximum increase in resistance (ΔR_{max}; 125 mmol/L K^+ plus 3 mg phenylephrine). NO synthase was blocked for 2 weeks with L-NAME (25mg/kg/day s.c.) in sham (n=7) and MI (n=7) rats and similar contractility experiments were performed. The effect of captopril treatment (12 mg/kg/day s.c., days 21-35) on vasoconstrictor responses was also tested (sham n=7; MI n=9).

K^+-induced vasoconstrictor responses and ΔR_{max} were reduced at 2 weeks after MI (125 mmol/L K^+: 15.7±1.0 vs. 21.9±0.9 mmHg.min/mL; ΔR_{max}: 22.2±1.3 vs. 27.7±1.6 mmHg.min/mL; Mean±S.E.M.; P<0.05, Kruskal Wallis). This reduction in vasoconstrictor ability was similar to that seen in L-NAME-treated sham rats (125 mmol/L K^+: 14.7±1.3; ΔR_{max}: 17.5±1.5 mmHg.min/mL), whereas L-NAME did not affect vasoconstrictor reactivity in MI rats (125 mmol/L K^+: 17.5±1.0; ΔR_{max}: 20.4±1.5 mmHg.min/mL).

At 5 weeks after MI, vasoconstriction to 125 mmol/L K^+ and ΔR_{max} was smaller in MI rats than in sham rats (125 mmol/L K^+: 15.7±0.7 vs. 19.3±0.9 mmHg.min/mL; ΔR_{max}: 21.7±1.4l vs. 26.1±1.3 mmHg.min/mL), but was partially restored after captopril treatment (125 mmol/L K^+: 18.7±0.7; ΔR_{max}: 23.5±1.1 mmHg.min/mL).

After MI in the rat, the development of hindlimb vascular contractile hyporeactivity may be due to reduced basal NO production. Treatment with captopril improves this abnormality.

5P THE SELECTIVE INHIBITOR OF INDUCIBLE NITRIC OXIDE SYNTHASE, 1400W, PREVENTS THE CIRCULATORY FAILURE BUT NOT THE ORGAN INJURY CAUSED BY ENDOTOXIN IN THE RAT

Gillian Wray, Colin Millar, Mary Leach, Charles Hinds & Christoph Thiemermann. The William Harvey Research Institute, St. Bartholomew's & the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1M 6BQ.

Inhibitors of nitric oxide synthase (NOS) can attenuate the hypotension and vascular hyporeactivity to pressor agents caused by endotoxin in animal models of septic shock, including the rat (Thiemermann, 1997). Furthermore, in a recently completed phase II clinical trial the NOS inhibitor N^G-methyl-L-arginine significantly increased the number of patients in whom shock resolved within 72 hours of commencing treatment. There is concern that the non selective inhibition of the constitutive, endothelial (eNOS) and the inducible isoform of NOS (iNOS) may cause excessive vasoconstriction and exacerbate organ injury. Here we investigate the effects of a highly selective inhibitor of iNOS (1400W) on haemodynamics and organ dysfunction in a rodent model of endotoxic shock. 1400W is 5000x more selective for human iNOS compared to eNOS and 50x more selective (iNOS vs. eNOS) in rat endotoxaemia. (Garvey *et al*, 1997)

Male Wistar rats (240-290g) were anaesthetised with sodium thiopentone (120 $mg.kg^{-1}$, i.p.). The trachea, carotid artery, femoral and jugular veins were cannulated to facilitate respiration, for measurement of mean arterial blood pressure (MAP) and heart rate (HR), for sampling and for administration of drugs. The bladder was also cannulated for collection of urine. At completion of surgery, cardiovascular parameters were allowed to stabilise for 15 min. Animals then received *E.Coli* lipopolysaccharide (LPS, 6 $mg.kg^{-1}$ i.v.) followed by an infusion of saline (6 $ml.kg^{-1}.h^{-1}$). At 2 h after LPS, this infusion was either maintained (control, n=10) or replaced by an infusion of either low dose (3 $mg.kg^{-1}.h^{-1}$, n=8) or high dose (10 $mg.kg^{-1}.h^{-1}$, n=7) of the iNOS inhibitor 1400W. The infusions were preceded by bolus injections of 3 or 10 $mg.kg^{-1}$ 1400W, respectively. Two groups of rats not receiving LPS (sham-operated animals; SOP) were given infusions of either vehicle (saline, n=10) or 1400W (10 $mg.kg^{-1}.h^{-1}$, n=3). Six hours after LPS/vehicle blood was taken for measurement of indices of organ injury. Creatinine (Crea) for renal injury, alanine aminotransferase (ALT) for liver injury and lipase for pancreatic injury. Plasma levels of nitrate + nitrite were measured at 6 h after LPS (t6) as an indication of iNOS activity. Results are given in the table below and values are expressed as mean ± s.e.mean.

Table 1: Effects of 1400W on organ injury and MAP

Group	Crea (μmol/l)	ALT (iu/l)	Lipase (iu/l)	Nitrate + nitrite (μM)	MAP,t6 (mmHg)
SOP	29 ± 10*	120± 27*	1 ± 0.5*	18 ± 2*	106 ±3*
SOP + 1400W (10)	36 ± 2*	81 ± 12*	1 ± 0.7*	49 ± 13*	108 ±1*
LPS control (LPS + saline)	51 ± 6	228 ± 54	59 ± 13	360 ± 19	83 ± 2
LPS + 1400W 3 $mg.kg^{-1}.h^{-1}$	48 ± 4	228 ± 32	45 ± 13	184±22*	112± 2*
LPS + 1400W 10 $mg.kg^{-1}.h^{-1}$	62 ± 9	210 ± 26	27± 6	82± 11*	118± 4*

(* $p<0.05$ vs. LPS + saline, ANOVA + Bonferoni's post test)

Thus, 1400W significantly attenuates the delayed hypotension, abolishes iNOS activity, but does not prevent the renal, liver or pancreatic injury caused by LPS in the rat.

CT is a recipient of a BHF Senior Fellowship (FS/96018)

Garvey, E.P., *et al.* (1997). *J. Biol. Chem.*, **269**, 26669-26676.
Thiemermann, C. (1997). *Gen. Pharmacol.*, **29**, 159-166.

6P 1*H*-[1,2,4]OXADIAZOLO[4,3-*A*]QUINOXALIN-1-ONE (ODQ) IS NOT A SELECTIVE INHIBITOR OF SOLUBLE GUANYLYL CYCLASE: INTERFERENCE WITH NO-SYNTHASE AND NITROVASODILATOR BIOTRANSFORMATION

H.H.H.W. Schmidt, J. Siebe*, B. Clement*, M. Feelisch#. (Introduced by Timothy D. Warner) Dept. of Pharmacology Julius-Maximilians-University, Würzburg; *Inst. of Pharmacy, Christian-Albrecht-University, Kiel; #Dept. of Medicine, Div. of Cardiology, Heinrich-Heine-University, Düsseldorf, Germany

We studied the vasomotor activity of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a novel possibly selective inhibitor of soluble guanylyl cyclase (Garthwaite *et al.*, 1995; Schrammel *et al.*, 1996), on isolated endothelium-intact rabbit aortic rings in organ baths. Rings were precontracted with phenylephrine (0.2 μM) at a resting tension of 2 g and subsequently relaxed using either endothelium-dependent (acetylcholine (ACh), histamine, ATP) or -independent vasodilators (nitrogen oxide donor compounds, papaverine, ANP). ODQ caused a concentration-dependent increase in vascular tone due to inhibition of basal NO release and an inhibition of endothelium-dependent and NO-induced relaxations, while endothelium- and NO-independent relaxations were unaffected. Maximal effects were seen at 3 μM ODQ. When rings were pretreated with 3 μM ODQ, the concentration response curve (CRC) for histamine, ATP and different NO donors (NO, GSNO, SNAP, SperNO) were equally shifted to the right by two orders of magnitude with no changes in the slope of the CRCs. Other NO donors such as Angeli's salt (Bonner & Akhtar, 1981), peroxynitrite (ONOO-), and SIN-1 were affected by ODQ to a similar extent (2-2.5 log orders). However, there were three interesting and noteworthy exceptions to this general effectiveness of ODQ observed with ACh (4.5 log order shift to the right) and the NO donors sodium nitroprusside (SNP; EC_{50} - ODQ: 1×10^{-8} M; +ODQ: 1.5×10^{-3} M) and glycerol trinitrate (GTN; EC50 - ODQ: 9×10^{-8} M; + ODQ: 3×10^{-4} M). The latter compound is known to require metabolic activation, possibly by cytochrome P450-type heme proteins, in vascular smooth muscle in order to release NO and relax blood vessels. Indeed, ODQ was shown to inhibit microsomal biotransformation of GTN to NO in a concentration-dependent manner with an IC_{50} of 33 and 15 μM in rat and human liver microsomes, respectively. The CRC for ACh in the presence of 3 μM ODQ was superimposable to that obtained after preincubation of rings with 100 μM N^G-nitro-L-arginine methylester, suggesting inhibition of NO-synthase activity by ODQ. These data indicate that ODQ not only affects NO-dependent activation of the heme-containing soluble guanylyl cyclase and thereby cGMP-dependent vasodilation but that its mechanism of action extends to other heme proteins as well. The results with ACh suggest that either the chemical composition of EDRF differs depending on the endothelium-dependent vasodilator used to trigger its release or that ODQ also inhibits NOS. Thus, care should be taken not to misinterpret results obtained with ODQ as a tool to probe for the possible involvement of soluble guanylyl cyclase in a given biological response.

Garthwaite, J., Southam, E., Boulton, C., Nielsen, E., Schmidt, K. & Mayer, B. (1995). *Molecular Pharmacology*, **48**, 184-188.
Schrammel, A., Behrends, S., Schmidt, K., Koesling, D. & Mayer, B. (1996). *Molecular Pharmacology*, **50**, 1-5.
Bonner, F.T. & Akhtar, M.J. (1981). *Inorg. Chem.*, **20**, 3155-3160.

7P FUNCTIONAL ENZYME KINETIC STUDIES ON BINDING SITE INTERACTIONS OF ACTIVE NEURONAL NITRIC OXIDE SYNTHASE

P.Kotsonis, L.G. Fröhlich, H.M. Bömmel, A.J. Reif & H.H.H.W. Schmidt (introduced by W. Martin), Dept. Pharmacol. Toxicol., Julius-Maximilians University, Würzburg D-97078, Germany.

Neuronal nitric oxide synthase (NOS-I) is dependent on calmodulin (CaM) for catalytic activity and requires for unknown reasons the redox-active cofactor (6R)-5,6,7,8-tetrahydrobiopterin (H_4Bip) for maximal activity. Previous binding studies generally report a 5-fold increase in the affinity of NOS-I for [^{3}H]-H_4Bip in the presence of *L*-arginine and a 2-fold increase in affinity for [^{3}H]-N$^\omega$-nitro-*L*-arginine due to H_4Bip suggesting a dual allosteric interaction (for review see Liu & Gross, 1996). However, few studies have examined the influence of *L*-arginine or CaM on endogenous H_4Bip content and the affinity of catalytically active NOS-I for H_4Bip and this was the aim of the present study. NOS-I from porcine cerebellum was isolated and purified by DEAE ion-exchange and ADP-sepharose affinity chromatography and enzyme activity was determined as the formation of [^{3}H]-*L*-citrulline from [^{3}H]-*L*-arginine at 37°C over 18 minutes. Concentration-response curves were constructed to either *L*-arginine (0 – 100 µM) or H_4Bip (0 – 100 µM) and the corresponding EC_{50} (K_m) determined by non-linear regression analysis using Biosoft UltraFit. The endogenous pterin content of NOS-I was measured by reverse phase HPLC coupled with fluorimetric detection (λ_{ex} = 352 nm, λ_{em} = 438 nm, Schmidt *et al.*, 1992). Results shown represent mean values ± sem of 4 -7 experiments each performed in triplicate and * indicates a statistical difference from control ($p<0.05$, unpaired 2-tailed Student's *t*-test with Bonferronni correction for multiple comparisons). The influence of H_4Bip on the K_m for *L*-arginine and enzyme activity was examined. Increasing the H_4Bip concentration from 0.1 µM to 3.0 µM decreased the K_m of *L*-arginine for NOS-I suggesting a positive allosteric influence of H_4Bip (at 0.1 µM H_4Bip, K_m of *L*-arginine = 8.62 ± 0.70 µM; at 3.0 µM H_4Bip, K_m = 4.93* ± 0.42 µM; * indicates difference from 0.1 µM H_4Bip, $p<0.05$). Similarly, increasing the *L*-arginine concentration from 0.3 µM to 30 µM decreased the K_m for H_4Bip revealing a dual interaction between both binding sites (at 0.3 µM *L*-arginine, K_m of H_4Bip = 389 ± 56 nM; at 30 µM *L*-arginine, K_m = 110* ± 22 nM; * indicates difference from 0.3 µM *L*-arginine, $p<0.05$). In agreement, incubation of NOS-I with 30 µM *L*-arginine under enzyme kinetic assay conditions prevented the spontaneous temperature-dependent dissociation of endogenous pterin (endogenous NOS-I pterin: at 4°C, 0.75* ± 0.09 pmoles; at 37°C, 0.23 ± 0.03 pmoles; at 37°C with 30 µM *L*-arginine, 1.10* ± 0.20 pmoles; * indicates a difference from 37°C , $p<0.05$). We attempted to antagonise this *L*-arginine-dependent effect using the specific *L*-arginine-based NOS inhibitor, N$^\omega$-nitro-*L*-arginine (NNA). However, NNA (0.3 µM) alone also prevented the loss of endogenous pterin from NOS-I (from 0.23 ± 0.03 pmoles to 0.83* ± 0.14 pmoles, * $p<0.05$) as did the combination of *L*-arginine (30 µM) and NNA (0.3 µM) (to 0.81 * ± 0.12 pmoles, * $p<0.05$) suggesting that *L*-arginine-based inhibitors also promote the allosteric incorporation of H_4Bip to NOS-I. In contrast, no apparent allosteric interaction was observed between the CaM and H_4Bip binding domains since CaM (0.3 – 100 nM) did not alter the K_m of H_4Bip ($p>0.05$). Moreover, a saturating concentration of CaM (50 nM) did not prevent the loss of endogenous pterin (from 0.23 ± 0.03 pmoles to 0.10 ± 0.06 pmoles, $p>0.05$). In conclusion, the present findings demonstrate a positive allosteric effect of *L*-arginine but not CaM on the binding of endogenous H_4Bip to NOS-I and its activating influence in NOS-I catalysis. Additionally, we show that *L*-arginine-based NOS inhibitors share this effect with *L*-arginine.

Liu, Q. & Gross, S.S. (1996). In *Methods in Enzymology.* ed. Packer, L. San Diego: Academic Press, **vol. 268**, pp 311-324.
Schmidt, H.H.H.W., Smith, R.M., Nakane, M. *et al.* (1992). *Biochemistry*, **31**, 3243-3249

8P NOVEL ANTI-PTERIN INHIBITORS OF NO SYNTHASE: EXCLUSION OF A CATALYTIC BUT EVIDENCE FOR A STABILISING FUNCTION IN NO SYNTHASE AND DIRECT LABELLING OF THE ENZYME'S PTERIN BINDING SITE

H. M. Bömmel, A. J. Reif, L. G. Fröhlich, H. Hofmann, D. M. Marecak*, V. Groehn,† A. Frey, M. Meinecke, P. Kotsonis, M. Bernhardt, G. D. Prestwich*, W. Pfleiderer,† and H.H.H.W. Schmidt. (Introduced by Timothy D. Warner) *Dept. of Pharmacol., Julius-Maximilians-Univ. Würzburg, Germany; *Dept. Med. Chem., Salt Lake City, Utah, U.S.A.; †Dept. Chem., Univ. Konstanz, Germany.

Nitric oxide (NO) is an important messenger molecule with multiple biological functions in blood vessels, neurones, macrophages and other tissue. The biosynthesis of NO is catalysed by a family of homodimeric NO synthases which require the redox-active cofactors (6R)-5,6,7,8-tetrahydro-biopterin (H_4Bip) and NADPH. However, while NADPH is stoichiometrically consumed, the function and binding site of H_4Bip in NO catalysis are still unknown. Basal NOS activity has been attributed to NOS-associated pterin.

A problem in understanding the function of H_4Bip in NOS catalysis is the difficulty in obtaining intact H_4Bip-free NOS since NOS copurifies with sub-stoichiometric amounts of H_4Bip. Therefore, we developed anti-pterin type inhibitors which specifically compete with H_4Bip in order to understand the function of H_4Bip in NOS catalysis and to identify the pterin binding site. Purified porcine cerebellum NOS-I (nNOS) activity (expressed as % of Vmax) was determined as formation of [^{3}H]L-citrulline from [^{3}H]L-arginine (Schmidt *et al.*, 1991). All results shown represent mean values ± SEM of at least 3 independent experiments each performed in triplicate. The endogenous pterin content was measured by reverse phase HPLC and the degree of NOS dimerisation by gel filtration chromatography (Schmidt et al., 1991).

Molecular modelling (Catalyst, version 2.2, Biocad, Mountain View, CA, USA) with these anti-pterins (APs) revealed for the first time a structure-activity relationship for the pterin binding site. APs with small substituents on their pterin backbone (type I) inserted into the hypothetical pterin binding pocket analogous to H_4Bip. Only APs with more complex substituents (type II) formed an additional hydrogen bond possibly adjacent to the the pterin binding site in an exosite. In agreement, enzyme kinetic analysis also revealed APs with distinct inhibitor profiles. That is, the type I APs antagonised only the H_4Bip stimulated NOS activity in a competitive manner but showed no effect on basal enzyme activity. In contrast, the type II APs completely inhibited both stimulated (control, 100.0 ± 0.6%, 100 µM PHS-72, a type II AP: 0.1±0.9%) and basal NOS activity (control, 20.01±5.14%, 100 µM PHS-72 (N2-Isobutyryl-6,7-di-phenyl-5-(1-benzoylnicotinoylium)-5,6,7,8-tetrahydropterin-bromid): 0.1±0.1%) in a partially competitive and irreversible manner. Surprisingly the type I APs displaced endogenous H_4Bip (control: 1.37 ± 0.107 pmol endogenous pterin; 100 µM PHS-32 (2-Amino-4,6-dioxo-3,4,5,6,7,8,8a,9,10-octahydro-oxazolo-[3,4-f]-pteridins), a type I AP: 0.428 ± 0.102 pmol endogenous pterin) without affecting basal activity (control, 20.01 ± 5.14%, 100 µM PHS-32, 24.8 ± 0.47%).

This important result demonstrates that basal activity is independent of endogenous pterin and rules out a catalytic role for H_4Bip. In support of this, linear regression analysis revealed that basal activity does not correlate with pterin-saturation (r2=0.000) but may be due to an initial pterin-independent enzyme conformation (NOS*). However, additional data suggest an essential stabilising function for H_4Bip since H_4Bip prevented the dissociation of NOS dimers into monomers and the corresponding initial loss of enzyme activity. Consistent with a pterin interaction with the catalytic centre, we also localised the pterin binding site using a [^{3}H]labelled type II AP as photoaffinity probe to the oxygenase domain in the smallest fragment yet reported (341 amino acids). Furthermore, since anti-pterins also inhibit NO^2-release in intact N_1E-115 cells (200 µM PHS-32, 25 ± 19.6% of control), they are promising pharmacological tools to down-regulate pathological NO formation.

Schmidt, HHHW, Pollock, J., Nakane, M, Gorsky, LD, Förstermann, U & Murad, F. (1991). Proc. Natl. Acad. Sci. U.S.A., 88, 365-369.

9P INTERACTION OF PEROXYNITRITE WITH L-CYSTEINE AND ITS ANALOGUES

Fiona J. Dowell & William Martin. CRI, West Medical Building, University of Glasgow, Glasgow, G12 8QQ.

Peroxynitrite (PN) is known to react with glutathione and L-cysteine (L-cys) to produce novel, NO-releasing vasorelaxants. Some reports suggest the reactions result in the formation of the respective S-nitrosothiols (Moro *et al.* 1994; Mayer *et al.* 1995) but this is disputed (Wu *et al.* 1994). We wished to investigate the functional groups on the L-cys molecule with which PN reacts to form novel vasoactive species.

PN was synthesised and stabilised at pH 12.4 (Dowell & Martin, 1996). To investigate the formation of stable vasodilator substances, PN (1 mM) was mixed (30 s) with the analogues of L-cys (each 10 mM). These solutions were then neutralised to remove unreacted PN and their relaxant actions assayed on endothelium-denuded rings of rat aorta contracted with phenylephrine (PE, 20 nM). Data are expressed as pEC_{50} values (mean ± s.e. mean, n≥6) and differences determined by ANOVA followed by the Bonferroni test.

None of the analogues of L-cys had any effect by themselves on the tone of PE-contracted rings. In contrast, when PN was mixed with L-cys and neutralised to remove any unreacted PN, a new more potent relaxant was formed (pEC_{50}: 6.43 ± 0.05 vs. 5.52 ± 0.05 for native PN, P<0.001). When neutralised PN was mixed with L-cys, however, no new relaxant was formed (5.32 ± 0.08); the relaxant response observed was similar to that seen with neutralised PN alone. In order to investigate which functional group of L-cys reacts with PN to form the new species, PN was mixed with analogues of L-cys. Those with a free thiol group, namely D-cys (6.40 ± 0.04), L-cysteine ethyl ester (6.34 ± 0.05), N-acetyl-L-cysteine (6.39 ± 0.06) and reduced glutathione (6.21 ± 0.05), reacted with PN to form new relaxant species with potencies similar to that of the product of PN and L-cys. When PN was mixed with analogues lacking a free thiol group, i.e. S-methyl-L-cysteine (5.87 ± 0.04) or L-alanine (5.80 ± 0.02), the products formed did exhibit new relaxant activity, but in each case this was significantly less than with L-cys (P<0.001). Analogues retaining the amino but lacking the free thiol or carboxylic acid functions i.e. L-cystine diethyl ester (5.99 ± 0.04) and L-alanine ethyl ester (5.52 ± 0.04), also reacted with PN to form products with a potency similar to that of analogues lacking only the thiol group. In contrast, N-acetyl-L-alanine (5.30 ± 0.04), which has a substituted amino group, lacks the free thiol but retains the carboxylic acid failed to generate new relaxant activity when mixed with PN. As with L-cys, when neutralised PN was mixed with any of the analogues no new relaxant activity was formed.

In summary, these results show that when peroxynitrite reacts directly with L-cysteine a potent NO-releasing vasodilator species is formed. It appears that the thiol is the preferred site of interaction, but peroxynitrite can also react with the amino functional group to generate novel vasorelaxant species.

This work was supported by the British Heart Foundation.

Mayer, B., *et al.* (1995). *J. Biol. Chem.*, 270, 17355-17360.
Moro, M.A., *et al.* (1994). *Proc. Natl. Acad. Sci. USA*, 91, 6702-6706.
Wu, M., *et al.* (1994). *Am. J. Physiol.*, 266, H2108-H2113.

10P A SUPEROXIDE DISMUTASE MIMETIC PROTECTS ACETYLCHOLINE-INDUCED RELAXATION FROM OXIDANT STRESS IN THE RABBIT AORTA

A. MacKenzie & W. Martin, Clinical Research Initiative, West Medical Building, University of Glasgow, Glasgow, G12 8QQ.

Nitric oxide (NO) is rapidly inactivated by superoxide anion (O_2^-). With the use of diethyldithiocarbamate (DETCA), which inhibits Cu/Zn superoxide dismutase (SOD) both intracellularly and extracellularly, we have previously demonstrated that endothelial NO is protected against destruction from O_2^- by this endogenous enzyme (MacKenzie & Martin, 1997). The aim of this study was to determine if, following inhibition of endogenous SOD and generation of O_2^-, NO-dependent relaxation could be restored by exogenously applied SOD and by the low molecular weight, cell permeable SOD mimetic Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; Faulkner *et al*, 1994).

Rabbits were killed with an i.v. injection of sodium pentobarbitone (200 mg Kg^{-1}). The thoracic aorta was removed and cut into rings. These were then suspended in tissue baths containing oxygenated Krebs solution at 37°C and contracted with phenylephrine (30 - 300 nM). NO-dependent relaxation was induced with acetylcholine (ACh; 10 nM - 3 μM). Endogenous SOD was inactivated by incubating aortic rings for 60 min with DETCA. Hypoxanthine (HX; 0.1 mM)/xanthine oxidase (XO; 4.8 mu ml^{-1}) was used to generate O_2^-. Data are expressed as mean ± s.e. mean of ≥ 6 observations and differences determined by ANOVA followed by the Bonferroni post test.

Maximal ACh-induced relaxation was reduced from 95 ± 1 % to 59 ± 3 % (P<0.001) following exposure to HX/XO in DETCA (0.3 mM)-treated tissues. This blockade was completely prevented following treatment with exogenous SOD (250 u ml^{-1}) or MnTMPyP (0.6 mM); maximal relaxation 92 ± 1 and 91 ± 4 %, respectively. Treatment with a higher concentration of DETCA (3 mM) alone reduced maximal relaxation from 89 ± 2 to 43 ± 3% (P<0.001). This blockade was unaffected following treatment with exogenous SOD (250 and 750 u ml^{-1}); maximal relaxation 40 ± 8 and 52 ± 7 %, respectively. It was, however, partially reversed following treatment with 0.6 mM MnTMPyP (maximal relaxation 69 ± 5 %, P<0.001) but further reversal was not observed with a higher concentration (1 mM; 67 ± 3 %).

In conclusion, the ability of DETCA to inhibit extracellular or intracellular SOD may differ with the concentration used. Specifically, treatment with a low concentration of DETCA (0.3 mM) may selectively inhibit extracellular SOD, since the inhibition of ACh-induced relaxation resulting from this coupled with HX/XO was reversed by membrane-impermeant SOD and by MnTMPyP. In contrast, treatment with a higher concentration of DETCA (3 mM) alone probably results in inhibition of SOD intracellularly, leading to intracellular destruction of NO, since relaxation was restored by MnTMPyP but not by SOD. Thus, the membrane permeant nature of SOD mimetics may provide them with greater therapeutic potential than SOD itself in pathologies associated with oxidant stress.

MacKenzie, A. & Martin, W. (1997) *Br. J. Pharmacol.* 120, 159P.
Faulkner, K.M. *et al.* (1994) *J. Biol. Chem.* 269, 23471-23476.

11P USE OF SELECTIVE ENZYME INHIBITORS PROVIDE NO EVIDENCE FOR CROSS-TALK BETWEEN INDUCBILE NITRIC OXIDE SYNTHASE AND CYCLOOXYGENASE-2 IN ENDOTOXIN-TREATED RATS

Lorna C. Hamilton, Annette Tomlinson, Lorne J. Dye, [1]Jane A. Mitchell and Timothy D. Warner. *The William Harvey Research Institute, London EC1M 6BQ, [1]Department of Anaesthesia and Critical Care Medicine, Royal Brompton Hospital, London, SW3 6NP.*

Exposure of cells and tissues to cytokines and/or endotoxin (LPS) *in vitro* and *in vivo* induces the expression of both nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). This has led to investigations of the interplay between these two systems, and in particular to suggestions that NO released by iNOS stimulates COX activity (Salvemini *et al.*, 1995). Here we have investigated in LPS-treated rats the possible cross talk between the iNOS and COX-2 systems *in vivo*.

Male Wistar rats (220-250 g) were anaesthetised with Inactin (120 mg kg^{-1}) and the carotid artery and jugular vein cannulated for the measurement of blood pressure and the administration of compounds or vehicle (saline). After a stabilisation period (15 min), animals received a bolus dose of the selective COX-2 inhibitor, SC 58635 (3 mg kg^{-1}) (Penning *et al.*, 1997) or vehicle followed 1h later by a continuous infusion of LPS (0.2 mg kg^{-1} hr^{-1}) or saline for 6 h. At the end of this period rats were given a bolus injection of arachidonic acid (AA) and blood collected 1 min later (Hamilton *et al.*, 1997a). To examine the effects of selective inhibition of iNOS animals were treated with 1400W (loading dose of 10 mg kg^{-1}, i.v., followed by infusion 10 mg kg^{-1}, i.v., for 4 h) (Garvey *et al.*, 1997). The plasma concentrations of 6-keto-$PGF_{1\alpha}$ (as a measure of PGI_2 production) and nitrite/nitrate (NO_2/NO_3) (as a measure of NO production) in blood samples taken at t=0 and following AA injection were then determined by radioimmunoassay and Griess reaction (after NO_3 reduction to NO_2), respectively. Expression of COX 2 and iNOS was analysed in sections of lung by immunohistochemistry.

LPS infusion for 6 h followed by AA injection caused marked elevations in the plasma levels of 6-keto-$PGF_{1\alpha}$, and NO_2/NO_3 (table 1) associated with induction of COX-2 and iNOS (Hamilton *et al.*, 1997b). SC 58635 inhibited elevations in plasma 6-keto-$PGF_{1\alpha}$, caused by LPS infusion and AA injection, but had no effect on the concentration of NO_2/NO_3. Conversely, 1400W greatly reduced the plasma levels of NO_2/NO_3 following administration of LPS but did not affect the formation of 6-keto-$PGF_{1\alpha}$ following AA injection.

In conclusion in the rat elevated production of prostanoids by COX-2 has no effect on the formation of NO by iNOS, and NO formed by iNOS has no effect on the production of prostanoids by COX-2.

LCH is the recipient of a BHF Ph.D. Studentship (FS/97013). JAM is Wellcome Career Development Fellow and TDW holds a BHF Lectureship (BS/95003).

Hamilton, L.C. *et al.* (1997a). *Br. J. Pharmacol.*, **122**, 22P.
Hamilton, L.C. *et al.* (1997b). *Br. J. Pharmacol.*, **120**, 49P.
Salvemini, D. *et al.* (1995). *Br. J. Pharmacol.*, **114**, 1171-1178.
Penning, T.D. *et al.* (1997). *J. Med. Chem.*, **40**, 1347-1365.
Garvey, E.P. *et al.* (1997). *J. Biol. Chem.*, **272**, 4959-4963.

	Vehicle (n=8)	LPS (n=8)	SC 58635 (n=5)	1400W (n=3)
6-keto-$PGF_{1\alpha}$, 0 h	0.1 ± 0.1	0.1 ± 0.1	0.05 ± 0.01	0.17 ± 0.08
6-keto-$PGF_{1\alpha}$, 6 h + AA	2.8 ± 1.0	374 ± 67*	3.8 ± 0.9†	395 ± 86*
NO_2/NO_3, 0 h	12 ± 1	9 ± 3	4 ± 1	8 ± 1
NO_2/NO_3, 6 h + AA	35 ± 3	213 ± 52*	270 ± 23*	78 ± 11†

Table 1. Plasma concentrations of 6-keto-$PGF_{1\alpha}$ (ng.ml), and NO_2/NO_3 (μM). * $p<0.05$ w.r.t. vehicle; † $p<0.05$ w.r.t. LPS; compared by ANOVA followed by Dunnett's test.

12P NITRIC OXIDE INHIBITS THE VASORELAXANT ENDOTHELIAL α_2-ADRENERGIC PATHWAY IN MICE

E. Thorin, P. Huang[1], M.C. Fishman[1], & J.A. Bevan[2], Montréal Heart Institute, Research Center, 5000 Belanger Street, Montréal, PQ, H1T 1C8, Canada, [1]Cardiovascular Research Center, Mass. General Hospital and Harvard Medical School, Boston, MA 02129 & [2]Department of Pharmacology, University of Vermont, Burlington, VT 05405, USA.

Activation of endothelial α_2-adrenergic receptors (α_2-AR) induces vascular relaxation in several species. The mechanism involved in large arteries implies the participation of nitric oxide (NO) whereas other yet undefined factors appear to mediate the α_2-AR-dependent response in small arteries. The aim of this study was to characterize the involvement of NO in response to α_2-AR agonists in isolated mesenteric arteries of the mouse.

Mice lacking the gene for endothelial nitric oxide synthase ($eNOS^-$; n=14) and their wild-type control (WT; n=41; male, a combination of C57BL/6 and SV-129 strains, 18-23g) were used. After cervical dislocation, mesenteric arteries were isolated and rings mounted on a microvessel myograph. Changes in isometric tension were recorded. Contractions are expressed as % of the maximal response (E_{max}) to 127mM KCl-physiological salt solution (K-PSS). Relaxations are expressed as % inhibition of the preaddition tone. In some experiments, the endothelium was removed by gentle scraping of the lumen with a human hair. All experiments were performed in the presence of indomethacin (10μM). Results are given as mean±s.e.

Arterial segments of similar diameter from both groups were used (157±6μm and 165±6μm external diameter from WT and $eNOS^-$ mice, respectively). Wall thickness was modestly increased in arteries isolated from $eNOS^-$ mice (36±1 μm *versus* 30±1μm in WT arteries, $P<0.05$). Contractions induced by phenylephrine (30μM) were 2.3 times higher in arteries from $eNOS^-$ than WT mice ($P<0.05$), without change in maximal contraction induced by 127mM K-PSS. Cumulative addition of oxymetazoline (OXY, 0.01-30μM; a selective α_2-AR agonist) significantly decreased phenylephrine- and serotonin-induced tone in $eNOS^-$ but not in WT arteries; OXY (10μM) induced 12±7% and 46±6% relaxation in WT and $eNOS^-$ mice arterial segments, respectively ($P<0.05$). In the presence of nitro-L-arginine (l-NNA, 100μM, inhibitor of NOS), OXY (10μM) induced 38±9% relaxation of arteries isolated from WT mice ($P<0.05$). l-NNA had no effect on the relaxation caused by OXY in $eNOS^-$ mice arterial rings. The relaxation caused by OXY was endothelium-dependent in both groups. To investigate possible interactions between NO and the α_2-adrenergic pathway, the effect of exogenous NO was tested in $eNOS^-$ and l-NNA treated WT mice mesenteric arteries. A threshold concentration of sodium nitroprusside (1nM, donor of NO) inhibited α_2-AR-mediated relaxation of $eNOS^-$ and l-NNA treated WT mice arteries. OXY-induced relaxation was restored upon readdition of methylene blue (1μM, inhibitor of guanylate cyclase) 20 minutes prior to α_2-adrenergic stimulation in the presence of sodium nitroprusside; the reversal effect of methylene blue was not affected by dimethylsulfoxide (10μM), a superoxide anion scavenger. We considered the involvement of an endothelium-derived factor that would mediate α_2-AR-dependent relaxation by activating smooth muscle K^+ channels: tetraethylammonium (1mM; inhibitor of Ca^{2+}-activated K^+ channels) prevented α_2-AR-mediated relaxation in the presence of l-NNA, whereas glibenclamide (1μM; inhibitor of ATP-sensitive K^+ channels) had no effect.

In summary, α_2-AR activation caused vascular relaxation in mice lacking the gene for endothelial NOS and after inhibition of NO formation in WT mice. This suggests that NO inhibits the endothelial α_2-adrenoceptor-dependent pathway through a mechanism that may involve activation of soluble guanylate cyclases. In mice mesenteric arteries, α_2-adrenergic-dependent relaxation is likely to be due to an endothelium-derived factor activating calcium-sensitive K^+ channels, whose release and/or production is reduced by concurrent NO formation.

13P THE EFFECT OF AMINOGUANIDINE ON ACETYLCHOLINE-INDUCED RELAXATION RESPONSES IN RAT BLOOD VESSELS

C. FORSTER, A. IRAL & H. RAZAVI, Department of Pharmacology, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

Relaxation of vascular smooth muscle following activation of nitric oxide synthase (eNOS) in endothelial cells represents an important mechanism for blood pressure regulation, whereas the inducible form of the enzyme (iNOS) most likely plays a role in the defense response to disease and infection. It has been suggested that inhibition of eNOS causes vasoconstriction. However, due to the non-selective nature of NOS inhibitors, it is difficult to establish whether the beneficial haemodynamic effects are due to iNOS or eNOS activation. Aminoguanidine is a more potent inhibitor of iNOS (Ruetten *et al.*,1996) in vascular smooth muscle. Therefore, we have compared the effects of aminoguanidine and N$^{\omega}$nitro-l-arginine (NNA) on relaxation response to acetylcholine in rat blood vessels.

Rat aorta and 3rd generation mesenteric arteries were obtained from ten male Sprague Dawley rats (weight range 300-320 g). The aorta was divided into ring segments and alternate rings had their endothelium carefully removed. The rings were mounted in organ baths and allowed to equilibrate. The mesentary was dissected and samples were taken at the region of the 3rd generation, they were then mounted in a microvascular chamber and allowed to equilibrate. In all preparations, rings were preconstricted with either noradrenaline (10^{-6}M) or phenylephrine (10^{-6}M) and without washing out concentration-effect curves were generated to acetylcholine (10^{-8}-10^{-4}M). Once completed preparations were washed and the experiments repeated in the presence of either aminoguanidine (10^{-5}-10^{-4}M) or NNA (10^{-5}-10^{-4}M).

Acetylcholine caused concentration-dependent relaxations in both types of blood vessels with intact endothelium. In the aorta, the mean maximum relaxation amounted to 48±8% following preconstriction with either noradrenaline or phenylephrine. Aminoguanidine failed to modify the relaxation response, whereas NNA significantly attenuated the relaxation response. With 10^{-4}M NNA, the relaxation response was totally abolished. In the small mesesenteric arteries, acetylcholine caused a complete relaxation of preconstricted segments which was concentration-dependent. NNA significantly reduced the relaxation response in a concentration-dependent fashion (10^{-5}M reduced the relaxation by 10±5%; $3x10^{-5}$M by 62±6%; and 10^{-4}M reduced the relaxation by 100%). In contrast to the aorta data, aminoguanidine was found to also inhibit the relaxations to acetylcholine in 3rd generation mesenteric arteries. This inhibition was similar to that seen with NNA, it was concentration-dependent and at 10^{-4}M caused a 100% decrease of the relaxation response.

These results indicate that, in rat aorta, the relaxation response seen in intact vessels to acetylcholine is strongly influenced by eNOS since aminoguanidine failed to modify the response. In contrast the relaxation seen in small mesenteric arteries seems to involve not only an eNOS component but also a, surprising iNOS component. Therefore, in small arteries, there seems to be an iNOS dependent mechanism which may contribute to a beneficial haemodynamic response in the microcirculation.

Supported by The Heart and Stroke Foundation of Ontario

Ruetten, H., Southan, G.J., Abate, A. *et al.* (1996) *Br. J. Pharmacol.* 118, 261-270.

14P PHARMACOLOGICAL CHARACTERIZATION OF NOCICEPTIVE COMPONENTS OF THE CORTICAL SOMATOSENSORY EVOKED POTENTIAL OF THE RAT

Ph. Lebrun, F. Colin, & J. Manil. Physiology Laboratory, Vrije Universiteit Brussel, B-1090 Brussels, Belgium

Nociception is currently measured by behavioral tests which are either easily measurable spinal reflexes lacking supraspinal modulation or complex supraspinally mediated behaviors with no clear end point and prone to observer bias. Both are dependent on motor function and false effects can be produced by substances affecting it. In human experimentation, cortical somatosensory evoked potentials (CSEP) have been shown to be an accurate measure of pain encompassing both nociception and associative processes such as emotions or memory. CSEPs are objective, readily quantifiable, and evoked by activity in afferent pathways only. We have applied this technique to rats in the aim of developing a better measure of changes of pain perception. In this study we measured the effect of substances with known analgesic properties on components of the rat CSEP to assess specificity and sensitivity and compared these effects to an equivalent behavioral test.

CSEPs evoked by electrical stimuli to the tail of male Wistar rats (>250 g) were recorded with permanent epidural electrodes (1-1Khz, sampling freq. 1KHz, 30 stim). Each test session consisted of 3 control runs, then drug injection and recordings every 10 minutes for 90 min. The CSEP amplitudes were plotted vs. time and the area between this curve and the baseline was expressed as % of max. effect for plotting dose-response curves. Curves were obtained in the same way on a 55° hot plate. (n=5). Morphine sc (2-50 mg/kg), ketamine ip (2-100 mg/kg), xylazine ip (0.5-10mg/kg), adinazolam ip (1-50 mg/kg) and haloperidol ip (0.5 - 20 mg/kg) (n=10) were tested.

Naloxone 0.5 mg/kg sc was given 75 min after 50 mg/kg sc morphine to test specificity. Only drug-naive animals were used.

Two major components of the rat CSEP were identified: a positive-negative (pn) wave complex from thalamocortical and early cortical activity, followed by a late negative (ln) cortical associative wave. A similar response was evoked by mechanical but not thermal stimulation. Analgesics morphine and ketamine caused a dose-related reduction of the amplitude of both waves (table 1). The hot plate was more sensitive for morphine and as sensitive as the ln for ketamine. The effect of morphine was reversed by naloxone. Sedatives (with some analgesic effect) adinazolam and xylazine had an effect on the ln but not on the pn or hot plate. Haloperidol (no analgesic effect) had no effect on either wave.

Conclusion: CSEPs are an objective measure of analgesia in the rat. They measure A∂ fiber function and are less sensitive than tests using heat stimuli to measure C-fiber mediated nociception. Differing effects on early and late waves allow differentiation of spinal from supraspinal effects. CSEPs are most useful for investigation of drugs also having sedative or motor effects.

Table 1: slopes ± s.e.m. of the linear portion of dose-response curves measured using pn and ln wave amplitudes and hot plate.

Drug	pn	ln	hot plate
morphine	54.90 - 18.89	32.09 - 5.45	91.89 - 7.91
ketamine	18.40 - 5.71	66.59 - 5.17	69.63 - 19.33
xylazine	21.41 - 9.92	61.15 - 6.41	-8.00 - 4.48
adinazolam	11.30 - 4.85	36.06 - 3.19	9.93 - 8.68
haloperidol	-2.62 - 6.95	5.83 - 4.91	-

15P ANTAGONISM OF METABOTROPIC GLUTAMATE RECEPTOR-MEDIATED RESPONSES AND NOCICEPTIVE RESPONSES BY THE mGluR1-SELECTIVE ANTAGONIST LY367385 IN THE RAT THALAMUS

T. E. Salt & J. P. Turner, Institute of Ophthalmology, University College London, 11-43 Bath Street, LONDON EC1V 9EL.

Previous work from this laboratory has shown that postsynaptic excitatory responses of rat ventrobasal thalamus neurones to mGluR agonists can be blocked by phenylglycine antagonists which act at Group I metabotropic glutamate receptors (*i.e.* mGluR1 and mGluR5). These antagonists also cause a parallel reduction of nociceptive responses of thalamic neurones, suggesting that Group I mGluRs participate in the mediation of such responses (Salt & Eaton, 1994). In order to distinguish which receptors are involved in these responses, we have carried out further experiments with the novel mGluR1-selective antagonist LY367385 {(+)-2-methyl-4-carboxyphenylglycine} (Baker *et. al.*, 1997).

Extracellular recordings were made from single neurones in the ventrobasal thalamus and immediately overlying dorsal thalamic nuclei of adult male Wistar rats anaesthetised with urethane (1.2 g/kg, I.P.) using multi-barrel electrodes (Salt & Eaton, 1994). Responses of neurones to iontophoretic applications of 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and (S)-3,5-dihydroxy phenylglycine (DHPG) were reduced during continuous iontophoretic application of LY367385 (20-40nA from a 50mM, pH8 aqueous solution) whereas responses to either N-methyl-D-aspartate (NMDA) or (±)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) were relatively unaffected (Table 1). In contrast, another antagonist reported to have mGluR1 activity, (R,S)-1-aminoindan-1,5-dicarboxylic acid (AIDA) (80-120nA from a 50mM, pH8 aqueous solution) proved to be much less selective when tested in similar experiments (Table 1). Nine of the neurones which had been studied with LY367385 were also investigated using noxious thermal sensory stimuli (52°C, 20sec, applied to either the tail or contralateral hindpaw). Responses to such stimuli were reduced to 51 ± 6.4% of control responses by LY367385 when the antagonist was applied with iontophoretic currents which reduced ACPD responses of the same neurones.

Table 1. Reduction of responses by LY367385 and AIDA. Values are % of control responses ± s.e.m. from *n* neurones.

	ACPD	DHPG	NMDA	AMPA
LY367385	15 ± 3.1	8 ± 1.6	119 ± 8.8	114 ± 6.4
n	14	4	18	18
AIDA	21 ± 3.5		55 ± 7.8	63 ± 11.1
n	10		10	6

These results suggest that excitatory responses of thalamic neurones to ACPD and DHPG may be mediated by mGluR1. Furthermore, the reduction of nociceptive responses by LY367385 indicates that mGluR1 is involved in thalamic nociceptive processing and that such antagonists may have analgesic properties.

Supported by The Wellcome Trust. LY367385 was a gift of Drs J.R. Harris & A.E. Kingston, Lilly Research.

Baker, S.R., Clark, B.P., Goldsworthy, J., Harris, J.R., Kingston, A.E. & Lodge, D. (1997). *Brit. J. Pharmacol., Proceedings*, IN PRESS.
Salt, T.E. & Eaton, S.A. (1994). *Neurochem. Intl.*, **24**, 451-458.

16P THE EFFECT OF 4030W92, A NOVEL SODIUM CHANNEL BLOCKER, ON THE TREATMENT OF NEUROPATHIC PAIN IN THE RAT

S.D. Collins, N.M. Clayton, M. Nobbs & C. Bountra. Neuroscience Unit, Glaxo Wellcome Research & Development Ltd., Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY

At present, painful neuropathies in the clinic are poorly treated by classical analgesics such as the Non Steroidal Anti-inflammatory Drugs (NSAIDs) or the opioids, resulting in a large unmet need for a suitable analgesic. A novel sodium channel blocker 4030W92 has been identified (Trezise et al., this meeting), [R (-) 2,4-Diamino-5-(2,3-dichlorophenyl)-6-fluromethyl)-pyrimidine] which we have examined in the Chronic Constriction Injury (CCI) model of neuropathic pain in the rat (Bennett & Xie, 1988). Briefly, under isoflurane anaesthesia, the common left sciatic nerve of male Random Hooded rats (180-200g) was exposed at mid-thigh level. Four ligatures of chromic gut (4.0) were tied loosely around the nerve with a 1mm spacing between each. The wound was then closed and secured with suture clips. The surgical procedure was identical for the sham operated animals except the sciatic nerve was not ligated. For each study, 40 animals were divided into groups of 10. The rats were allowed a period of seven days to recover from the surgery before behavioural testing began. The effect of 4030W92 on CCI-induced decrease in mechanical paw withdrawal threshold was measured using an algesymeter (Randall & Selitto, 1957). The presence of mechanical allodynia was assessed using Von Frey Hair monofilaments (range: 4.19-84.96g). The rats were lightly restrained and placed upon a metal grid floor, from below which the monofilaments were applied to the plantar surface of the hindpaws. The lowest monofilament to produce a withdrawal was the response recorded. In each study, a period of two weeks was allowed following surgery to enable the neuropathy to become established before chronic dosing commenced. Statistical analysis was carried out to compare the difference between the drug treated group and vehicle treated group using unpaired Student's t test ($P<0.05$ considered significant).

Chronic dosing with 4030W92 ($10mgkg^{-1}$ b.i.d. p.o. days 13-27 post surgery) fully reversed, by day 26, the CCI-induced decrease in paw withdrawal threshold to that of sham operated animals (106 ± 7g vs 95 ± 7g respectively, both n=10; $P>0.05$). A highly significant reversal (50 ± 11%, $P<0.01$) of the mechanical allodynia was achieved following 10 days of chronic dosing with 4030W92 ($10mgkg^{-1}$ b.i.d. p.o.). Following cessation of the treatment the analgesic effects of 4030W92 were maintained for the remainder of the study, as the drug treated group did not return to vehicle treated baseline values. Morphine ($3mgkg^{-1}$ t.i.d. s.c. days 12-16 post-surgery) produced a maximal reversal of about 12%, whereas ibuprofen ($5mgkg^{-1}$ t.i.d. p.o. days 11-15 post-operative) was ineffective in this model (0% reversal). In conclusion, 4030W92 reverses the hypersensitivity associated with neuropathic pain. Current analgesics are relatively ineffective in treating neuropathic pain in the clinic, therefore, 4030W92 may be a major advance in the treatment of neuropathic pain in man.

Bennett, G.J. & Xie, Y.K. (1988). Pain, 33 87-107.
Randall, L.O. & Selitto, J.J. (1957). Arch. Int. Pharmacodyn., 61 409-419.
Trezise, D.J., John, V.H., Nobbs, M. et al. (1997). This meeting.

17P ELECTROPHYSIOLOGICAL CHARACTERISATION OF 1-AMINOINDAN-1,5-DICARBOXYLIC ACID ON THE NEONATAL RAT SPINAL CORD PREPARATION

J.M. Pinkney[1], W.P. Kingston[1], N.K. Thomas[2], D.E. Jane[2] & P.C-K. Pook[1]. [1]School of Health Sciences, University of Sunderland, Sunderland, SR1 3SD. [2]Dept of Pharmacology, University of Bristol, Bristol, BS8 1TD.

(RS)-1-Aminoindan-1,5-dicarboxylic acid (AIDA) is a conformationally restricted analogue of (S)-α-methyl-4-carboxyphenylglycine (MCPG). MCPG has been reported to be an antagonist of both pre- and post-synaptic mGlu receptors. In preliminary studies, Pellicciari *et al.*, (1995) reported that AIDA is a relatively potent and selective antagonist of $mGlu_{1\alpha}$ but has no effect on $mGlu_2$ and $mGlu_4$ up to a concentration of 100 μM. In the present study, the activity of AIDA was investigated at both pre-and post-synaptic receptors in the isolated hemisected neonatal rat spinal cord preparation.

To investigate activity post-synaptically, recordings were made on motoneurones of a ventral root in a Mg^{2+} free medium containing 0.1 μM tetrodotoxin (TTX). (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD) and (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG) were used as the standard agonists (30-75 μM, 1min applications). Results indicate that AIDA is a weak antagonist of postsynaptic mGlu receptors, with an apparent K_D ± s.e.mean of 495 ± 21 μM (n=5) calculated for the antagonism of (1S,3R)-ACPD-induced depolarisations and a K_D ± s.e.mean of 517 ± 36 μM (n=5) for the antagonism of (S)-3,5-DHPG-induced depolarisations.

To investigate activity at pre-synaptic mGlu receptors recordings of monosynaptic responses of motoneurones were made from a ventral root evoked by stimulation of the corresponding dorsal root (30V, 2 pulses min^{-1}). The medium was TTX free but contained 2 mM $MgSO_4$ and 50μ M D-2-amino-5-phosphonopentanoate. The group III mGlu receptor agonists, L-2-amino-4-phosphonobutanoic acid (L-AP4), L-serine-O-phosphate (L-SOP) and the group II mGlu receptor agonists (1S,3S)- and (1S,3R)-ACPD, (2S,1'S,2'S)-2-(2-carboxycycloprop-1-yl)glycine (L-CCG-I), (2S,2'R,3'R)-2-(2,3-dicarboxycycloprop-1-yl)glycine (DCG-IV) and (S)-3-carboxy-4-hydroxyphenylglycine ((S)-3C4HPG) (0.1-500 μM, 5 min applications) were used to determine control levels of agonist-induced depression of the monosynaptic response. At a concentration of 100μM, AIDA has a weak agonist activity at pre-synaptic mGlu receptors, and more interestingly also potentiates the depression of the monosynaptic responses induced by L-AP4 and L-SOP (1-500 μM) to 2.4 ± 0.16, n=8 and 2.00 ± 0.17, n=5 times the control respectively, an effect lasting up to 2 hours. In comparison, depressions induced by group II agonists (1-200μ M) were not greatly potentiated, (1S,3S)-ACPD 1.21 ± 0.08, n=4, (1S,3R)-ACPD 1.17 ± 0.08, n=4, L-CCG-I 1.20 ± 0.07, n=4, DCG-IV 1.32 ± 0.07, n=5 and (S)-3C4HPG 1.13 ± 0.15, n=4 times the control.

Furthermore, the AIDA-induced potentiation of the L-AP4-induced depression of the monosynaptic response is partially inhibited by the prior addition of group II mGlu receptor agonists (1-200μM): (1S,3S)-ACPD, 1.47 ± 0.12, n=7, (1S,3R)-ACPD, 1.27 ± 0.06 n=4, L-CCG-I, 1.67 ± 0.16, n=5, DCG-IV, 1.04 ± 0.03, n=3 and (S)-3C4HPG, 1.50 ± 0.04, n=5 times the control. Finally, this inhibition by (1S,3S)-ACPD (3-7.5μM) can be antagonised by the selective antagonist for (1S,3S)-ACPD-induced depressions (Jane *et al*, 1996), (S)-α-ethylglutamate (1mM); the AIDA-induced (100μM) potentiation of L-AP4-induced (1-5μM) depression being 2.47 ± 0.36, n=6 times the control.

There clearly appears to be an interaction between group II and III mGlu receptors and the AIDA binding site. Group II and III mGlu receptors are negatively coupled to cAMP formation, but the mechanism of the AIDA-induced potentiation of the depression of the monosynaptic response mediated by group III mGlu receptor agonists is not clear from this study.

Pellicciari, R. *et al.* (1995) *J. Med. Chem.* 38: 3717.
Jane, D.E. *et al.* (1996) *Neuropharmacology* 35: 1029.

18P SOMATOSTATIN (SRIF) ACTIVATES A K^+ CONDUCTANCE IN ANTERIOR CINGULATE CORTICAL PYRAMIDAL NEURONES BY AN ACTION ON sst_2 RECEPTORS

G.A. Hicks, W. Feniuk and P.P.A. Humphrey. Glaxo Institute of Applied Pharmacology, University of Cambridge, Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1QJ.

The anterior cingulate cortex, a region thought to be involved in the affective response to pain (Devinsky *et al.*, 1995), contains a high density of SRIF receptors. Radioligand binding studies (Holloway *et al*, 1996) suggest that at least one subtype of SRIF receptor (sst_2) is present in this area. Using a selective antibody, Schindler *et al.* (1997) localised the sst_{2a} isoform to pyramidal neurone cell bodies and apical dendrites. We have studied the effects of SRIF and some related analogues (see Chessell *et al*, 1996) in deep layer pyramidal cells of the anterior cingulate cortex.

Slices of forebrain containing the anterior cingulate cortex were prepared using methods similar to Chessell *et al.*, (1996). Whole-cell patch clamp recordings were made from visualised neurones in the deep layers (V & VI) using fire-polished electrodes (3-6 MΩ) containing (mM): Kgluconate 140, $MgCl_2$ 2, $CaCl_2$ .57, EGTA 1, HEPES 10, MgATP 4, NaGTP 0.3, pH 7.4. Drugs were applied by bath perfusion. Due to desensitisation of responses to SRIF and variations in current amplitude in different neurones, concentration-effect relationships were determined from currents evoked in many slices expressed as a percentage of the outward current produced by the $GABA_B$ receptor agonist baclofen (10 μM) in the same neurone.

When neurones were voltage-clamped at -60 mV, application of SRIF (1-1000 nM) caused a concentration-dependent outward current which was sensitive to Ba^{2+} (300 μM, 80 ± 3% inhibition, n=4) and accompanied by an increased membrane conductance. In the presence of a maximal concentration of baclofen (100 μM), co-application of SRIF (300 nM, n=3) produced no further outward current.

Concentration-effect relationships were determined for a range of SRIF analogues with known receptor selectivities. The rank order of potency of these ligands was (EC50): Octreotide (1.8 nM) >BIM 23027 (3.7 nM) >SRIF (20 nM) =L-362,855 (20 nM) which suggests an action at sst_2 receptors. The sst_5 receptor antagonist, BIM 23056 (see Wilkinson *et al*, 1996), was without antagonist (1 μM, n=3) or agonist (100 nM or 1 μM, n=4) effect. The selective sst_2 receptor antagonist, L-Tyr^8-Cyanamid 154806 (1 μM; see Feniuk *et al*, this meeting), partially reversed responses to sub-maximal concentrations of SRIF (100 nM, 44 ± 6% reversal n=5) and L-362,855 (100 nM, 70 ± 6% reversal n=5) but was without activity alone or against baclofen (10 μM, n=4). In addition, sst_2 receptor desensitisation with a prolonged application of BIM 23027 (100 nM), resulted in an abolition of the response to a second BIM 23027 application and reduced responses to L-362,855 (1 μM, n=3) by 73% compared to those observed in non-desensitised neurones. Interestingly, the responses to L-362,855 (1 μM) showed no desensitisation during applications for up to 10 min. This may account for the greater L-362,855 maximal response (52.4 ± 3% baclofen) compared to SRIF (40.4 ± 2.7% baclofen) or BIM 23027 (39.1 ± 1.8% baclofen).

These data suggest that SRIF inhibits pyramidal neurones of the anterior cingulate cortex by an action predominantly at sst_2 receptors, which activate a K^+ conductance. It appears that SRIF and $GABA_B$ receptors couple to the same population of K^+ channels in these cells.

Chessell, I.P. *et al.* (1996). *Br. J. Pharmacol.* **117**, 1673-1678.
Devinsky, O. *et al.* (1995). *Brain* **118**, 279-306.
Holloway, S. *et al.* (1996). *Neuropharmacology* **35**, 1109-1120.
Schindler, M. *et al.* (1997). *Neuroscience* **76**, 225-240.
Wilkinson, G. *et al.* (1996). *Br. J. Pharmacol.* **118**, 445-447.

19P QUANTITATIVE AUTORADIOGRAPHY OF OPIOID RECEPTOR SUBTYPES IN THE BRAINS OF κ-RECEPTOR KNOCKOUT MICE

S.J.Slowe, I.Kitchen, F.Simonin[1] & B.Kieffer[1]. School of Biological Sciences, University of Surrey, Guildford, Surrey, GU2 5XH and [1]UPR 9050 CNRS, ESBS Universite Louis Pasteur, 67400 Illkirch, Strasbourg, France.

The mouse κ-opioid receptor (KOR) gene has been successfully disrupted by homologous recombination and animals deficient in both copies of the gene found to exhibit a loss of behavioural responses to U-50,488 effects. There are no significant changes in morphine induced antinociception in mice homozygous for the mutation but the severity of morphine withdrawal is attenuated. In order to determine if there is complete loss of κ-receptors or any compensatory changes in the expression of μ- and δ-opioid receptor subtypes we have carried out a complete autoradiographic mapping of opioid receptors in wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mouse brains. Tissue sections from +/+, +/- and -/- animals were processed for autoradiography as described previously (Kitchen *et al.*, 1997). Adjacent coronal sections (20 μm) were cut (300 μm apart) for the determination of total and non-specific binding of μ-, δ- and κ-receptors labelled with [^{3}H]-D-Ala^{2}MePhe^{4}Gly-ol^5 (DAMGO) (4 nM), [^{3}H]-D-Ala^2 deltorphin-I (7 nM) and [^{3}H]CI-977 (2.5 nM) respectively. Sections were apposed to [^{3}H]hyperfilm for three (μ- and δ-) and six (κ) weeks. Slides from +/+, +/- and -/- brains were laid against the same film and developed and analysed in parallel.

In brains from -/- mice there were no detectable κ-receptors labelled with [^{3}H]CI-977 in any brain region. There were also no significant changes in the levels of μ- and δ-opioid receptors in mutant mice. There was a 50-60% reduction in κ-receptors in +/- mice suggesting there was no compensatory expression of the remaining KOR gene in these animals (Table 1).

Table 1. κ-, μ- and δ-opioid receptor expression in selected brain regions of wild-type, heterozygous and homozygous mice.

	Caudate putamen	Frontal parietal cortex	Hypothalamus	Basolateral amygdala
κ (+/+)	18 ± 2.6	25 ± 3.3	25 ±1.2	22 ± 3.4
κ (+/-)	9 ± 0.9	11 ± 1.2	14 ± 2.5	14 ± 1.2
κ (-/-)	n.d.	n.d.	n.d.	n.d.
μ (+/+)	58 ± 1.9	25 ±2.5	49 ± 5.6	47 ± 2.7
μ (+/-)	61 ± 3.0	30 ± 0.9	56 ± 5.2	52 ± 3.2
μ (-/-)	59 ± 7.2	27 ± 3.8	54 ± 4.8	49 ± 2.8
δ (+/+)	113 ± 4.9	62 ± 2.7	15 ± 1.7	68 ± 3.2
δ (+/-)	124 ± 7.9	63 ± 4.5	16 ± 1.6	66 ± 5.8
δ (-/-)	127 ± 5.5	64 ± 3.9	18 ± 1.7	64 ± 4.5

Values are means (fmol mg^{-1} tissue) ± s.e. mean for 5-6 determinations. n.d. = not detectable.

The results indicate that there is no compensatory expression of μ- and δ-opioid receptor sites in any brain region of mice deficient in the κ-opioid receptor gene and contrast with our previous studies in μ-opioid receptor deficient mice where we have observed small regional down-regulation of κ- and δ-sites (Kitchen *et al*, 1997).

Kitchen, I., Slowe, S.J., Matthes, H.W.D. & Kieffer, B.L. (1997). *Brain Res.* In press.

20P BEHAVIOURAL RESPONSIVITY TO THE SELECTIVE D_1-LIKE AGONIST A 68930 IN TRANSGENIC MICE WITH D_{1A} DOPAMINE RECEPTOR 'KNOCKOUT'

J.J. Clifford[1], O. Tighe[2], D.T. Croke[2], J. Drago[3], D.R. Sibley[4] & J.L. Waddington[1]. Departments of [1]Clinical Pharmacology & [2]Biochemistry, Royal College of Surgeons in Ireland, Dublin 2; [3]Department of Anatomy, Monash University, Australia; [4]NINDS, Bethesda, USA.

Among D_1-like dopamine receptors it is the adenylyl cyclase-coupled D_{1A} receptor that has been assumed to play a fundamental role in the regulation of psychomotor behaviour; however, there is indirect evidence which indicates a functional role for another D_1-like receptor that may be coupled to a transduction system other than/additional to adenylyl cyclase (Waddington et al., 1995). Described here is the profile of responsivity to the D_1-like agonist A 68930 (Daly & Waddington, 1993) in transgenic mice with targeted gene deletion of the D_{1A} receptor.

D_{1A} 'knockout' mice were constructed, bred and genotyped as described previously (Drago et al., 1994; Clifford et al., 1997). At approximately 12 weeks, black females [n=8 per group] were challenged with 0.068 - 2.0 mg/kg s.c. A 68930 [now redesignated as A 75734; A 68930 retained for historical continuity] and assessed by direct observation over three successive 1h periods using an ethologically-based rapid time-sampling behavioural checklist procedure (Clifford et al., 1997). Statistical analysis was by analysis of variance (ANOVA), followed by Student's t-test or Mann-Whitney U-test. In wildtypes [$D_{1A}^{+/+}$], A 68930 dose-dependently induced sniffing, sifting and rearing, with prominent induction of grooming and intense grooming. Relative to wildtypes, 'knockouts' [$D_{1A}^{-/-}$] evidenced unaltered sniffing and sifting responses, with only rearing to the highest dose of A 68930 being attenuated substantially [-70%, P<0.001]; grooming was not altered, while intense grooming to the highest dose was attenuated [-51%, P<0.05] but still occurred to significant excess relative to vehicle controls [+284%, P<0.05]; there was a prominent increase in locomotion to the highest dose of A 68930 [+512%, P<0.001].

Mice with targeted gene deletion of the D_{1A} receptor showed a preservation of several behavioural responses to the highly potent and selective full efficacy D_1-like agonist A 68930; only rearing was attenuated substantially in reciprocity with substantial heightening of locomotion. Grooming, including intense grooming, is the most widely accepted behavioural index of D_1-like receptor activation (Waddington et al., 1995) and this response was not abolished in $D_{1A}^{-/-}$ mice; they were still able to manifest responses similar to those of their $D_{1A}^{+/+}$ counterparts. These findings suggest either that several elements of behavioural responsivity to D_1-like stimulation involve at least in part not the D_{1A} but rather some other D_1-like receptor, or that compensatory processes consequent to developmental absence of D_{1A} receptors can mediate these responses.

These studies were supported by RCSI. We thank Abbott for making A 68930 [as A 75734] available to us.

Clifford, J.J., Tighe, O., Croke, D.T. et al. (1997) *Br. J. Pharmacol.* 120,134P.
Daly, S.A. & Waddington, J.L. (1993) *Psychopharmacology* 113, 45 - 50.
Drago, J., Gerfen, C.R., Lachowicz, J.E. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 12564-12568.
Waddington, J.L., Daly, S.A., Downes, R.P. et al. (1995) *Prog. Neuro-Psychopharmacol. Biol. Psychiat.* 19, 811-831.

21P CYANOTRIPHENYLBORATE IS AN UNCOMPETITIVE INHIBITOR OF HUMAN $GABA_A$ RECOMBINANT RECEPTORS

Julian R. A. Wooltorton, Paul Whiting* & Trevor G. Smart, Department of Pharmacology, The School of Pharmacy, 29-39 Brunswick Square, London, WC1N 1AX, & *Merck, Sharp & Dohme, Terlings Park, Harlow, Essex, CM20 2QR.

The inhibitory neurotransmitter, GABA, directly gates Cl^- selective channels which form an integral part of $GABA_A$ receptors. These are proposed to be hetero-oligomeric in structure, and composed of 5 subunits selected from the groups: α, β, γ, δ and ε (Sieghart, 1995; Davies *et al.*, 1997). Each polypeptide subunit consists of 4 proposed transmembrane domains, the second of which (TM2) is thought to line the pore of the ion channel. The ability of the ion channel to permit Cl^- ion flux can be restricted by the presence of modulators that bind to amino acid residues in TM2 either from the membrane or from within the ion channel lumen. At present only the weak inhibitor, penicillin-G, possesses the profile of an open channel blocker for the $GABA_A$ receptor (Twyman *et al.*, 1992). This study characterizes the inhibitory properties of cyanotriphenylborate (CTB), a compound possessing net negative charge, on human $GABA_A$ α1β1γ2s recombinant receptors expressed in *Xenopus* oocytes. Oocytes were injected with human GABA receptor α1, β1, and γ2s cDNAs in an equimolar combination and studied using a two-electrode voltage clamp technique. Membrane conductances were determined from 10 mV hyperpolarizing steps from a holding potential of -25 mV.

GABA concentration-response curves were depressed in the presence of 1-50 μM CTB. Data were normalized with respect to the response to 20 μM GABA. The EC_{50} values for GABA determined in 1-50 μM CTB were relatively unchanged until a near saturating concentration of CTB (Control: EC_{50}=21.6±1.0 μM [mean±s.e.mean; n=17-19 oocytes]; +1 μM CTB: 27.5±9.6 [4-6]; +10 μM CTB: 20.3±5.6 [4-9]; +50 μM CTB: 6.9±2.3 [4-5]). In comparison, CTB significantly reduced the maximal normalized responses from the control, 2.19±0.05, to 1.69±0.24, 0.96±0.09 and 0.17±0.02, respectively ($P<0.05$, *t*-test). The inhibition induced by CTB was considerably greater at the higher GABA concentrations (%block: 1 μM CTB: +10 μM GABA, -2.2±3.1%; +100 μM GABA, 40.3±3.3% [n=4]; 10 μM CTB: 16.8±18.1%, 76.0±6.9% [5]; 50 μM CTB: 83.2±2.7%, 90.6±2.7% [4]). The concentration-inhibition relationship for CTB on the 20 μM GABA response produced an IC_{50} of 6.73±0.57 μM (n=3-14). Over 95% inhibition was achieved at 100 μM CTB. The current-voltage (I-V) relationship was analyzed for 20 μM GABA in the absence and presence of 10 μM CTB. Voltage ramps of 600 ms were applied to oocytes from -100 and +50 mV. Whilst the reversal potential (E_{rev}) remained unchanged by CTB, this agent induced significant inward rectification at positive membrane potentials ($P<0.05$; -CTB: E_{rev}=-28.8±0.7 mV [n=4]; +CTB: -28.8±1.0 mV, %block[-60 mV]=45.4±2.9%, [+40 mV]=60.7±3.1%), consistent with a voltage-sensitive block.

These data are all in accordance with CTB being a potent, agonist dependent blocker of the human recombinant $GABA_A$ α1β1γ2s channel. It is conceivable that glycine residues located near the cytoplasmic end of TM2, critical for CTB sensitivity in the glycine α1 receptors (Rundström *et al.*, 1994), are also involved in CTB binding in $GABA_A$ receptors: these residues are conserved in the 3 $GABA_A$ subunits expressed in this study. Such accessibility by CTB to residues presumably located deep in TM2 suggests that the $GABA_A$ receptor-ion channel has a relatively wide external vestibule.

This work was supported by the Medical Research Council and Merck, Sharp & Dohme.

Davies, P.A., Hanna, M.C., Hales, T.G. & Kirkness, E.F. (1997) *Nature* **385**, 820-823.
Rundström, N., Schmieden,V., Betz, H., Bormann, J. & Langosch, D. (1994) *Proc. Natl. Acad. Sci.* **91**, 8950-8954.
Sieghart, W. (1995) *Pharmacol. Rev.* **47**, 181-234.
Twyman, R.E., Green, R.M. & MacDonald, R.L. (1992) *J. Physiol.* **445**, 97-127.

22P PHARMACOKINETIC-PHARMACODYNAMIC MODELLING OF THE EEG EFFECT OF THE GABA UPTAKE INHIBITORS R- AND S-TIAGABINE IN RATS

A. Cleton, P.M. Edelbroek, R.A. Voskuyl, M. Danhof
Division of Pharmacology, Leiden Amsterdam Center for Drug Research, Leiden University, P.O. Box 9503, 2300 RA Leiden, The Netherlands

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. The synaptic action of GABA is terminated by rapid uptake into the presynaptic terminals and surrounding glial cells. Pharmacological inhibition of transport provides a mechanisms for increasing GABA-ergic transmission, which may be useful in the treatment of various neurologic disorders. Recently, a number of lipophilic GABA transporters have been designed and synthesized, which are capable of crossing the blood-brain-barrier.

The purpose of our investigations was to develop an animal model for the quantification of the concentration-pharmacological effect relationship of R- and S-1-*N*-(4,4-di-(3-methyltien-2-yl)but-3-enyl) nipecotic acid (tiagabine) in individual rats using quantitative EEG parameters as pharmacodynamic endpoint.

Chronically instrumentated male Wistar rats, weighing 225-275 gram, were randomly assigned to three groups which received 3, 10 and 30 $mg.kg^{-1}$ of R-tiagabine intravenously over 10 in. In an additional group, the R- and S-isomer (10 and 20 $mg.kg^{-1}$, respectively) were investigated in a cross-over design. The EEG was continuously recorded and frequent arterial blood samples were taken for determination of the tiagabine concentration by HPLC.

The pharmacokinetics of tiagabine could be described by a two-compartment pharmacokinetic models. No stereoselective differences in the pharmacokinetic of the R- and S-isomers of tiagabine were observed. The values for clearance, volume of distribution at steady-state and terminal half-life were (mean ±s.e.m.) 101 ± 6 $ml.min^{-1}.kg^{-1}$, 1.9 ± 0.1 $l.kg^{-1}$ and 24 ± 1 min for R-tiagabine (n=23) and 103 ± 8 $ml.min^{-1}.kg^{-1}$, 1.5 ± 0.1 $l.kg^{-1}$ and 17 ± 3 min for S-tiagabine (n=9), respectively. Amplitudes in the 11.5 - 30 Hz (ß) frequency band of the EEG power spectrum were used to describe the concentration - EEG effect relationships. A physiologic indirect response model was developed to account for the time delay in maximum response and the occurrence of maximum plasma drug concentrations. On the basis of this pharmacokinetic-pharmacodynamic model the individual plasma concentration-EEG effect relationships of R- and S-tiagabine were successfully quantified.

Nu statistic differences in the pharmacodynamic parameters were observed for the different doses of R-tiagabine. The averaged pharmacodynamic parameters of R-tiagabine were (mean ± s.e.m., n=23) E_0 188 ± 10 μV, E_{max} 39 ± 3 %, EC_{50} 100 ± 22 $ng.ml^{-1}$, Hill factor 1.9 ± 0.3, k_{out} 0.73 ± 0.14 min^{-1} and k_{in} 105 ± 19 $\mu V.min^{-1}$, respectively. For the S-isomer an significantly higher EC_{50} of 192 ± 34 $ng.ml^{-1}$ was observed ($p<0.05$, paired t-test), while the values of the other pharmacodynamic parameters estimates were identical. The *in vivo* potency is in line with the receptor affinity determined on basis of the inhibition of [^{3}H]GABA uptake in a rat forebrain synaptosomal preparation, 131 ± 23 nM and 374 ± 85 nM for R and S-tiagabine ($p<0.05$, paired t-test), respectively.

The results of the present study show that the concentration-EEG effect relationships of tiagabine can be characterized in individual rats using the amplitude changes in the 11,5- - 30 Hz (ß) fre-quency band of the EEG and that it reflects the differences in potency of the R- and S-isomers. The described pharmacokinetic-pharmacodynamic model can be very useful for detailed studies into the pharmacodynamics of GABA uptake inhibitors *in vivo*.

23P NMDA-SENSITIVE BINDING AND mRNA FOR THE NR1 SUBUNIT OF THE NMDA RECEPTOR COMPLEX ARE DIFFERENTIALLY AFFECTED IN STRIATUM AND ASSOCIATIVE CORTEX OF PARKINSON'S DISEASE PATIENTS

P. Meoni, B. H. Bunnemann[1], A.E. Kingsbury[2], D. G. Trist[1] and N.G. Bowery, Dept of Pharmacology, Medical School, University of Birmingham, Birmingham, B15 2TT, [1]GlaxoWellcome Medicines Research Center, Verona, Italy, and [2]Parkinson's Disease Society Brain Bank, London WC1N 1PJ.

Glutamate (GLU) and dopamine (DA) interact at several forebrain sites in a mutual and reciprocal way. Dopaminergic innervation to the telencephalon originates from neurons located in the substantia nigra (SN) and ventral tegmental area (VTA) which project to the striatum (ST), the limbic system and the cerebral cortex. The prefrontal cortex (PF) and ST also receive glutamatergic inputs from different areas of the cortex and mediodorsal thalamic nuclei respectively (Smith et al., 1994). At these sites, DA has been demonstrated to exert an important modulatory effect on the glutamatergic system (Iversen, 1995). Degeneration of pigmented, DA-containing neurons in the SN and VTA is one of the pathological hallmarks of Parkinson's Disease (PD). The aim of the present study was to elucidate changes in the NMDA system in areas innervated by dopaminergic projections of the SN and VTA in PD.

Post-mortem PF and ST from patients affected by PD or with no history of neurological disorders was obtained from the Parkinson's Disease Society Brain Bank. Samples were matched by sex, age, post-mortem delay and tissue pH. 12µm sections were fixed with 4% paraformadehyde in phosphate buffered saline (PBS) pH 7.0 for 15 min and and washed in PBS twice for 10 min. Sections were then hybridised with a 45 mer oligonucleotide directed against the NR1pan subunit labelled with [^{35}S]ATP and diluted in hybridization buffer. Sections were hybridized overnight at 42°, and then washed 5 min at 20°C in standard saline citrate (SSC, 2 times normal concentration), 60 min at 55°C and 60 min at 20°C in SSC. Washed sections were dehydrated, air-dried and apposed to ^{3}H-Hyperfilm (Amersham) for 3-7 days. For receptor autoradiography, sections were pre-washed for 30 min at 4°C and twice for 15 min at 30°C in Tris-acetate buffer, 50 mM. Washed sections were incubated for 20 min at 4°C with [^{3}H]glutamate 100nM (NEN, 49.4 Ci/mmol) in tris acetate buffer 50 mM containing 5µM AMPA, 1µM kainate and 100 µM AISD (inhibitor of anion transport). Non-specific binding was assessed in the presence of 200 µM NMDA. After the incubation, sections were washed for 2 min in ice-cold buffer, air dried and apposed to ^{3}H-Hyperfilm (Amersham) for 5 weeks.

Levels of mRNA encoding for the NMDA NR1pan subunit were detected by in-situ hybridization at both a macro and a microscopical level. In the ST, analysis of autoradiographic films showed no difference in mRNA levels for the NR1pan subunit, while in the same area a 70% decrease (Student'st test, $p<0.05$) in NMDA-sensitive binding could be detected in the PD group. In the PF, no differences were detected at the macroscopical level in both the mRNA levels for the NR1pan subunit and NMDA-sensitive binding, while microscopical analysis of the localization of the *in-situ* signal revealed a decrease in the number of grains per cell in layer IV of the cortex that was significant in small (-43%) and medium sized (-22%) neurons (ANOVA, $p<0.05$).

An overactivation of the glutamatergic system in PD has been proposed to result from the neuronal changes observed in this pathology (Blandini et al., 1996). The present observation of a selective decrease in specific neuronal population of the associative cortex and in NMDA-sensitive GLU binding in the ST suggest that overactivation of the glutamatergic system might result in the down regulation of mRNA for the NR1pan subunit or GLU NMDA-sensitive binding in discrete brain areas.

Blandini, F.. *et al*, (1996) *Funct. Neurol.* 11, 3-15.
Iversen, S.D.., (1995) *Behaviour. Pharmacol.* 6, 478-491.
Smith, Y. et al., (1994) *J.Comp. Neurol.* 344, 1-19.

24P THE EFFECT OF PPI-368 ON ALZHEIMER'S DISEASE-RELATED AMYLOID β-PEPTIDE POLYMERIZATION

N.J. Hayward, M.A. Findeis, K.J. Chin, M.L. Grace, M.R. Huff, M.S. Kelley, J.J. Lee, G.M. Musso, D.E. Shufrin, J.D. Wakefield, P.J. Ward & S.M. Molineaux. PRAECIS Pharmaceuticals Inc., I Hampshire Street, Cambridge, MA 02139, U.S.A.

Polymerization of amyloid β-peptide (Aβ) results in neuronal toxicity *in vitro*, and the formation *in vivo* of Aβ-peptide plaque is associated with the onset and progression of Alzheimer's disease (Lorenzo & Yanker, 1994). Inhibition of the process by which Aβ assembles into extended arrays of antiparallel β-sheet and eventually into cross β-fibril-like structure is an appropriate target for therapeutic intervention to delay or prevent the progression of the disease.

Based upon the high affinity of Aβ for itself, we have rationally designed and screened, via *in vitro* assays, a series of Aβ-derived peptides for their ability to interact with Aβ and thus prevent polymerization. The data from the resulting lead compound, PPI-368 (cholyl-Leu-Val-Phe-Phe-Ala-OH) is described.

In the nucleation-dependent polymerization assay PPI-368 (1.5 - 5 µM) was mixed with 5 µM monomeric Aβ(1-40) in 96 well plates and incubated at room temperature with constant agitation. Under these conditions Aβ rapidly polymerizes to fibrils following a defined lag period. At various time points, formation of β-sheet fibrils was measured by the addition of 10 µM thioflavin-T to three samples and the enhanced fluorescence signal that occurs when thioflavin-T binds to β-sheet containing fibrils relative to monomeric Aβ peptide was quantified. PPI-368 inhibited the formation of fibrils in a dose dependent manner (Table 1).

Neurotoxicity of the Aβ peptide solution at each time point was assessed in a 3,(4,5-dimethylthiazol-2-yl),2,5-diphenyltetrazolium bromide toxicity assay using NT2 human neuronal cell line. Co-ordinate inhibition of the formation of neurotoxic polymers and fibrils was observed. Electron microscopy confirmed that fibril formation did not occur in the presence of PPI-368. The ability of PPI-368 to inhibit the polymerization of monomeric Aβ suggests that it bound directly to monomers or soluble oligomers. Radiolabeled PPI-368 was incorporated into fibrils during polymerization, demonstrating that the inhibitor can also bind to Aβ fibrils.

Table 1. PPI-368 inhibits polymerization of monomeric Aβ(1-40) (5 µM) in a dose dependent manner (n=3/dose).

[PPI-368] (µM)	Time to 50% max. fibril formation (min)	Fold increase in Lag time
0	60	1
0.5	90	1.5
1.5	110	1.8
5.0	210	3.5

In the polymerization extension assay PPI-368 was mixed with 100 µM monomeric Aβ(1-40) and 5 µM Aβ(1-40) as pre-polymerized fibrils and incubated at 37°C without agitation. The growth rate of fibril formation measured by thioflavin-T is linear over 5 h. PPI-368 was shown to inhibit the growth rate of fibrils by 50% at a molar ratio of 1:3, PPI-368:Aβ (n=3).

PPI-368, a low molecular weight peptido-organic compound, is a potent, selective inhibitor of Aβ polymerization that coordinately blocks the formation of all neurotoxic species of Aβ oligomers and fibril growth.

Lorenzo, A. & Yanker, B.A. (1994). *Proc. Natl. Acad. Sci. U.S.A.*, 91, 12243-12248.

25P IDENTIFICATION OF A NOVEL CLASS OF INHIBITOR OF β-AMYLOID PEPTIDE AGGREGATION

D.R. Howlett, R.E. Markwell & S.J.Wood[1], SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Harlow, Essex CM19 5AW and [1]709 Swedeland Road, King of Prussia PA 19406, USA.

The beta-amyloid peptide has been shown to be toxic to neuronal cells in culture, but only after an ageing process of several days, corresponding to the kinetics of aggregation of the peptide (Howlett *et al.*, 1995). Preventing the aggregation of beta-amyloid can, therefore, be considered to be a viable therapeutic approach for preventing the progression of beta-amyloid dependent neurodegeneration in Alzheimer's disease. This communication reports several structurally related inhibitors of beta-amyloid aggregate formation and describes the biological effect of this inhibition.

Compounds were obtained from the SmithKline Beecham compound collection and were identified by a Congo red binding assay, as previously described (Wood *et al.*, 1996). Beta-amyloid 1-40 peptide (Bachem, UK) was dissolved in 0.1% acetic acid at 460 μM and diluted to 11 μM in phosphate buffered saline pH 7.4. Peptide was aggregated by incubating overnight at 37°C in the presence or absence of competing compounds. In an immunoassay, using a monoclonal antibody raised to the 1-16 sequence of beta-amyloid, in a conformation which selectively recognises aggregated beta-amyloid, it was demonstrated that SKF-74652 ([5-chloro-2-(4-methoxy phenyl)-3-benzofuranyl][4-[3-(diethylamino)-propoxy]phenyl]methanone hydrochloride inhibited aggregation of beta-amyloid 1-40 (11μM) peptide with an IC_{50} of 28+/-8μM (mean +/- s.d.; n=6). The immunoassay also allowed the identification of a number of structurally related active compounds, such as SKF-64346 ([2-[4-[3-(diethylamino) propoxy]phenyl]-3-benzofuranyl](4-methyl phenyl) methanone hydrochloride) with an IC_{50} of 56 +/- 12μM (n=4). A related compound, SKF-73033 ([2-[4-[2-(diethylamino) ethoxy]phenyl]-3-benzofuranyl](3,5-diethylphenyl)methanone hydrochloride, was totally inactive at concentrations up to 1mM indicating a tight structure-activity requirement for inhibition of aggregation within the class of compound.

The relationship between inhibition of aggregation by the benzofurans and the prevention of formation of a toxic aggregate was investigated by exposing IMR-32 human neuroblastoma cells (ECACC, Porton Down, UK) to peptide, which had been incubated (at 11uM) over 24h at 37°C in the presence or absence of competing compound. Aggregated peptide produced a concentration-related (200pM to 1μM) decrease in cell viability, as assessed by measuring the reduction of MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) to formazan product (Howlett *et al.*, 1995), with an EC_{50} of 5+/-1 nM (n=3)(40% max decrease). Peptide which had been incubated in the presence of SKF-74652 (100μM) showed a diminished capacity for MTT reduction (EC_{50} = 480+/-40 nM; n=3). Thus, SKF-74652 appears to inhibit the formation of a toxic aggregate of beta amyloid. Conversely SKF-73033 was ineffective (at 1mM) at preventing the formation of the toxic aggregate.

Compounds such as SKF-74652, which inhibit beta-amyloid aggregate formation and thus reduce the formation of a neurotoxic species of the peptide, may prove effective in preventing the deposition of beta-amyloid and subsequent neurodegeneration observed in Alzheimer's disease.

[1]Present address: AMGEN, Thousand Oaks, CA 91320-1789, USA

Howlett, D.R., *et al.* (1995) *Neurodegeneration* 4, 23-32.
Wood, S.J., *et al.* (1996) *J. Biol. Chem.* 271(8), 4086-4092.

26P SUBCLONING, EXPRESSION AND PURIFICATION OF MURINE Syk SH2 DOMAIN PROTEINS

R. Ruijtenbeek, C.M. Gibbs, F.A.M. Redegeld, M. Flück[1], R.M.J. Liskamp[2] and F.P. Nijkamp, Utrecht Institute for Pharmaceutical Sciences, Department of Pharmacology and Pathophysiology, [2]Department of Medicinal Chemistry, Utrecht University, PO Box 80.082, 3508TB Utrecht, The Netherlands, and [1]Laboratory for Clinical and Experimental Research, Department of Clinical Research, University of Bern, Tiefenaustrasse 120, CH-3004 Berne, Switzerland.

Activation of the high affinity receptor for immunoglobulin E (IgE), FcεRI, which is expressed on the surface of mast cells and basophils, plays a central role in the initiation of an allergic response. One of the early events in the signal transduction pathway is recruitment and activation of Syk. This protein tyrosine kinase binds via its SH2 domains to the phosphorylated gamma chain of the FcεRI receptor. Binding and subsequent activation of Syk appears to be critical for activation of downstream events and ultimately, mast cell degranulation (Benhamou, 1997).

We want to investigate the possibilities of inhibiting mast cell activation by using phosphopeptides and peptidomimetics that bind to one or both SH2 domains of Syk, thereby preventing Syk to activate downstream enzymes. To determine the binding properties of (modified) phosphopeptides to Syk SH2 domains dual and single SH2 domains of murine Syk were expressed as biotinylated proteins and glutathione S-transferase (GST) fusion proteins.

Murine $p72^{syk}$ cDNA was initially cloned by reverse transcriptase-PCR from a spleen cDNA library (Flück *et al.*, 1995). Plasmid DNA from this clone was used as a template in PCR reactions to obtain the single N-terminal, C-terminal and tandem SH2 domain PCR fragments (encoding amino acid residues 1-125, 153-259 and 1-259, respectively). The PCR fragments were subcloned into both the PinPoint Xa (biotinylation) vector and the pGex-3X (GST) vector. The proteins were produced in BL21(DE3)pLys cells by IPTG (isopropyl-β-D-thiogalacto-pyranoside) induction. In case of the biotinylated proteins this was done in the presence of 5 μM biotin. The fusion proteins were purified by affinity chromatography.

Expression from the PinPoint vector resulted in murine Syk SH2 proteins with a 13 kDa peptide leader sequence at the N-terminus which is a substrate for *in vivo* biotinylation by *E.coli* biotin holoenzyme synthase (Chen *et al.*,1996). The proteins could be visualized after electrophoresis by coomassie staining or after blotting on PVDF membrane by horseradish peroxidase coupled to streptavidin or coupled to an anti GST antibody, respectively. The purified N-SH2, C-SH2 and TD-SH2 GST fusion proteins or biotinylated proteins migrated as single bands with apparent molecular masses of 41, 40 and 56 kDa, and 28, 27 and 44 kDa, respectively. The yield of purified proteins was 1-2 mg.liter^{-1} culture.

The tagged SH2 domain proteins from murine Syk will be used in binding assays (ELISA, Surface Plasmon Resonance) in order to screen compounds for their ability to interfere with binding of Syk to the IgE receptor. In this way we try to find potential inhibitors of mast cell degranulation.

Supported by Glaxo Wellcome The Netherlands

Benhamou, M. (1997) in *IgE Receptor (FceRI) Function in Mast Cells and Basophils*, ed. Hamawy, France, pp33-55. Heidelberg: Springer-Verlag
Chen, T., Repetto, B., Chizzonite, R. *et al.* (1996) *J. Biol. Chem.*, 271, pp25308-25315
Flück, M., Zürcher, G., Andres, A.C. *et al.* (1995), *Biochem. Biophys. Res. Comm.*, 213, pp273-281

27P INVOLVEMENT OF P-GLYCOPROTEIN ON THE APICAL OUTWARD TRANSFER OF L-3,4-DIHYDROXYPHENYLALANINE IN PORCINE RENAL LLC-PK$_1$ AND LLC-GA5 Col300 CELLS

P. Soares-da-Silva, M.P. Serrão, M.A. Vieira-Coelho & P. Gomes.
Inst. Pharmacol. & Therap., Fac. Medicine, 4200 Porto, Portugal.

Cyclosporine-A, a P-glycoprotein inhibitor, produces marked changes in the handling of L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of natriuretic dopamine (Soares-da-Silva et al., 1996). The present work was aimed at studying the role of P-glycoprotein on the basal-to-apical uptake and flux of L-DOPA in LLC-PK$_1$ cells and in LLC-GA5 Col300 cells, a renal cell line expressing the human P-glycoprotein in the apical membrane (Saeki et al., 1993). LLC-PK$_1$ cells (ATCC CRL 1392; passages 198-206) and LLC-GA5 Col300 cells were grown at 37° C in a humidified atmosphere (5% CO_2) on polycarbonate filters (Costar, 3413) in Medium 199 supplemented with 10% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin; the medium used for LLC-GA5 Col300 cells (passages 10-14) contained 300 ng ml^{-1} colchicine. 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, LLC-PK$_1$ cells were preincubated (30 min) with Hanks medium with added benserazide (50 μM), pargyline (100 μM) and tolcapone (1 μM) and, thereafter, incubated for 6 min with L-DOPA (0.25 μM) applied from the basal cell border. L-DOPA was assayed by h.p.l.c. with electrochemical detection. P-glycoprotein activity was measured as the rate of accumulation of rhodamine 123 (rh123; 100 μM) in the presence and absence of verapamil or UIC2, a mouse monoclonal antibody that recognises an extracellular epitope of human P-glycoprotein (Mechetner & Roninson, 1992). Results are arithmetic means with s.e.mean, n=4-6. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. LLC-GA5 Col300 cells accumulate less rh123 (2.9±0.1 nmol mg protein^{-1}) than LLC-PK$_1$ cells (8.1±0.6 nmol mg protein^{-1}). In LLC-PK$_1$ cells the effect of 10 and 25 μM verapamil and 3 μg ml^{-1} UIC2 was a 14% increase in rh123 accumulation. In LLC-GA5 Col300 cells, 10 and 25 μM verapamil increased rh123 accumulation by 32% and 74%, respectively; UIC2 did not change rh123 accumulation. The accumulation of L-DOPA in LLC-PK$_1$ cells (97±6 pmol mg protein^{-1}) did not differ from that observed in LLC-GA5 Col300 cells (104±3 pmol mg protein^{-1}). The basal-to-apical flux of L-DOPA in LLC-PK$_1$ cells (9.6±0.8 nmol mg protein^{-1}) was twice that in LLC-GA5 Col300 cells (4.1±0.1 nmol mg protein^{-1}). The effects of verapamil (Ver; 10 and 25 μM) and UIC2 (3 μg ml^{-1}) on the uptake and flux of L-DOPA in both cell lines are shown in the table; values are percent of control (n=6).

	LLC-PK$_1$	LLC-GA5 Col300
	L-DOPA uptake (% of control)	
UIC 2	126.6±3.7*	111.3±4.0 *
Ver 10 μM	278.8±6.5 *	200.5±40.6
Ver 25 μM	188.3±13.0 *	120.8±9.4 *
	L-DOPA flux (% of control)	
UIC 2	92.6±4.9	117.6±2.4 *
Ver 10 μM	150.3±1.1 *	131.3±26.9
Ver 25 μM	146.9±0.4 *	168.7±10.1 *

* significantly different from corresponding controls (P<0.05)

It is suggested that LLC-GA5 Col300 cells are endowed with more P-glycoprotein activity than LLC-PK$_1$ cells, but both cell lines extrude L-DOPA through a verapamil-sensitive mechanism.

Mechetner, E.B. & Roninson, I.B. (1992) Proc. Natl. Acad. Sci. USA, 89, 5824-5828.
Saeki, N. et al. (1993) J. Biol. Chem., 268, 6077-6080.
Soares-da-Silva P, et al. (1996) Br. J. Pharmacol., 117, 123P.
Supported by grant PSAU/P/SAU/123/96.

28P ACTION OF GR79236 ON MESENTERIC AFFERENT DISCHARGE IN THE ANAESTHETISED RAT

A.J. Kirkup, D. Grundy, I.P. Chessell* and P.P.A. Humphrey*, Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN and *Glaxo Institute of Applied Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ.

Adenosine stimulates mesenteric afferent discharge in the anaesthetised rat (Kirkup *et al.*, 1997a, b). Since this excitatory action is insensitive to the A_1-selective antagonist, 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX; Kirkup *et al.*, 1997a), and is not mimicked by agonists selective for A_{2A}- and A_3-receptors (Kirkup *et al.*, 1997b), it suggested that A_{2B} receptors mediate this effect. In an attempt to confirm the apparent lack of involvement of the A_1-receptor, we have, in the present study, examined the actions of the A_1-selective agonist, N-[(1S, *trans*)-2-hydroxycyclopentyl]adenosine (GR79236; Gurden *et al.*, 1993), on mesenteric afferent discharge. In addition, the effects of either DPCPX or the A_{2A}-selective antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5ylamino] ethyl) phenol (ZM241385; Poucher *et al.*, 1995), on responses to GR79236, were determined.

Experiments were performed with sodium pentobarbitone-anaesthetised (60mg.kg^{-1}, i.p.) male Wistar rats (330-450g). A jugular vein and carotid artery were cannulated for systemic delivery of drugs and for continuous monitoring of haemodynamic parameters, respectively. Extracellular recordings were made from nerve bundles supplying a segment of mid-jejunum which was cannulated to facilitate the measurement of intraluminal pressure. GR79236 or the A_{2A}-selective agonist, 2-[*p*-(carboxyethyl) phenylethylamine]-5-N-ethyl-carboxamido-adenosine (CGS21680), were each administered cumulatively 15min after animals had been treated with either vehicle (5% *v/v* dimethylsulphoxide and 5% *v/v* 1M NaOH in saline, i.v.) or antagonist. Unless otherwise stated, data are shown as the mean±s.e.mean from 4-7 animals and tested by ANOVA, followed by Dunnett's analysis, for statistical significance.

GR79236 (0.3-1000μg.kg^{-1}, i.v.) induced a dose-dependent increase in afferent discharge (-$\log_{10}$ ED_{50} g.kg^{-1} = 4.42±0.14), an increase in intrajejunal pressure (-$\log_{10}$ ED_{50} g.kg^{-1} = 4.80±0.18), bradycardia (-$\log_{10}$ ED_{50} g.kg^{-1} = 5.00±0.08) and hypotension (-$\log_{10}$ ED_{50} g.kg^{-1} = 5.03±0.08). The effects of GR79236 (3-3000μg.kg^{-1}, i.v.) on afferent discharge, intrajejunal pressure, heart rate and blood pressure were antagonised by DPCPX (3mg.kg^{-1}, i.v.; Table 1). ZM241385 (3mg.kg^{-1}, i.v.) did not modify the effects of GR79236 (0.3-1000μg.kg^{-1}, i.v.; Table 1). Nevertheless, ZM241385 (3mg.kg^{-1}, i.v.) antagonised the hypotensive action of CGS21680 (0.3-3000μg.kg^{-1}, i.v.) with a geometric mean dose ratio (95% confidence limits) of 120 (35, 411).

Table 1. Geometric mean dose ratios (95% confidence limits) for the effect of DPCPX and ZM241385 on the actions of GR79236 on mean arterial pressure (M.A.P.), heart rate (H.R.), intraluminal pressure (I.P.) and afferent discharge (A.D.).

	M.A.P.	H.R.	I.P.	A.D.
DPCPX	16 (11, 26)*	88 (56,139)	12 (6, 25)*	6 (3, 9)*
ZM241385	2 (0.8, 5.1)	1.4 (1.0, 1.9)	1.4 (0.3, 5.7)	1.2 (0.6, 2)

*P<0.05 *versus* H.R.

In conclusion, these data suggest that both A_1 and A_{2B} receptors are functional in this preparation and that activation of either can lead to the excitation of mesenteric afferents.

A. J. Kirkup is a GlaxoWellcome Research Fellow.

Gurden, M.F. *et al.* (1993). *Br. J. Pharmacol.*, **109**, 693-698.
Kirkup, A.J., Grundy, D. *et al.* (1997a). *Br. J. Pharmacol.*, **120**, 18P.
Kirkup, A.J. *et al.* (1997b). *Br. J. Pharmacol.*, **122**, 24P
Poucher, S.M. *et al.* (1995). *Br. J. Pharmacol.*, **115**, 1096-1102.

29P INDUCTION OF A C-FOS PROMOTER-REGULATED LUCIFERASE REPORTER GENE BY N^6-CYCLOPENTYL-ADENOSINE IN CHO-K1 CELLS TRANSFECTED WITH THE HUMAN ADENOSINE A_1-RECEPTOR

A. C. Megson, P. E. Shaw & S.J. Hill, Institute of Cell Signalling & School of Biomedical Sciences, University of Nottingham Medical School, Nottingham, NG7 2UH.

Reporter genes can be transfected into cells to provide a simple method of monitoring changes in gene expression (Cooper et al., 1996; Yalkinoglu et al., 1995). In this study we have transfected a reporter gene encoding firefly luciferase (pGL3 Basic, Promega), under the control of the full human c-fos promoter (-711 to +1 bases; Shaw et al., 1989), into CHO-K1 cells (containing the cDNA for the human A_1-adenosine receptor). Here we have investigated the effect of stimulation of transfected human adenosine A_1-receptors and endogenously expressed G-protein coupled receptors on c-fos-promoter regulated gene expression in these cells.

CHO-K1 cells expressing the human A_1-adenosine receptor (Specific [^{3}H]-1-3-dipropyl-8-cyclopentylxanthine, DPCPX, binding; B_{MAX} 277 ± 68 fmol/mg protein, K_D 3.5 ± 0.7 nM; n=6) were secondarily transfected with the c-fos-pGL3 reporter vector and a zeocin selectable vector pZeoSV (Invitrogen), and cells selected with zeocin (200μg/ml). Cells were incubated for 24h in serum-free DMEM/F12 media prior to assay and then stimulated with agonist for 6h. Luciferase was then measured using the Promega luciferase assay system according to the manufacturer's instructions.

The A_1-receptor-selective agonist N^6-cyclopentyladenosine (CPA; 10^{-5}M) produced a maximal stimulation of luciferase expression of 2.14 ± 0.08 fold over basal levels (n=17). Fetal calf serum (FCS; 10%; n=23), thrombin (1 U/ml; n=8), ATP (0.1mM; n=5), forskolin (FK; 3μM;n=5), cholecystokinin (CCK-8, 1μM; n=4) and phorbol 12,13-dibutyrate (PDBu; 1μM;n=7) produced stimulations (fold over basal responses obtained in the absence of FCS) of 5.86 ± 0.24, 4.72 ± 0.50, 1.14 ± 0.06, 2.52 ± 0.51, 1.27 ± 0.09 and 5.84 ± 0.60 respectively. The response to CPA was concentration-dependent (EC_{50} 20.5 ± 2.9 nM; n=9) and antagonised by 100nM DPCPX (apparent K_D 2.5 ± 1.6 nM; n=3). The response to CPA was completely attenuated by 24h pre- incubation with pertussis toxin (PTX;100ng/ml; n=3) , or prior treatment (added 30 min prior to agonist) with the MAP kinase kinase (MEK) inhibitor PD98059 (50μM; n=2; Alessi et al., 1995). In contrast responses to FCS (10%) were only partially inhibited by PTX (by 38.7 ± 4.4%; n=3) and PD98059 (46%, 36%; n=2). Significant ($p < 0.01$; 2-way ANOVA) induction of the c-fos-regulated luciferase reporter was detectable at 1h in response to FCS (10%) and thrombin (1U/ml) and at 2h in response to CPA (10μM) (n=3).

These studies confirm that adenosine A_1-receptor stimulation can stimulate gene transcription via activation of PTX-sensitive $G_{i/o}$-proteins. Furthermore, the data obtained in the present study with G-protein-coupled receptors, FCS, FK and PDBu indicate that co-expression of recombinant cells with a reporter protein, regulated by the human c-fos promoter, provides a simple and reliable method for screening functional responses mediated by a number of different intracellular signalling pathways.

We wish to thank the MRC for financial support

Alessi,D.R. et al., (1995) J. Biol. Chem. **270**,.27489-27494.
Cooper, J. et al., (1996) Br. J. Pharmacol. **120**, 291P.
Shaw, P.E. et al (1989) Cell, **56**, 563-572.
Yalkincglu, A.O. et al., (1995) J. Receptor & Signal Trans Res. **15**, 117-130.

30P REGULATION OF BRAIN ENDOTHELIAL CELL CYCLIC AMP BY ADENOSINE AND ATP MAY INDICATE THE PRESENCE OF A P3 RECEPTOR

M.R. Boarder, S. Gubby, Z., Anwar , J.A. Albert, J.A. Roberts, & J.P.Boyle, Dept of Cell Physiology and Pharmacology, University of Leicester, P.O. Box 138, Leicester LE1 9HN, UK.

The control of cyclic AMP has long been recognised as important for the regulation of the blood brain barrier by brain capillary endothelial cells. We have recently reported that ATP, acting at an undefined receptor, stimulated a very large increase in cyclic AMP accumulation in the presence of forskolin (Albert et al, 1997). Here we provide more information relating to the nature of this response, and consider the possibility that ATP and adenosine may be acting at the same receptor, similar to that called P3 by Shinozuka et al (1988).

Primary cultures of brain capillary endothelial cells were used without passage, as described in Albert et al (1997). Cells in 96 well plates were stimulated in the presence of agonists (at 300μM except where indicated) and forskolin (5μM) for 5 min. Antagonists were present during a 10 min preincubation. Levels of cyclic AMP accumulation were measured by a protein binding assay.

The following P2 agonists were tested: ATP, ATPγS, UTP, UDP, 2-methylthio ATP and α,β-methylene ATP. None of these applied alone gave a reproducible increase in cyclic AMP levels. Forskolin at 5μM gave only a modest increase in cylcic AMP (Table 1). None of the P2 agonists decreased cyclic AMP, as suggested by Webb et al (1996). In the presence of forskolin both ATP and ATPγS gave a substantial and similar increase in cyclic AMP accumulation (Table 1). Adenosine also increased cyclic AMP. Responses to adenosine, ATPγS and ATP were attenuated by 100μM 8-sulphophenyl-theophylline (8-SPT); e.g. 300μM ATP + 5μM forskolin, 16.5 ± 0.5; with 100μM 8-SPT, 4.7 ± 0.7 (pmol cyclic AMP/well, n = 4). Concentration response curves showed that ATP, ATPγS and adenosine each gave similar maximal responses and similar EC_{50} (approx. 30μM). When stimulations were for 1 min instead of for 5 min the maximal responses were reduced but the relationship between stimulations with these 3 agonists was unchanged; i.e. they still had very similar maxima and EC_{50} values.

Table 1 Stimulation of cyclic AMP accumulation
Values are pmol cyclic AMP/well, mean ± s.e. mean, n = 4

	Control	*ATP*	*ATPγS*	*UTP*
No forskolin	1.02 ± 0.9	1.14 ± 0.18	1.16 ± 0.11	0.83 ± 0.08
Forskolin	2.85 ± 0.88	20.6 ± 5.1	20.9 ± 4.2	3.34 ± 1.18

The agonist and 8-SPT profile of these responses are not consistent with a described P2 receptor, and the responses of ATP and ATPγS are unlikely to be mainly due to conversion to adenosine. One possible explanation that is worthy of further study is the presence of a P3 receptor.

Albert, J.L., Boyle, J.B., Roberts, J.A. et al (1997) *Br. J. Pharmacol.* In press.
Shinozuka, K., Bjur, R.A. & Westfall D.P. (1988) *N.S. Arch. Pharm.* 338, 221-227.
Webb, T.E., Feolde, E., Vigne, P. et al (1996) *Brit. J. Pharmacol.* 119. 1385-1392

31P A METHOD FOR MEASURING INTRACELLULAR CYCLIC AMP LEVELS WITH IMMUNOASSAY TECHNOLOGY AND NOVEL, CELLULAR LYSIS REAGENTS

J.K. Horton, L.S. Seddon, A.S. Williams and P.M. Baxendale
Amersham International, Forest Farm, Whitchurch, Cardiff, CF4 7YT, UK

Adrenergic receptors are linked to adenyl cyclase via either inhibitory or stimulatory G-proteins. These receptors, when activated by agonist occupancy of their ligand binding site, influence the intracellular production of cAMP. Established methods for preparing cellular extracts for cAMP measurement involve tedious processes, such as acid or solvent extraction, in order to obtain samples in a suitable form for subsequent assay (Horton & Baxendale, 1995; Hancock *et al*, 1995). These techniques involve cell lysis, removal of extracting agent and assay. Here we describe a novel, direct, homogeneous, assay technique eliminating sample preparation methods and the need to separate bound from free radioactivity.

Antibodies to cAMP were raised in New Zealand White rabbits after immunization with antigen in which a 2'-O-succinyl derivative was conjugated to human serum albumin (Horton *et al*, 1992). Adenosine 3'5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodo-tyrosine methyl ester (2000Ci/mmol) and scintillation proximity assay (SPA) beads were prepared as described previously (Horton & Baxendale, 1995). CHO cells were seeded into 96-well cluster plates suitable for both cell culture and scintillation counting. Cells were stimulated with forskolin (1-100μM) for 20 minutes (37°C, 95% humidity, 5% CO_2). The culture supernatant was gently aspirated and the cells lysed with 1% (w/v) dodecyltrimethylammonium bromide. Working standards (4-512pmol/ml, 50μl aliquots) were pipetted into empty wells of the plate used to culture cells. Equal volumes of specific antisera, [^{125}I] cAMP and anti-rabbit polyvinyltoluene SPA beads were thoroughly mixed to prepare a single reagent. 150μl was added to the microtitre wells containing standards and samples. The plates were sealed and incubated at room temperature overnight. The amount of [^{125}I] cAMP bound to the SPA beads was determined by counting in a TopCount scintillation counter.

The cell lysis reagent used in the method described here had no effect on antibody: antigen binding. Forskolin increased levels of intracellular cAMP in a dose-dependent manner. Basal levels of cAMP were 4.88±3.01pmol/10^6 cells in the absence of forskolin. Cyclic AMP levels significantly increased ($p<0.001$) to 141.42±40.83pmol/10^6 cells in the presence of 100μM forskolin. Statistical analysis was carried out by Student's unpaired t-test. These data are expressed as mean±S.D. (n=12). Intracellular levels of cAMP were in accordance with other published data (Hancock *et al*, 1995) where cAMP levels were estimated with more complex procedures. In a separate series of experiments, acid or solvent extraction methods were used to prepare samples. There was a high level of agreement between results obtained with the established methods and the technique described here (Figure 1).

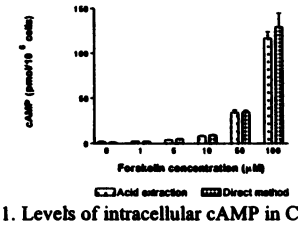

Figure 1. Levels of intracellular cAMP in CHO cells. Cells were exposed to varying concentrations of forskolin (1-100μM) for 20 minutes. Intracellular cAMP was measured following extraction with hydrochloric acid or with the direct assay method. These data are expressed as means ± S.D. (n=5).

The results indicate this one-stage method is well suited for cAMP screening enabling the direct measurement of intracellular cAMP without inconvenient, time-consuming extraction procedures.

Hancock A.A., Vodenlich A.D., Maldonado C., *et al* (1995). *J. Recep. Sig. Transduction Res.*, 15, 557-559.
Horton, J.K., Martin, R.C., Kalinka, S., *et al* (1992). *J. Immunol. Meth.*,155, 31-40.
Horton, J.K. and Baxendale, P.M. (1995). *Methods in Molecular Biology*, 41, 91-105.

32P PHARMACOLOGICAL MODULATION OF CHLORIDE ION EFFLUX FROM T84 CELLS

W. P. Bowen, H. F. McMurray, S. Lall, G. S. Baxter and R. A. Coleman, Pharmagene Laboratories, 2A Orchard Road, Royston, Herts., SG8 5HD, UK.

A microplate chloride channel assay has recently been described, in which the adenylate cyclase activator, forskolin, has been shown to enhance chloride ion efflux from T84 cells (a human colonic epithelial cell line), using the fluorescent marker, N-(6-methoxyquinolyl) acetoethyl ester (MQAE, West & Molloy, 1996). These authors demonstrated that forskolin (0.1-10μM) caused concentration-related increases in rate of chloride ion efflux, and speculated that this effect involved increases in intracellular cAMP. In the present study, we have used this assay to investigate the effect of a range of pharmacological agents on chloride ion efflux in this cell line and determined whether any such effects are in line with their possible effects on intracellular levels of cAMP.

T84 cells (provided by Dr Mike West, Glaxo-Wellcome) were seeded at 2 x 10^4 cells per well in black-walled 96-well plates (Costar), and cultured for 7 days before use. They were loaded overnight with 7 mM MQAE, and then washed with DMEM:F12 medium. Each concentration of every test agonist was added to 6 separate wells. Chloride ion efflux, seen as a change in fluorescence, was measured from time zero for each individual well, and means determined. Agonist-induced efflux was quantified 6 min post drug addition, and corrected for basal efflux. In studies with propranolol, cells were pre-incubated with propranolol in medium for 30 min prior to addition of agonist. Indomethacin (3μM) was included in all buffers to inhibit endogenous production of prostanoids.

In initial studies, the effects of a range of agonists on chloride ion efflux were compared. Forskolin (0.3-10μM), isoprenaline (0.1-10μM) and PGE_2 (0.1-10μM) all caused concentration-related increases in chloride ion efflux of similar magnitude (mean pEC_{50} value ± s.e.mean: 6.39 ± 0.14 (n=5), 6.91 ± 0.06 (n=8) & 7.43 ± 0.07 (n=18), respectively). In each case, the effect was predominantly on the initial rate of efflux. Bradykinin, histamine and substance P (10μM, $n\geq3$) were all ineffective, and carbachol (10μM, n=1) caused a small increase in efflux (23% of isoprenaline maximum). A range of prostanoid agonists, PGD_2, $PGF_{2\alpha}$, the PGI_2 analogue, cicaprost, and the thromboxane A_2 mimetic, U-46619, agonists at DP-, FP-, IP- and TP-receptors (10μM, $n\geq3$), were all at least 100-fold less potent than PGE_2, none causing more than 50% of the maximum stimulation seen with PGE_2. In the presence of propranolol (100nM), the concentration-effect curves to isoprenaline were shifted to the right by 100-fold (mean pEC_{35} value ± s.e.mean: 7.03 ± 0.02 (n=3) and 5.01 ± 0.003 (n=3) in absence and presence of propranolol, respectively), whereas similar curves to PGE_2 were unaffected (mean pEC_{50} value ± s.e.mean: 7.19 ± 0.15 (n=3) and 7.25 ± 0.14 (n=3) in absence and presence of propranolol, respectively).

Forskolin, isoprenaline and PGE_2, agents well known to increase levels of intracellular cAMP in many other types of cell, increase chloride ion efflux from T84 cells through actions at adenylate cyclase, ß-adrenoceptors and prostanoid EP-receptors respectively. In contrast, bradykinin, histamine, substance P and carbachol, agonists not usually associated with elevations of intracellular cAMP, were weak or inactive. It remains to be determined whether agonist-induced increases in chloride ion efflux from T-84 cells are causally related to elevations of intracellular cAMP.

West, M. R. & Molloy C. R. (1996). Anal. Biochem, 241, 51-58.

33P G-PROTEIN ACTIVATION THROUGH D_1 RECEPTORS IN A HAMSTER KIDNEY CELL LINE

Noreen O' Connor and Kathy M. O'Boyle, Department of Pharmacology, University College Dublin, Belfield, Dublin 4., Ireland.

$D_{1\text{-Like}}$ Dopamine receptors (D_1, D_5) are G-protein coupled receptors known to stimulate adenylyl cyclase via activation of G_s G-protein. The present study investigated the direct activation of G_s in D_1/BHK cells by dopamine, the D_1-selective full and partial agonists, A68930 (Kebabian et al., 1992) and SKF38393 (Arnt et al., 1992) using a ^{35}S-GTPγS binding assay. In addition, the agonist/antagonist properties of the non-adenylyl cyclase stimulating D_1 agonist SKF83959 (Arnt et al.,1992) was investigated.

Membranes were prepared from D_1/BHK cells grown in culture. D_1 receptor characteristics were studied in radioligand binding experiments using ^{3}H-SCH23390 and 10μM flupenthixol to determine non-specific binding. G-protein activation was investigated by measuring the binding of ^{35}S-GTPγS (Lawlor et al., 1995). Changes in cAMP levels were measured using a cAMP protein binding method.

The binding of ^{3}H-SCH23390 to membranes prepared from D_1/BHK cells was saturable and to a single site with an apparent K_D of 0.90 ± 0.14 nM and B_{max} of 523 ± 27 fmol/mg protein (n=3). In intact cells, dopamine (0.1nM - 10μM) increased production of cAMP from a basal level of 156 ± 94 to 1117 ± 130 pmol/mg protein (n=3, p<0.0005, t-test).

Dopamine (10nM - 10μM) caused a dose-dependent increase in the binding of ^{35}S-GTPγS in D_1/BHK membranes. 10μM dopamine produced a maximum effect of 21.3 ± 2.2% (n=5) increase over basal binding levels. The D_1-selective agonists A68930 and SKF38393 also produced dose-dependent increases in ^{35}S-GTPγS binding. A maximum increase in binding over basal levels of 29.0 ± 3.4% (n=5) was produced by 10μM A68930. SKF38393 produced only low levels of G-protein activation. 10μM SKF38393 resulted in 7.6 ± 1.7% (n=4) increase in ^{35}S-GTPγS binding over basal. Responses to all agonists were reduced by 1μM SCH23390 but unaffected by 1μM eticlopride. 10μM SKF83959, which is reported to be a non-adenylyl cyclase stimulating D_1 agonist (Arnt et al., 1992) produced a 17.2 ± 1.7 % (n=3) increase over basal ^{35}S-GTPγS binding which was not reduced by either 1μM SCH23390 or eticlopride. However 1μM SKF83959 antagonised the increase in ^{35}S-GTPγS binding mediated by 10μM dopamine (27.8 ± 2.5 to 4.4 ± 2.5 %, n=3, p<0.005, t-test).

These results demonstrate that the ^{35}S-GTPγS assay is suitable for measuring activation of G_s G-protein and is sensitive to agonists with different intrinsic activities. The nature of the response to SKF83959 requires further investigation. However, these data indicate that SKF83959 may stimulate G-protein activation. The possibility of a $D_{1\text{-like}}$ site which is not linked to adenylyl cyclase has been suggested (Arnt et al, 1992).

This work was supported by Health Research Board of Ireland, University College Dublin and Forbairt.

Arnt, J., Hyttel, J., Sanchez, C. (1992) *Eur.J.Pharmacol.*, 213, 259-267.
Lawlor, M., O'Boyle, K.M. (1995) *Br.J.Pharmacol.*, 115:36P.
Kebabian, J.W., DeNinno, M.P., Schoenleber, R. (1992) *Neurochem.Int.* 20:157S-160S

34P THE EFFECTS OF CARBACHOL AND PDGF-BB ON DNA SYNTHESIS AND THE PROLIFERATION OF HUMAN CULTURED AIRWAY SMOOTH MUSCLE CELLS

Charlotte K Billington & Ian P Hall, introduced by P.C Rubin. Department of Therapeutics, University Hospital of Nottingham, Nottingham NG7 2UH

Cultured human airway smooth muscle (HASM) cells express muscarinic M_2 receptors (Widdop et al 1993) and have been shown to proliferate in response to a range of growth factors such as platelet derived growth factor (PDGF-BB). We have investigated the effect of stimulation with carbachol on DNA synthesis and cell number in these cells.

HASM cells were grown as previously described (Widdop et al 1993). DNA synthesis was measured by [^{3}H]-thymidine incorporation in 24 well plates in the presence of 0.1%FCS (Danielpour et al 1989). Cell number was assessed by coulter counting after 48h stimulation with agonists in the presence of 1%FCS.

PDGF-BB (20ng/ml) alone induced a 7.6±0.4 fold increase (cf unstimulated) in [^{3}H]-thymidine incorporation in HASM cells (n=4). Carbachol (1μM) alone did not significantly increase DNA synthesis, but did increase the response seen with PDGF to 12.1±1.4 fold (59.5% increase, p<0.05 cf PDGF-BB alone, n=4). The EC_{50} for this response to carbachol was 5.6 ±1.0 nM (n=4). The response to carbachol, but not PDGF, was reversed by prior incubation with pertussis toxin for 10min (50ng/ml, n=4).

PDGF-BB (20ng/ml) for 48h also induced a 38.7±20.0 % increase (n=6, p<0.05) in HASM cell number above the initial count (20350±2915 HASM cells per well). Under the conditions of these experiments (1%FCS, 48h incubation) carbachol did not increase the response to PDGF-BB but did produce a significant increase in cell number when incubated with cells in the absence of PDGF-BB (% increase = 14.6±0.2, n=6, p<0.05). In the absence of either agonist cell number did not change significantly. The increase in cell number seen with carbachol was completely abrogated by prior incubation with 50ng/ml pertussis toxin (n=6, p<0.05).

These results demonstrate that under defined conditions carbachol can increase HASM cell number and also increase PDGF-BB driven DNA synthesis. These responses appear to be mediated through a Gi dependent pathway and probably are mediated through stimulation of the M_2 receptor present on these cells.

IPH is a National Asthma Campaign Senior Research Fellow.

Widdop S., Daykin K., Hall IP.(1993) Am J. Resp Cell and Mol Biol.9;5;541-546

Danielpour D, Dart LL, Flanders KC, et al, J. Cell. Physiol.(1989) 139; 79-86

35P *IN VITRO* BINDING CHARACTERISTICS AND DISTRIBUTION OF THE GROUP II METABOTROPIC RECEPTOR AGONIST [^{3}H]LY354740 IN RAT BRAIN

J.G. Richards, H. Schaffhauser, J. Messer, Z. Bleuel, S. Chaboz, A. Klingelschmidt, J. Wichmann, J.A. Kemp & V. Mutel, Pharma Division, Preclinical CNS Research, F.Hoffmann-La Roche Ltd, CH-4070 Basel, Switzerland.

Metabotropic glutamate (mGlu) receptors are a family of G-protein coupled receptors which have been implied in diverse physiological functions, from synaptic plasticity to excitotoxicity, and are thus potential targets for the therapy of neurodegenerative diseases (Conn and Pin, 1997). mGlu receptors are classified into three groups, according to sequence homologies, signal transduction and agonist rank order of potency. For group II receptors (mGlu 2 and 3, which are negatively coupled to cAMP production), LY354740 ((+)-2-aminobicyclo-[3.1.0]hexane-2,6-dicarboxylate) is reported to be a selective agonist. We now describe the *in vitro* binding properties of [^{3}H]LY354740, the first subtype-selective mGlu receptor radioligand, in rat brain (Schaffhauser et al., in press) and compare these with the reported distribution of mGlu2/3 mRNA and proteins, as well as with agonist-induced stimulation of GTPγ[^{35}S] binding.

[^{3}H]LY354740 (s.a. $1.554.10^{12}$ Bq/mmol) was synthesized at the Roche Isotope Laboratory. Brain membranes, from male Füllinsdorf albino rats (120-180g), were prepared by homogenization of cerebral cortex in cold buffer (50mM Tris-HCl pH 7.1), centrifugation at 48'000g for 10min, then washed twice, incubated at 37°C for 10min and finally frozen at -80°C. After thawing, the homogenate was washed three times in cold binding buffer (50mM Tris-HCl +2mM $MgCl_2$, pH 7.4). For competition experiments, using 3nM [^{3}H]LY354740, the incubation time was 1hour at 22°C and the homogenate was filtered onto Whatman GF/B filters followed by 5 washes with cold binding buffer; nonspecific binding was defined in the presence of 10μM DCG-IV. For quantitative receptor radioautography, cryostat sections of fresh-frozen rat brain were pre-washed (2x 10min) in buffer + EDTA then incubated for 1hour at 22°C in 50nM [^{3}H]LY354740 in buffer (+ 2mM $MgCl_2$ and $CaCl_2$), rinsed in buffer (2x 30sec + 1min), dried and exposed to tritium-sensitive Ultrofilm (Amersham) for 2wks. then subjected to image analysis (MCID M2, Imaging Res., Ontario). LY354740-stimulation of GTPγ[^{35}S] binding was studied as previously described (Sim et al., 1995).

Specific binding to homogenates (determined in the presence of 10μM DCG-IV) was ~80%. The radioligand bound with a high affinity and capacity (K_D= 7-10 nM; B_{max}= 250-700 fmoles/mg prot). In competition binding experiments, the rank order of affinities (IC_{50}, μM) of various ligands was: LY354740 (0.009), LY341495 (0.01), DCG-IV (0.06), LCCG-I (0.07), glutamate (1.1), (1S,3R)-ACPD (2), MCCG (12), quisqualate (40), ibotenate (65), L-AP3 = MCPG (120), L-AP4 (350). In parasagittal brain sections, we observed a high density of specific binding to the accessory olfactory bulb, cortex (layers 1-3 > 4-6), striatum, molecular layers of the hippocampal formation, subiculum, presubiculum, retrosplenial cortex, anterodorsal thalamic nucleus and cerebellar granular layer. LY354740 (1μM) stimulated GTPγ[^{35}S] binding by 40-60% of basal in several of these brain regions. We conclude that, the pharmacology and tissue distribution of binding sites shows that [^{3}H]LY354740 interacts with group II receptors (preferentially, but not exclusively, with mGlu2) in rat brain.

Conn, P.J. and Pin, J.-P. (1997) *Ann Rev Pharm Tox* 37: 205-237.

Sim, L.J. et al., (1995) *Proc Natl Acad Sci USA* 92: 7242-7246.

Schaffhauser, H. et al., *Mol Pharmacol* (in press)

36P EXAMINATION OF THE MECHANISMS UNDERLYING INHIBITION OF CAPACITATIVE CALCIUM ENTRY BY SODIUM NITROPRUSSIDE IN THE MOUSE ANOCOCCYGEUS

C.P. Wayman, I. McFadzean, A. Gibson & P. Wallace, Pharmacology Group, Division of Biomedical Sciences, King's College London, Manresa Road, London SW3 6LX.

In the mouse anococcygeus, both sodium nitroprusside (SNP) and cyclic GMP inhibit a non-selective cation current activated by depletion of intracellular calcium stores (I_{DOC}; Wayman *et al.*, 1996a). This inhibition of capacitative calcium entry could result from two potential mechanisms; SNP may act directly at the level of the store-operated cation channel or it may act to refill the depleted stores, thus removing the primary stimulus for channel opening. In this study, we have investigated the effects of SNP on I_{ClCa}, a current that reflects the release of stored calcium (Wayman *et al.*, 1996b), and on I_{DOC} in the absence of calcium when store refilling would be prevented.

The methodology for the isolation of, and patch-clamp recording from, anococcygeus single muscle cells is described elsewhere (Wayman *et al.*, 1996b). The physiological salt solution bathing the cells contained *inter alia* $CaCl_2$ (10mM) and nifedipine (1μM). The intracellular solution contained (in mM) CsCl 130, tetraethyl-ammonium chloride 20, HEPES 10 and was supplemented with ATP 0.5 and GTP 0.5 (pH 7.20). For "calcium-free" conditions, extracellular $CaCl_2$ was replaced with EGTA (1mM) and BAPTA (10mM) was included in the intracellular solution.

Caffeine (1-10mM) and carbachol (1-50μM) produced concentration-dependent activation of I_{ClCa} in cells held at a membrane potential of -40mV. Two consecutive applications of caffeine (2mM) or carbachol (10μM), separated by a 3 or 5 minute wash period respectively, produced repeatable I_{ClCa} responses; the amplitudes of the second carbachol- and caffeine-induced responses were 83±17% (n=4; mean±s.e.mean) and 65±7% (n=6) of the appropriate initial application. Addition of SNP (10μM) during the wash and during the subsequent application of caffeine (2mM) or carbachol (10μM) produced significant increases ($P<0.001$; Student's t-test) in both the caffeine- (154±9%, n=9) and the carbachol-induced I_{ClCa} (441±85%, n=4) relative to the respective initial application. Under zero calcium conditions, caffeine (2mM), carbachol (10μM) and cyclopiazonic acid (CPA, 10μM; a sarcoplasmic reticulum [SR] calcium uptake pump inhibitor) failed to activate I_{ClCa} but induced I_{DOC} with amplitudes of 6.2±1.3pA (n=9), 4.3±0.4pA (n=8) and 3.9±0.5pA (n=9), respectively. Concomitant application of SNP (10μM) with caffeine, carbachol or CPA produced significant increases ($P<0.001$) in I_{DOC} (45±9% n=9, 47±11% n=8, 46±10% n=9, increases respectively). I_{DOC} was abolished by further addition of SKF96365 (10μM) or cadmium (100μM) in all cells tested.

These data suggest that SNP can increase the calcium content of the SR, possibly by enhancing sequestration, and that this might underly the drugs ability to inhibit I_{DOC}. However the finding that SNP increases I_{DOC} under "calcium-free" conditions may point to a more complex regulatory mechanism.

The authors thank the Wellcome Trust and MRC for support.

Wayman, C.P., McFadzean, I., Gibson, A. *et al.* (1996a) *Br. J. Pharmacol.* 118, 2001-2008.

Wayman, C.P., McFadzean, I., Gibson, A. *et al.* (1996b) *Br. J. Pharmacol.* 117, 566-572.

37P CONSERVED CYSTEINE RESIDUES ARE CRITICAL FOR THE FUNCTION OF THE INWARDLY RECTIFYING POTASSIUM CHANNEL IRK3

J.P.A. Bannister, B.A. Young, A. Sivaprasadarao & D. Wray, Department of Pharmacology, Worsley Medical and Dental building, University of Leeds, Leeds LS2 9JT

Sequences of members of the inwardly rectifying potassium channel family (Nichols & Lopatin, 1997) indicate the presence of at least three conserved cysteine residues. Since cysteine residues are known to play a role in the assembly of oligomeric proteins by means of disulphide bonds (Lesage *et al*, 1996), we mutated these residues in IRK3 to serines and investigated the consequences of these changes on the functional properties of the channel using two electrode voltage clamp techniques.

Cysteine residues at positions 113, 140 and 145 were mutated to serine using site-directed mutagenesis (Kunkel *et al*, 1987). Each mutant was confirmed by sequencing. The cRNA for wild type (wt) and mutant channels was injected into *Xenopus* oocytes either alone or in combination with the various cysteine mutants in a volume of 50 nl. The wt cRNA concentration was kept constant at 1 ng per oocyte in all combinations of wt and cysteine mutant. Electrophysiological recordings were made in symmetrical potassium solutions (100 mM K^+) using two electrode voltage clamp techniques 18-24 hours post injection.

The C113S and C145S mutants when expressed individually, in uninjected oocytes, gave currents that were not significantly different from control currents. For instance, in steps to -60 mV from a holding potential of 0 mV, uninjected oocytes gave currents of 120 $\pm$ 34 nA while C113S and C145S mutant injected oocytes gave currents of 143 $\pm$ 40 nA and 174 $\pm$ 57 nA respectively ($P>0.05$, n=9 for test and control). C140S mutants when expressed in oocytes, gave currents which were not significantly different from wt channel currents ($P>0.05$). For instance, in steps to -60 mV from a holding potential of 0 mV, mutant channels gave currents of 10.01 $\pm$ 0.98 μA (n = 9) and wt 12.32 $\pm$ 0.78 μA (n = 9).

When co-expressed with wt channels, both the C113S and C145S mutant channels significantly inhibited wt currents. The wt and C113S mixtures of 10:1 and 1:1 wt:mutant gave a % inhibition of wt currents of 31 $\pm$ 7 ($P<0.05$) and 73 $\pm$ 2 ($P<0.01$) respectively. The wt and C145S mixtures of 10:1 and 1:1 wt:mutant gave similar % inhibitions of 39 $\pm$ 13 ($P<0.05$) and 71 $\pm$ 2 ($P<0.01$) respectively (n=9 for each batch of oocytes).

The results show that C113 and C145, but not C140, are critical for channel function. The results suggest that the roles of C113 and C145 might be in assembly of tetrameric channels but does not preclude the possibility that they may be involved in some other critical functional role. Biochemical investigations are currently under way to distinguish between these two possibilities.

Supported by the Welcome Trust.

Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) *Methods in Enzymology* 154, 367-382.
Lesage, F., Reyes, R., Fink, M. *et al.* (1996) *EMBO* 15, 6400-6407
Nichols, C.G. and Lopatin, A.N. (1997) *Annu Rev. Physiol.* 59, 171-191

38P LCB 2183 INHIBITS DELAYED-TYPE HYPERSENSITIVITY-ASSOCIATED LATE PHASE MUCOSAL MAST CELL ACTIVATION IN MICE

P.A.J. Henricks, A.D. Kraneveld, C. van Petersen & F.P. Nijkamp, Dept. Pharmacology & Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O. Box 80.082, 3508 TB Utrecht, The Netherlands.

In recent years, we have developed pulmonary delayed-type hypersensitivity (DTH) models for non-IgE-mediated asthma by skin-sensitizing mice with low molecular weight haptens such as 2,4-dinitrofluorobenzene (DNFB), toluene diisocyanate and picryl chloride. In these models tracheal hyperreactivity and pulmonary inflammation were found 24 to 48 h after the challenge with the same hapten intranasally (Garssen *et al.*, 1991; Buckley & Nijkamp, 1994; Scheerens *et al.*, 1996). Moreover, it was shown that mouse mast cell protease-1 (mMCP-1; a marker for mucosal mast cells) was significantly enhanced in the serum of sensitized mice 24 h after challenge. LCB 2183 (2-(ethoxymethyl)-4(3*H*)-pteridinione; Lipha, Lyon, France) has anti-allergic activities and inhibits tracheal hyperreactivity and pulmonary inflammation induced by picryl chloride in mice (Buckley *et al.*, 1995). The aim of this study was to examine the effects of LCB 2183 on mucosal mast cell activation 24 h after challenge in DNFB-sensitized mice.

Male balb/c mice (6-8 wk) were skin-sensitized on day 0 and 1 with DNFB (0.5%, 100 μl) or vehicle (acetone:olive oil= 4:1) and all animals were challenged on day 5 intranasally with dinitrobenzene sulphonic acid (0.6% in PBS, 50 μl; Buckley & Nijkamp, 1994). Mice were treated with LCB 2183 (25 mg/kg intragastric administration in tap water) or water according to four different protocols (Table 1). mMCP-1 was measured in sera obtained from mice 24 h after challenge using an ELISA kit (Moredun Animal Health LtD).

Table 1. Administration regimens of LCB 2183 (25 mg/kg).

protocol	time points (h) in relation to the challenge						
1	-50,		-26,		-2,	+6,	+22
2			-26,	-18,	-2,	+6,	+22
3	-50,	-42,	-26,	-18,	-2,	+6,	+22
4			-26,		-2,	+6,	+22

Amounts of mMCP-1 were significantly increased in sera from DNFB-sensitized mice 24 h after challenge (16.7 $\pm$ 2.8 ng mMCP-1/ml serum) compared with sera from non-sensitized mice (5.1 $\pm$ 0.7 ng mMCP-1/ml serum; n=5-7, $P<0.05$; ANOVA followed by Bonferroni's test). Treatment of mice with LCB 2183 according to protocol 1, 2 or 4 did not influence the DNFB-induced mucosal mast cell activation. However, treatment with LCB 2183 according to protocol 3 was effective in inhibiting the DNFB-induced increase in mMCP-1 serum levels 24 h after the challenge (non-sensitized + LCB 2183: 6.2 $\pm$ 1.0 and DNFB-sensitized + LCB 2183: 9.1 $\pm$ 1.9 ng mMCP-1/ml serum; n=5-7). These results demonstrate that repeated treatment with LCB 2183 can decrease mucosal mast cell activation.

This study was financially supported by Lipha, Lyon, France.

Buckley, T.L. & Nijkamp, F.P. (1994) *Am. J. Respir. Crit. Care Med.* 149, 400-407.
Buckley, T.L. *et al.* (1995) *Eur. J. Pharmacol.* 282, 103-109.
Garssen, J. *et al.* (1991) *Am. Rev. Respir. Dis.* 144, 931-938.
Scheerens, H. *et al.* (1996) *Am. J. Respir. Crit. Care Med.* 154, 858-865.

39P ISOLATION AND BIOLOGICAL ACTIVITY OF ANTIGEN-SPECIFIC LYMPHOCYTE FACTORS IN A MURINE MODEL FOR TOLUENE DIISOCYANATE-INDUCED OCCUPATIONAL ASTHMA

H. Scheerens, T.L. Buckley, T. Muis, C.E. Vallinga, F.A. Redegeld, #H. Van Loveren, F.P. Nijkamp. Dept. Pharmacology & Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O.Box 80082, 3508 TB Utrecht, The Netherlands. #National Institute of Public Health and the Environment, P.O.Box 1, 3720 BA Bilthoven, The Netherlands.

Toluene diisocyanate (TDI) is a low molecular weight hapten which is a well known cause of occupational asthma. Recently, we developed a murine model to investigate TDI-induced occupational asthma. Skin-sensitization on day 0 and day 1 with 200 µl 1% TDI followed by intranasal challenge with 20 µl 1% TDI on day 8 resulted in a nonspecific *in vitro* tracheal hyperreactivity 24 hr after the challenge (Scheerens *et al.*, 1996). This response was IgE-independent and resembled a delayed-type hypersensitivity (DTH) reaction. DTH reactions are characterized by the production of antigen-specific lymphocyte factors which are responsible for the early phase found in DTH reactions (Van Loveren *et al.*, 1987).

In the present study, the TDI-induced DTH-like reaction was further characterized. A TDI-specific lymphocyte factor was isolated from cultured spleen cells of TDI-sensitized mice (male BALB/c, 6-10 weeks old) after 2 days and purified using a TDI-gammaglobulin-sepharose column. Next, the biological activity of this TDI-specific lymphocyte factor was tested in the skin and airways. Sensitization with TDI-factor (60 µg, i.v.) followed after 30 min. by topical application of 0.1% TDI on the ears induced a significant ear swelling 2 hr after the challenge when compared with PBS-treated mice (PBS 1.16 ± 0.59 x 10^{-2} mm; TDI-factor 4.71 ± 0.69 x 10^{-2} mm, mean ± sem for n=7 mice/group, P<0.01, student's t-test). This response was TDI-specific because topical challenge with 0.5% dinitrofluorobenzene, another low molecular weight hapten, failed to induce an increased ear swelling response in TDI-factor sensitized mice.

With respect to airway responses, mice sensitized with TDI-factor (60 µg, i.v.) exhibited *in vitro* tracheal hyperreactivity to carbachol 2 hr after intranasal challenge with 1% TDI when compared with PBS-treated mice (Emax: PBS 1670 ± 114 mg; TDI-factor 2115 ± 164 mg, mean ± sem for n=8-9 mice/group, P<0.01, student's t-test). At the same time point a significant increase in mouse mast cell protease-1 (MMCP-1; a specific protease found in mucosal mast cells, measured by ELISA) was found in lung tissue of TDI-factor sensitized mice when compared with PBS-treated mice (PBS 2.32 ± 0.41 ng/g lung; TDI-factor 3.89 ± 0.16 ng/g lung, mean ± sem for n=5 mice/group, P<0.05, student's t-test). These results suggested that TDI-specific lymphocyte factors were capable of activating mast cells. Furthermore, 2.5 min. after the challenge an *in vivo* bronchoconstriction was observed in TDI-factor sensitized mice when compared with PBS-treated mice (340% increase in bronchoconstriction compared with baseline).

In conclusion, our results demonstrate that TDI-specific lymphocyte factors are produced after *in vivo* exposure to TDI. These factors are hapten-specific and are biologically active. Moreover, they mimic some of the effects observed after active TDI-sensitization.

Scheerens, H., Buckley, T.L., Garssen, J., *et al.* (1996). Am. J. Resp. Crit. Care Med., 154, 858-865.
Van Loveren, H., Ptak, W. & Askenase, P.W. (1987). Lymphokines, 14, 405-429.

40P PHARMACOLOGICAL PROFILING OF PHOSPHODIESTERASE 4 (PDE4) INHIBITORS AND ANALYSIS OF THE THERAPEUTIC RATIO IN RATS AND DOGS

K.J.Escott, S.J. McMillan, M. Birrell, S.E. Webber, J.E. Souness, C. Maslen, R. Temple, S. Iqbal, M. Mariucci, J. Rose, L.E. Geiger, S. Thurairatnam, S. Bower, D. Aldous and C.A. Sargent. Rhône-Poulenc Rorer Ltd., Dagenham, Essex

PDE4 inhibitors are effective in animal models of asthma and arthritis, but their clinical efficacy is limited by side effects (De Brito *et al.*, 1997). In this study we investigated the pharmacological effects of the PDE4 inhibitors, RP 73401 (Ashton *et al.*, 1994) and RPR 132294 (4-Methoxy-3-tetra-hydrofuran-3-yloxybenzoic acid (3,5-dimethylisoxazol-4-yl) amide). The data was analysed to determine the most appropriate measurements to calculate the separation between efficacy and toxicity (therapeutic ratio).

In vitro inhibition of PDE 4 in guinea pig macrophages was determined for both compounds (Ashton *et al.*, 1994). Plasma protein binding was estimated *in vitro* by measuring lipopolysaccharide (LPS) induced TNF-α release in dog blood. Vehicle or PDE 4 inhibitors were added 1 hour prior to LPS (0.1 µg/ml) and incubated for a further 23 hours. Plasma TNF-α was analysed by ELISA. Ovalbumin (OA)-induced bronchospasm in anaesthetised rats was the primary *in vivo* model. Compounds were administered p.o. 1 hour prior to inducing bronchospasm (Raeburn *et al.*, 1994). As emesis is one of the most potent side effects of PDE 4 inhibitors this was the *in vivo* model of toxicity. Following oral administration, the compounds ability to induce emesis in dogs was assessed for 48 hours. The data was calculated as the non-emetic dose, 0.5 log unit lower than the minimum dose required to induce emesis in 1/4 dogs. The efficacy model in the dog was ex vivo (LPS)-induced TNF-α release in dog blood after oral administration of compound. A dose ten times lower than the non-emetic dose was given, blood samples were taken after 1 hour and TNF-α measured as described by Sekut *et al.*, 1995.

RP 73401 was 100 fold more potent than RPR 132294 in inhibiting PDE4 *in vitro* (table 1). Both compounds produced a similar inhibition of *in vitro* LPS induced TNF-α release (table 1). In ovabumin-induced bronchospasm RPR 132294 was ten fold more potent than RP73401 (table 1). The non emetic dose of RPR 132294 was 3 fold higher than RP 73401. Both RP 73401 and RPR 132294 produced a similar inhibition of *ex vivo* LPS-induced TNF-α release (table 1).

The novel PDE4 inhibitor, RPR132294, displayed activity in both *in vitro* and *in vivo* systems. This data highlights that efficacy and therapeutic ratio varies in different pharmacological systems. The most predictive measurements appear to be in the same species. This study illustrates the importance of comparing efficacy and toxicity in the same animal species.

Ashton M.J., *et al.*, 1994, *J. Med. Chem.*, 37, 1696-1703.
De Brito F.B. *et al.*, 1997, *Emerging Drugs*, 2, 249-268.
Raeburn D. *et al.*, 1994, *Br. J. Pharmacol.*, 113, 1423-1431.
Sekut L. *et al.*, 1995, *Lab. Animal Sci.*, 45, 647 - 651.

Table 1. Pharmacological profile of RP 73401 and RPR 132294

Treatment	Inhibition of PDE4 - IC50 (nM)	Inhibition of *in vitro* TNF-α IC50 (nM)	Inhibition of bronchospasm ED50 ($mgkg^{-1}$)	Non-emetic dose ($mgkg^{-1}$)	Inhibition of *ex vivo* TNF-α (% untreated)	Therapeutic Ratio (emesis/ex vivo TNFα)
Vehicle	-	-	-	-	2.9 ± 2.9	-
RP 73401	0.48 ± 0.1	22.9 ± 2.4	0.99 (0.66, 14.2)	0.1	38.5 ± 14.2	10
RPR 132294	76.0 ± 1.8	71.6 ± 28.3	0.096 (0.01, 0.1)	0.3	43.5 ± 13.2	10

Data expressed as mean ± sem, except ED50 values (geometric mean and 95 % confidence limits) , n = 4 - 6 in each group.

41P INHIBITION OF PEROXYNITRITE FORMATION BY APOCYNIN IN A MURINE MACROPHAGE CELL-LINE

R.B.R. Muijsers, G. Folkerts, E. van den Worm[1], C.J. Beukelman[1], D.S. Postma[2] and F. P. Nijkamp, Department of Pharmacology and Pathophysiology, and [1]Department of Medicinal Chemistry, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, 3508TB Utrecht, The Netherlands, and [2]Department of Pulmonology, University Hospital Groningen, Oostersingel 59, 9700RB Groningen, The Netherlands.

Peroxynitrite ($ONOO^-$), the coupling product of nitric oxide (NO) and superoxide is believed to be responsible for some of the detrimental effects on tissues formerly contributed to nitric oxide (Reviewed by Muijsers *et al.* 1997). Inhibition of peroxynitrite formation could therefore have therapeutical potency in inflammatory conditions. Apocynin (acetovanillon) is a known selective inhibitor of NADPH oxidase and thereby prevents superoxide formation. Here we assessed the *in vitro* ability of apocynin to inhibit peroxynitrite formation.

The murine macrophage cell-line J774.A (ATCC) was used as a model. Cells were incubated (10^6 cells.ml^{-1}) in supplemented RPMI 1640 in the presence of 25 μM 123-Dihydrorhodamine with and without stimuli and inhibitors for 24 h. (5% CO_2, 37°C). Dose response curves of the inhibitors were made non-cumulatively. Oxidation of 123-dihydrorhodamine to 123-rhodamine was quantified using a fluorimetric analyzer (ex. 485 nm, em. 530 nm) as an index for peroxynitrite formation (Kooy *et al.*, 1994). Nitrite accumulation in the medium was determined using the Griess assay, providing an index for nitric oxide release.

Upon stimulation with rmIFNγ (500 U/ml) and LPS (*E. coli* O.111:B4; 10 μg/ml) the 123-rhodamine concentration in the medium increased from 761 ± 12* nM (basal oxidation) to 1727 ± 15 nM (n=3, $P<0.01$, *t*-test). The nitrite concentration increased from 3.5 ± 0.09 μM to 57.7 ± 0.33 μM (n=3, $P<0.01$) in response to stimulation. The nitric oxide synthase inhibitor aminoguanidine dose dependently inhibited stimulated 123-rhodamine and nitrite accumulation with a $logIC_{50}$ of -3.9 ± 0.05 M and -2.9 ± 0.24 M respectively. The superoxide scavenger TEMPO (2,2,6,6-Tetramethylpiperidin) inhibited 123-dihydrorhodamine oxidation ($logIC_{50}$ -3.6 ± 0.14) but did not affect nitrite accumulation. Apocynin also inhibited 123-rhodamine accumulation ($logIC_{50}$ -4.4 ± 0.03), but showed a minor inhibition of nitrite formation at the highest concentration used. Apocynin (300 μM) did not inhibit direct 123-dihydrorhodamine oxidation by the peroxynitrite donor SIN-1 (3-morpholinosydnonimine) and is therefore not a scavenger of peroxynitrite.

It is concluded that J774 macrophages form peroxynitrite upon stimulation with IFNγ and LPS. Furthermore, these data demonstrate that apocynin is a potent inhibitor of peroxynitrite formation in the model used, most likely via inhibition of superoxide release. Apocynin is not a scavenger of peroxynitrite.

*All values ±SEM

Supported by the Dutch Asthma Foundation #95.28

Kooy, N.W., Royall, J.A., Ischiropoulos, H., *et al.* (1994) *Free. Radical. Biol. Med.*, 16, pp. 149-156
Muijsers, R.B.R, Folkerts, G, Henricks, P.A.J. *et al.* (1997) *Life Sciences*, 60, pp. 1833-1845

42P DIFFERENTIAL EFFECTS OF POLY-CATIONIC PEPTIDES ON L-ARGININE UTILIZATION BY NO SYNTHASE AND ARGINASE IN RAT ALVEOLAR MACROPHAGES (AMφ)

R. Hammermann, J. Hirschmann, J.G. Folkerts*, F.P. Nijkamp*, I. Wessler# & K. Racké, Institute of Pharmacology & Toxicology, University of Bonn, Reuterstr. 2b, D-53113 Bonn, Germany; *Fac. Farmacie, Univ. Utrecht, Netherland; #Dept. Pharmacol., Univ. Mainz, Germany.

L-Arginine (L-Arg) serves as substrate of NO synthase (NOS) and arginase, enzymes which play a particular role in AMΦ (Hey *et al.*, 1995). Since the cellular uptake of L-Arg in AMΦ can be inhibited by cationic peptides (Hirschmann *et al.*, 1997), it was tested whether cationic peptides affect L-Arg utilization by NOS and arginase in AMΦ.

Rat AMΦ ($2.5*10^6$ cells per well) were cultured for 20 h in DMEM-F12 medium containing 5 % FCS in the absence or presence of LPS (see Hey *et al.*, 1995). Thereafter, utilization of L-Arg by NOS and arginase was determined by measuring the accumulation of ^{3}H-L-citrulline (^{3}H-L-Cit) and ^{3}H-L-ornithine (^{3}H-L-Orn) in incubation media during 1 h incubation with ^{3}H-L-Arg (37 kBq, 0.1 μM) in the absence or presence of poly-L-arginine (p-L-Arg, 5 000-15 000 MW) or poly-L-lysine (p-L-Lys, 4 000-15 000 MW).

As summarized in Table 1, in naive AMΦ p-L-Arg inhibited both ^{3}H-L-Cit and ^{3}H-L-Orn formation, although effects on ^{3}H-L-Cit were more pronounced. In AMΦ cultured in the presence of LPS (i.e. after marked induction of iNOS) p-Arg only inhibited ^{3}H-L-Cit formation, whereas ^{3}H-L-Orn formation was facilitated. p-L-Lys, although inhibiting L-Arg uptake (Hirschmann et al., 1997) did not inhibit ^{3}H-L-Cit and ^{3}H-L-Orn formation.

The lack of simple relationship between effects on L-Arg uptake and its metabolism by NOS and arginase suggests multiple cellular compartments for L-Arg.

Hey, C., Wessler, I. & Racké, K. (1995) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 351, 651-659.
Hirschmann, J., Hey, C., Hammermann, R. *et al.*, (1997) This meeting.

Table 1. Effects of poly-L-arginine (p-L-Arg) or poly-L-lysine (p-L-Lys) on ^{3}H-L-Arg utilization by NOS and arginase in rat AMΦ.

	^{3}H-L-Cit	^{3}H-L-Orn	^{3}H-L-Cit	^{3}H-L-Orn	^{3}H-L-Cit	^{3}H-L-Orn	^{3}H-L-Cit	^{3}H-L-Orn	
	Controls		30		100		300		μg/ml p-L-Arg
No LPS	90±13	128±6.1	19±5.4**	64±6.0**	4.8±1.4**	46±9.2**	2.2±0.7**	33±6.9**	d.p.m.*1000
LPS 1 μg/ml	445±26	27±1.9	241±9.4**	34±3.8	100±4.7**	62±4.2**	53±2.3*	82±12**	d.p.m.*1000
					100		300		μg/ml p-L-Lys
No LPS					106±11	136±21	112±10	123±17	d.p.m.*1000
LPS 1 μg/ml					594±10	55±2.4*	561±14	52±1.5**	d.p.m.*1000

means±s.e.mean of 4-12 experiments, *P<0.05, **P<0.01 vs respective control values (Dunnett's *t* test).

43P LCB 2183 PROFOUNDLY INHIBITS DELAYED-TYPE HYPERSENSITIVITY-ASSOCIATED EARLY PHASE MUCOSAL MAST CELL ACTIVATION IN MOUSE LUNG

A.D. Kraneveld, P.A.J. Henricks, C. van Petersen & F.P. Nijkamp. Dept. Pharmacology & Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O. Box 80.082, 3508 TB Utrecht, the Netherlands.

Low molecular weight compounds are the most common agents causing occupational asthma, induced by unknown mechanisms, because specific IgE antibodies are not involved. A murine pulmonary delayed-type hypersensitivity (DTH) reaction induced by skin-sensitization with low molecular weight molecules picryl chloride, toluene diisocyanate and dinitrofluorobenzene (DNFB) has been characterized. The features observed in this non-IgE-mediated animal model were pulmonary inflammation and tracheal hyperreactivity 24 to 48 h after challenge (Buckley *et al.*, 1995; Buckley & Nijkamp, 1994; Scheerens *et al.*, 1996). LCB 2183 (2-(ethoxymethyl)-4(3*H*)-pteridinione, LIPHA, Lyon, France) has anti-allergic activities and has been shown to inhibit picryl chloride-induced pulmonary inflammation and tracheal hyperreactivity (Buckley *et al.*, 1995). Recently, mucosal mast cell activation was demonstrated shortly (<1 h) after the challenge (Kraneveld *et al.*, 1997), suggesting an initiating role for mast cells in pulmonary DTH reactions. The aim of this study was to examine the effects of LCB 2183 on mucosal mast cells in normal mice and on DTH-associated early phase mucosal mast cell activation.

Male Balb/c mice (6-8 wk) were treated with LCB 2183 (25 mg/kg intragastrically) or vehicle (tap water): protocols 1 & 2 (Table 1). DTH reaction was induced by skin-sensitization with DNFB (0.5%, 100 µl) or vehicle (acetone:olive oil=4:1) on day 0 & 1. On day 5 mice were challenged intranasally with dinitrobenzene sulphonic acid (0.6% in PBS, 50 µl).The animals were treated with LCB 2183 or vehicle according to protocols 1, 3 & 4 (Table 1). After 30 min, serum samples and lung tissue homogenate samples were obtained for measurement of mouse mucosal mast cell protease-1 (mMCP-1, a marker for mucosal mast cells) using an ELISA kit (Moredun Animal Health , UK).

Table 1. Administration regimens of LCB 2183 (25 mg/kg)

protocol	time (h) in relation to challenge time			
1	single dose			-2
2	4 days twice daily			
3	-50	-26		-2
4		-26	-18	-2

Single or repeated LCB 2183 administration did not influence mMCP-1 serum or lung tissue levels in normal mice.
30 Min after the challenge of DNFB-sensitized mice an increase in serum mMCP-1 was found compared to controls (DTH: 17.3 ± 3.0 & control: 5.4 ± 1.7 ng mMCP-1/ml serum, n=5, $P<0.05$, ANOVA, Bonferonni). Concomitantly, a reduction in lung tissue mMCP-1 content was found in DNFB-sensitized mice (DTH: 0.38 ± 0.04 & control: 0.83 ± 0.10 ng mMCP-1/mg tissue protein, n=6, $P<0.05$, ANOVA, Bonferonni).
Pretreatment of mice with LCB 2183 according to protocol 1 or 4 did not influence the DNFB-induced mucosal mast cell activation. However, pretreatment with LCB 2183 according to protocol 3 profoundly inhibited DTH-induced mucosal mast cell activation (serum: LCB/DTH: 9.7 ± 2.1 & LCB/control: 6.1 ± 1.0 ng mMCP-1/ml serum; lung tissue: LCB/DTH: 0.68 ± 0.12 & LCB/control: 0.65 ± 0.13 ng mMCP-1/mg tissue protein, n=6, ANOVA, Bonferonni).

In conclusion, LCB 2183 does not induce mucosal mast cell activation or changes in number of tissue resident mucosal mast cells in lungs of normal mice. LCB 2183 - given three times over 2 days - prevents the DTH-associated "early phase" mucosal mast cell activation in airways of mice.

This study was financially supported by LIPHA, Lyon, France.
Buckley, T.L. *et al.*(1994) *Am.J.Respir.Crit.Care Med.* 149, 400-407.
Buckley, T.L. *et al.* (1995) *Eur. J. Pharmacol.* 282, 103-109.
Kraneveld A.D., *et al.* (1997) *Immunol. Lett.* 56, 181.
Scheerens H. *et al.* (1996) *Am J.Respir.Crit.Care Med.* 154, 858-865.

44P EFFECT OF TYPE IV PHOSPHODIESTERASE INHIBITORS AGAINST ANTIGEN-INDUCED EOSINOPHILIA IN SENSITISED GUINEA-PIGS

P.Newbold, M.Mather, & A.Young. Department of Pharmacology, Astra Charnwood, Bakewell Road, Loughborough, Leicestershire, LE11 5RH. (Introduced by P Leff)

In this study we have compared a number of PDEIV inhibitors given as a single dose by the oral route prior to antigen challenge. The study aimed to provide, for the first time, a measure of potency for these compounds when given by a standard (oral) route in an antigen -induced eosinophilia model.
Male Dunkin-Hartley guinea-pigs (250-300 g) were sensitised to ovalbumin (50 mg sc and 50 mg ip) and left for 2 weeks. The animals were then dosed with mepyramine (0.5 ml ip of a 10 $mg.ml^{-1}$ solution) 30 min prior to ovalbumin (OA) challenge. Each guinea-pig was individually exposed to 0.1 % OA aerosol in a nose only exposure chamber for 10 min. At 24 h post OA challenge they were killed and the lungs lavaged with three 5 ml washings of phosphate buffered saline containing 0.05 mM EDTA (PBS/EDTA). The BAL fluid was centrifuged and the cell pellet resuspended in 1 ml of PBS/EDTA. Differential cell counts were determined in Kimura's stain. The compounds were dosed orally to groups of guinea-pigs (n = 10) 90 min prior to aerosol challenge. Rolipram, RP73401, LAS31025, SKB207499, RS25344 (Alvarez *et al.*, 1995), CDP840 and ORG20241 (a PDEIII/IV inhibitor) were tested. Control animals received vehicle (50 % PEG400/saline, 1 $ml.kg^{-1}$ po).
Table 1 shows the effect of the PDEIV inhibitors when tested against OA-induced eosinophilia in the BAL fluid. The most potent compound tested was RS25344, followed by RP73401. However, both of these exhibited dose-related anorectic effects. LAS31025 significantly inhibited antigen-induced eosinophilia at 10 $mg.kg^{-1}$ but by less than 50%. CDP840 had no effect at doses up to 10 $mg.kg^{-1}$. ORG20241 also had no effect at doses up to 10 $mg.kg^{-1}$. SKB207499 reduced eosinophil infiltration by 35% at 30 $mg.kg^{-1}$, but this was not significant. In conclusion certain PDEIV inhibitors display oral activity in the guinea-pig with an apparent rank order of potency of RS25344 (ED_{50}<0.1$mg.kg^{-1}$) > RP73401 (ED_{50} ≈3 $mg.kg^{-1}$) > LAS31025 (ED_{50} ≈10$mg.kg^{-1}$) > SKB207499 (ED_{50} > 30 $mg.kg^{-1}$).

Table 1. Effect of PDEIV inhibitors on eosinophil levels in the BAL fluid of guinea-pigs 24 h post antigen challenge. Data (n = 10 per group) were analysed by ANOVA and Bonferroni's t test.

Treatment	Dose po $mg.kg^{-1}$	Eosinophils x 10^6/ml (±s.e.mean)	% inhibited	P
Basal	-	1.2±0.2		-
Vehicle	-	5.2±0.9		
Rolipram	20	2.7±0.8	48	NS
Vehicle	-	9.1±1.1		
RP73401	0.3	7.3±1.1	20	NS
	1	6.0±1.7	34	NS
	3	4.0±1.1	56	<0.05
	10	2.8±0.6	69	<0.01
Vehicle	-	6.7±1.1		
RS25344	0.1	2.4±0.7	64	<0.01
	0.3	1.6±0.4	76	<0.01
	1	2.2±0.9	67	<0.01
	3	0.9±0.3	90	<0.01
Vehicle	-	5.1±0.6		
SKB207499	3	5.4±0.9	0	NS
	10	4.5±1.0	12	NS
	30	3.3±0.8	35	NS
Vehicle	-	5.5±0.4		
LAS31025	1	6.5±0.8	0	NS
	3	3.5±0.7	36	NS
	10	3.0±0.7	45	<0.05

References
Alvarez, R., *et al.*, (1995), Mol. Pharmacol., **48**, 616-22.
Acknowledgements
We thank Dr. D.R. Cheshire of Medicinal Chemistry, Astra Charnwood for the synthesis of compounds.

45P INTERLEUKIN-1β INHIBITS PROLIFERATION OF HUMAN PULMONARY ARTERY SMOOTH MUSCLE CELLS VIA A CYCLOOXYGENASE-2 DEPENDENT PATHWAY

Jourdan K.B., Evans T.W. and Mitchell J.A.
Unit of Critical Care, Imperial College at NHLI, Royal Brompton Hospital, Sydney Street, London, SW3 6NP, UK.

Cyclooxygenase 2 (COX-2) is known to be induced by cytokines such as IL-1β under inflammatory conditions such as sepsis and systemic inflammatory response syndrome (SIRS). Moreover, IL-1β modulates the growth of a number of cellular preparations. Thus we have investigated the effect of IL-1β, COX inhibitors and prostaglandin (PG) E_2 on proliferation of human pulmonary artery (PA) smooth muscle cell growth.

Human PA smooth muscle cells were cultured by explant from vessels obtained from lung cancer surgery. Smooth muscle phenotype was confirmed by immunostaining for smooth muscle α-actin. Cells were first treated for 24 hours with or without the COX-1/2 inhibitor indomethacin (100μM), the specific COX-2 inhibitor L-745,337 (10μM) or PGE_2 (1 μM). In some experiments IL-1β (10ng/ml) was added alone or in combination with the inhibitors. Proliferation of cells was measured after 24 hour stimulation with foetal calf serum (10%) by the incorporation of ^3H thymidine into the cells over the following 6 hour period (Dicker and Rozengurt, 1980).

Incubation with either indomethacin or L-745,337 increased the serum induced proliferation of human PA smooth muscle cells to above basal (Figure 1A). In contrast PGE_2 inhibited ^3H thymidine incorporation (IC_{50} = 186nM). Cicaprost, an IP receptor agonist, also inhibited proliferation but less potently than PGE_2 (IC_{50} = 1.9μM). IL-1β significantly inhibited proliferation of the smooth muscle cells (Figure 1B). Furthermore this inhibition was completely reversed by the COX inhibitors indomethacin and L-745,337.

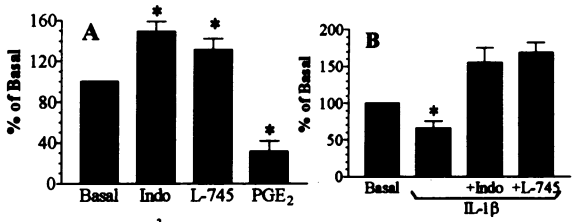

*Figure 1 shows ^3H thymidine uptake as % of basal (A) after indomethacin, L-745,337 and PGE_2 treatment alone or (B) with IL-1β. Data are the mean ± s.e.mean (n=6-12, cells from 4 patients). *p<0.05 cf. basal by one sample t test.*

Human PA smooth muscle cells are known to release low levels of PGE_2 under basal conditions which are greatly increased after stimulation with IL-1β (See Jourdan *et. al.* this meeting). In the same cells when basal levels of prostaglandins are inhibited proliferation is significantly increased. Exogenous PGE_2 had the opposing effect, arresting proliferation. IL-1β alone inhibited proliferation, an effect that was reversed by COX-2 inhibition. In conclusion the inflammatory cytokine IL-1β which is elevated in inflammatory diseases such as sepsis and SIRS, leads to induction of COX and release of PGE_2 which inhibits proliferation of human vascular smooth muscle cells.

KBJ is a MRC research assistant and JAM is a Wellcome Trust Career Development Fellow.

Dicker, P., and Rozengurt, E. (1980). *Nature* **287**,607-612.

46P INTERLEUKIN-1β INHIBITS PROLIFERATION OF RAT PULMONARY ARTERY SMOOTH MUSCLE CELLS THROUGH A NITRIC OXIDE-DEPENDENT PATHWAY

Jourdan K.B., Mitchell J.A. and Evans T.W.
Unit of Critical Care, Imperial College at NHLI, Royal Brompton Hospital, Sydney Street, London, SW3 6NP, UK.

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are known to be expressed under inflammatory conditions by cytokines such as interleukin 1β (IL-1β). Moreover, IL-1β modulates the growth of a number of cellular preparations. Thus, here we have assessed the role of iNOS and/or COX-2 on the effects of IL-1β on rat pulmonary artery (PA) smooth muscle cell growth.

Rat (male, Wistar, 300g) PA smooth muscle cells were cultured by explant from vessels denuded of endothelium. Smooth muscle phenotype was confirmed by immunostaining for smooth muscle α-actin. Cells were first stimulated for 24 hours with serum and IL-1β (10ng/ml) with or without the non-specific NOS inhibitor N^G-nitro L-arginine methyl ester (L-NAME) or the iNOS selective inhibitor aminoguanidine (AG) (both 1mM). After 24 hours nitrite release was measured using the Greiss assay as an index of NO formation (Bishop-Bailey *et al.* 1997) and proliferation of cells was measured by the incorporation of ^3H thymidine into the cells over a 6 hour period (Dicker and Rozengurt, 1980).

IL-1β stimulated rat cells to release nitrite (EC_{50} =1.97 ng/ml) (Figure 1A). Similarly IL-1β stimulated release of PGE_2 (EC_{50} =1.03 ng/ml, E_{max}= 18.63±0.46 ng/ml). Nitrite release was reversed to basal levels by L-NAME with an IC_{50} of 100.7±23.6 μM and by AG with an IC_{50} of 140.6±26.3 μM (Figure 1A). In the proliferation experiments, IL-1β had an inhibitory effect reducing the amount of ^3H thymidine incorporated into the cells (IC_{50}=0.504ng/ml), an effect that was reversed by the addition of L-NAME (1mM) (Figure 1B). Neither inhibition of COX-1/2 by indomethacin (100μM) nor COX-2 by L-745,337 (10μM) had any effect on the IL-1β induced inhibition of rat PA smooth muscle proliferation. This is in contrast to observations made in human cells. (See Jourdan *et. al.* this meeting).

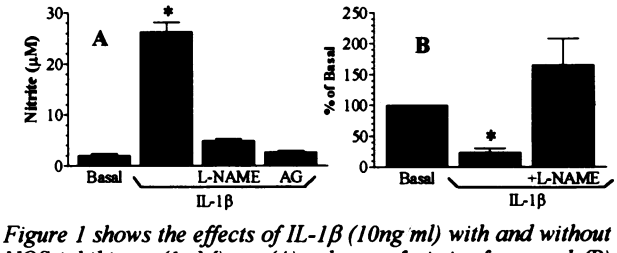

*Figure 1 shows the effects of IL-1β (10ng ml) with and without NOS inhibitors (1mM) on (A) release of nitrite from and (B) proliferation of rat PA smooth muscles cells. Data are mean ± s.e.mean. (n=4-10). *p<0.05 cf. basal. (A) ANOVA, (B) One sample t test.*

Thus, NO released from rat PA smooth muscle cells after stimulation by IL-1β, inhibits proliferation of the cells in an autocrine pathway. By contrast COX-2 products did not contribute to the effects of IL-1β on rat PA smooth muscle cell growth. These observations together with our other studies (see Jourdan *et. al.* this meeting) suggest that rat and human evolution has diverged such that NOS and COX, although induced by the same stimulus have different levels of activity and functions in the two species.

KBJ is a MRC research assistant and JAM is a Wellcome Trust Career Development Fellow.

Bishop-Bailey, D. *et al.* (1997). *Br. J. Pharmacol.* **121**, 125-133.
Dicker, P., and Rozengurt, E. (1980). *Nature* **287**,607-612.

47P CAPTOPRIL IS NECESSSARY FOR BRADYKININ-INDUCED AIRWAY HYPERRESPONSIVENESS

Annick de Vries, Stefan Faas, Ferdi Engels, Paul A.J. Henricks & Frans P. Nijkamp. Department of Pharmacology and Pathophysiology, Utrecht Institute of Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands

Sensory neurons play a modulatory role in airway hyperresponsiveness. We have previously shown that the lipid mediator 13-hydroxy-linoleic acid (13-HODE) induces airway hyperresponsiveness both in vivo and in vitro. This effect can be blocked by either depleting the sensory neurons or specifically blocking NK_1 receptors (Engels *et al.*, 1995). Apparently 13-HODE is able to induce airway hyperresponsiveness by sensitizing nerve endings. It is known that bradykinin is also able to sensitize nerve endings (Kopp *et al.*, 1997). Therefore, the present study focused on the effect of bradykinin on airway reactivity.

Male Dunkin Hartley guinea pigs (450-550g) were treated with the angiotensin converting enzyme (ACE) inhibitor captopril, 0.3mg/ml in drinking water for 14 days. This treatment had no effect on the reactivity of the airways to histamine in anesthetized, spontaneously breathing guinea pigs. Bradykinin aerosol (100nM aerosol during 8 breaths, every 5 minutes for 5 times) on its own, had no effect either. However, after captopril treatment bradykinin aerosol induced a significant hyperresponsiveness to histamine. A summary of the results is given in Table 1.

Table 1. Pulmonary resistance (H_2O/ml/s mean ± s.e.m.) measured after 2μg histamine/100g body weight

	control	captopril	bradykinin	captopril + bradykinin
pulmonary resistance	2.73 ±- 0.17 n=4	5.09 ± 1.47 n=3	2.55 ± 0.71 n=4	12.07 ± 2.72 n=5

The effect of captopril on bradykinin induced pulmonary resistance can be explained by its inhibitory effect on ACE that is able to break down the inhaled braykinin. Alternatively, ACE inhibition might result in a decrease of substance P metabolism. This notion is substantiated by the observation that 13-HODE induced airway hyperresponsiveness is also mediated by the release of substance P from sensory nerve endings.

Taken together, we suggest that 13-HODE and bradykinin share a similar mode of action in inducing airway hyperresponsiveness.

Engels, F., van Houwelingen, A.H., Buckley, T.L. *et al.* (1995) *Advances in Prostaglandins, Thromboxane, and Leukotriene Research*, 23: 361-363
Kopp, U.C., Farley, D.M. and Smith, L.A. (1997) *Am. J. Physiol.*, R2009-R2016

48P 5-HYDROXYTRYPTAMINE AND HISTAMINE ARE INVOLVED IN AIRWAY HYPERRESPONSIVENESS AND EOSINOPHILIA IN A MURINE MODEL OF ALLERGIC ASTHMA

J.J. De Bie, P.A.J. Henricks, E.H. Jonker, G.A. Hofman, T. Muis, F.P. Nijkamp & A.J.M. Van Oosterhout

Dept. of Pharmacology and Pathophysiology, Utrecht Institute of Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB, Utrecht, The Netherlands.

In a murine model of allergic asthma *in vivo* airway hyperresponsiveness to methacholine, eosinophil infiltration and antigen-specific IgE in serum, have been observed after ovalbumin challenge. Furthermore, mast cell degranulation was detected within 30 min after challenge. Histamine (H) and serotonin (5-Hydroxy-tryptamine, 5-HT) are released by mast cells and can cause an acute bronchoconstrictive reaction. In this study we investigated the role of histamine and serotonin in the induction of airway eosinophilia and hyperresponsiveness as well as antigen-specific IgE levels in serum. Sensitized male BALB/C mice (weight: approximately 25g) were challenged with an aerosol (once a day for 8 days) of either ovalbumin (OA) or saline (SAL). Prior to - and 8h after challenge, animals were injected i.p. with either the selective $5\text{-}HT_2$ receptor antagonist ketanserin (12 mg kg^{-1}), the selective H_2 receptor antagonist cimetidine (10 mg kg^{-1}), a combination of both antagonists or vehicle (n=6 per group). Dose response curves of airway responsiveness to methacholine, eosinophil numbers in bronchoalveolar lavage fluid and serum-IgE levels were determined at 24h after the last challenge.

A 9-fold increase in airway responsiveness was observed at the highest dose (640 λg kg^{-1}) methacholine in vehicle-treated OA-challenged mice when compared to saline-challenged animals (127.7 ± 1.4 cmH_2O ml^{-1} sec^{-1} and 13.6 ± 2.7 cmH_2O ml^{-1} sec^{-1} respectively). Treatment with ketanserin or cimetidine significantly reduced airway responsiveness (36%, $P<0.05$ and 49%, $P<0.05$ respectively) when compared to vehicle-treated OA-challenged animals. However, the responsiveness was still significantly enhanced when compared to saline-challenged animals. The combination of cimetidine and ketanserin demon-strated an additional decrease of airway hyperresponsiveness (69%, $P<0.05$), which was not significantly different from saline-challenged animals. Eosinophil numbers in bronchoalveloar lavage fluid were also reduced after the different treatments, although not significantly (ketanserin 26%, cimetidine 33% and 35% for the combination) when compared to vehicle-treated OA-challenged animals (15.0 ± 5.9 x 10^4 cells). No effects of either drug on serum OA-specific IgE levels were observed. Selective H_1 (mepyramine; 12 mg kg^{-1}) and $5\text{-}HT_1$ (methiotepine; 1.3 mg kg-1) receptor antagonists as well as an α-adrenoceptor antagonist (phentolamine; 5 mg kg^{-1}) showed no effect on airway hyperresponsiveness and eosinophil infiltration. It can therefore be concluded that both histamine and 5-HT are involved in ovalbumin-induced airway hyperresponsiveness and eosinophil infiltration.

This study is funded by: Glaxo Wellcome B.V. The Netherlands.

49P EVIDENCE FOR 5-HT$_{1B/1D}$ RECEPTOR-MEDIATED VASOCONSTRICTION IN HUMAN ISOLATED PULMONARY RESISTANCE ARTERIES

M.R. MacLean & I. Morecroft. Division of Neuroscience and Biomedical Systems, IBLS, University of Glasgow, Glasgow G12 8QQ.

Elevated plasma 5-hydroxytryptamine (5-HT) has been implicated in pulmonary hypertension (Herve *et al.*, 1995). We have recently shown that in human large intra-pulmonary arteries there exists a functional population of h 5-HT$_{1B/1D}$ receptors (MacLean, *et al*, 1996). Here we examined the 5-HT receptors mediating vasoconstriction in human pulmonary resistance arteries (PRAs). PRAs (internal diameter ~300μm) were dissected out from macroscopically normal sections of lung tissue removed with bronchial carcinomas, mounted as ring preparations (2mm length) on a wire myograph (under tension) in Krebs (at 37°C). The PRAs were bubbled with 16% O_2/6%CO_2 balance N_2. Cumulative concentration response curves were constructed (0.1nM-0.3mM) to 5-HT or sumatriptan (agonist selective for 5-HT$_{1D/1B}$ receptors) in the presence/absence of 1μM (3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl]benzamide (GR55562) a selective 5-HT$_{1D/1B}$ antagonist (Walsh *et al.*, 1995). Antagonist incubation time was 45 minutes prior to further experimentation. The results are shown in Table 1. These results show that 5-HT and sumatriptan are equipotent vasoconstrictors of human PRAs. 1μM GR55562 antagonized the response to 5-HT [pK_B = 7.7 ± 0.3] whilst virtually abolishing the response to sumatriptan within the concentration range studied. Preliminary results also indicate neither the response to 5-HT nor sumatriptan is affected by 0.1mM ketanserin (5-HT$_{2A}$ antagonist).Together these observations provide the first evidence for the existence of a significant, functional population of h 5-HT$_{1B/1D}$ receptors which mediate vasoconstriction in human PRAs suggesting that a selective 5-HT$_{1B/1D}$ antagonist may be useful in the treatment of pulmonary hypertension.

Herve, P., Launay, J.M., Scrobohaci, M.L., *et al.* (1995) *Am. J. Med.* 99, 249-254.
MacLean, M.R., Clayton, R.A., Templeton, A.G.B.,*et al* (1996) *Br. J. Pharmacol.* 119, 277-282.
Walsh, D.M., Beattie, D.T., Conner, H.E. (1995). *Eur. J. Pharmacol.* 287, 79-84.

Table1. Effect of GR55562 on 5-HT and sumatriptan-induced vasoconstriction in human PRAs.

Group		pEC_{50}	n,n	Maximum response (% 50mM KCl)
5-HT	-control	7.1 ± 0.1	7,6	110 ± 24
	+ 1mM GR55562	5.4 ± 0.2***	6,5	75 ± 18
Sumatriptan	-control	6.7 ± 0.2	6,6	102 ± 13
	+ 1mM GR55562	<<5.0	6,4	no maximum.

Mean data ± s.e.mean.n,n = number of rings, number of tissue samples. Significance of difference from relevant control response in absence of antagonist. ***P< 0.0001. (Students unpaired t-test).

50P EFFECT OF DEVELOPMENTAL AGE ON 5-HT RECEPTOR-MEDIATED VASORELAXATION IN PERINATAL RABBIT ISOLATED PULMONARY RESISTANCE ARTERIES

I. Morecroft & M.R. MacLean. Division of Neuroscience and Biomedical Systems, IBLS, University of Glasgow, Glasgow G12 8QQ.

At birth, the pulmonary circulation undergoes rapid structural and functional changes in adapting to extrauterine life and we have recently shown that rabbit pulmonary arteries undergo developmental changes in 5-HT-mediated vasoconstriction (Morecroft, *et al..*, 1995). Here we examined the 5-HT receptors mediating vasodilation in perinatal rabbit pulmonary resistance arteries (PRAs). PRAs (internal diameter 250~350μm) were dissected out from lungs of fetal and neonatal NZW rabbits (killed with 200mg kg^{-1} sodium pentobarbitone). Vessels were mounted on a wire myograph under tension (in Krebs at 37°C). They were bubbled with 16% O_2/6%CO_2 balance N_2 (3% O_2/6%CO_2 balance N_2 for fetal tissue). In the presence of 0.1μM ketanserin (5-HT$_{2A}$ antagonist), the PRAs were pre-constricted with sarafotoxin S6c (1-3nM) and cumulative responses obtained (0.1nM-0.3mM) to 5-carboxamidotryptamine (5-CT) (agonist at 5-HT$_1$ & 5-HT$_7$ receptors) in the presence/absence of 1μM (3-[3-(dimethylamino)propyl]-4-hydroxy-N-[4-(4-pyridinyl)phenyl] benzamide (GR55562) a selective 5-HT$_{1B/1D}$ antagonist (Walsh, *et al.*, 1995) or 0.1mM L-NAME or 0.1mM L-NAME + 1μM spiperone. All antagonists were incubated for 45 minutes prior to further experimentation. The neonatal results are shown in Table 1. 5-CT-induced relaxation was absent in the fetal and 0-24 hr vessels but was very marked at 4 and 7 days. At 4 days L-NAME virtually blocks the relaxation suggesting 5-CT mediates vasorelaxation indirectly via nitric oxide (NO) release, possibly through 5-HT$_{1B/1D}$ receptors. At 7 days vasorelaxation to 5-CT is partly mediated directly via a different 5-HT receptor which is NO-independent. Taken together, these observations show marked developmental changes in the 5-HT receptors responsible for mediating vasorelaxation in perinatal rabbit PRAs. Selective agonists for these receptors may be useful in the treatment of neonatal pulmonary hypertension.

Morecroft, I., Docherty, C., MacLean (1995). *Br. J. Pharmacol.* 115, SS P33.
Walsh, D.M., Beattie, D.T., Conner, H.E. (1995). *Eur. J. Phar..acol.* 287, 79-84.

Table1. Effect of GR55562, spiperone and L-NAME on 5-CT-induced vasorelaxation in neonatal rabbit PRAs.

Treatment	4 days pEC_{50}	E_{max}.	n,n	7 days pEC_{50}	E_{max}.	n,n
5-CT-control	7.1 ± 0.2	91 ± 5	7,7	7.5 ± 0.2	91 ± 2	6,6
+ 0.1mM L-NAME		21 ± 6**	5,5	7.3 ± 0.3	43 ± 5**	6,6
+ 1mM GR55562	5.7 ± 0.2**	98 ± 5	6,6	7.7 ± 0.1	70 ± 7	6,5
+0.1mM L-NAME + 1mM spiperone				5.6 ± 0.3†	44 ± 3	6,4

Mean data ± s.e.mean.n,n = number of rings, number of tissue samples. E_{max} = maximum response (% 50mM KCl) Significance of difference a) from relevant control response in absence of antagonist/L-NAME. *P <0.01**P< 0.001; b) from 7 day in presence of L-NAME, †P <0.01. (ANOVA)

51P *IN VIVO* CHARACTERISATION OF THE TACHYKININ RECEPTOR SUBTYPES IN SQUIRREL MONKEY AIRWAYS

J.S. Chainey, E. Braun, C. Bertrand, H.A. Ball. Department of Respiratory Diseases, Novartis AG, Basel, Switzerland.

The airway pharmacology of the tachykinins (neurokinin A, NKA and substance P, SP) is of interest since they may have a role in asthma. NKA (NK_2-receptor preferring) induces bronchoconstriction and hyper-responsiveness in non-asthmatic human airways (in the presence of thiorphan) while substance P (NK_1-receptor preferring) is essentially inactive (Cheung et al., 1992; Joos et al., 1987). These, together with *in vitro* studies, indicate the relative importance of the NK_2 rather than the NK_1 subtype in man. The present study aimed to characterise the receptor subtypes responsible for tachykinin-induced bronchoconstriction in the squirrel monkey *in vivo*.

Following overnight fasting, female squirrel monkeys (*Saimiri sciureus;* 650-850 g) were anaesthetised by injection of 1 $mlkg^{-1}$ Saffan (Pitman-Moore, UK) and 0.5 $mgkg^{-1}$ valium (Roche, CH) i.m. Airway parameters (intubated & spontaneously breathing) were monitored by means of a head-out plethysmograph box to measure airflow, and a fluid-filled polyethylene catheter inserted into the oesophagus to approximate transpulmonary pressure changes. Total airways resistance (R_L) and dynamic lung compliance (C_{dyn}) were calculated according to the method of Amdur and Mead (1958).

In the first part of the study, concentration-response curves to aerosols of SP or NKA (5 min; 0.1 - 3.0 mM) were obtained in the presence of the neutral endopeptidase inhibitor thiorphan (maximally effective concentration of 2.0 $mgml^{-1}$ for 5 mins, 10 minutes prior to challenge) to inhibit their catabolism.

Secondly, the role of the NK_2 receptor was further investigated using the endopeptidase-resistant NK_2 agonist [β-ala^8]-NKA(4-10) (β-ala^8-NKA) and the selective NK_2 antagonist SR 48 968 (Emonds et al., 1993). Monkeys were pretreated with SR 48 968 (3-30 $mgkg^{-1}$, 2 h p.o.; 0.3 $mgkg^{-1}$, 30 min i.v.) or its vehicle (1 $mlkg^{-1}$ methylcellulose containing 2% DMSO), and β-ala^8-NKA administered as an aerosol (1.0 mM for 5 min; an ED_{80} concentration) in a multiple crossover fashion (every 2 weeks).

NKA and SP caused a dose-related increase in R_L (Fig 1A) and fall in C_{dyn} (not shown). However, SP was > 10-fold less potent than NKA with a shallower slope. β-ala^8-NKA elicited a 2.2-fold in crease in R_L (vehicle, Fig 1B) and fall in C_{dyn} (not shown). Oral pretreatment with SR 48 968 dose-dependantly inhibited responses to β-ala^8-NKA, with complete blockade at 0.3 mg kg^{-1} i.v. (Fig 1B).

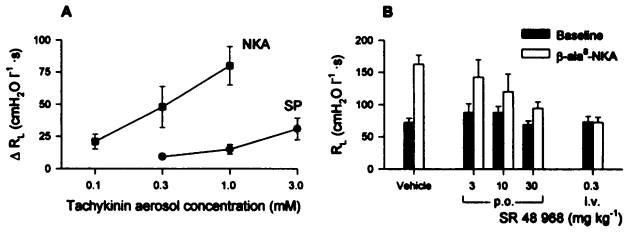

Fig 1. A. Effects of NKA and SP (both in presence of thiorphan) on airways resistance (R_L). **B.** Effect of SR 48 968 pretreatment on β-ala^8-NKA-induced changes in R_L. Values are mean ± s.e.mean with n = 4-12 monkeys per concentration/ dose.

In summary, NKA in contrast to SP was highly effective in eliciting bronchoconstriction suggesting an important role of the NK_2 receptor subtype. This was confirmed by the finding that the NK_2 agonist β-ala^8-NKA induced a potent bronchoconstriction which could be completely inhibited by the selective NK_2 antagonist SR 48 968. As such, these results indicate that the squirrel monkey is similar to man and provides a suitable model for the evaluation of NK_2 receptor antagonists.

Amdur M.O. & Mead J. (1958). *Am. J. Physiol.*, **192**, 364-368.
Cheung D., Bel E., Den Hartigh J. et al (1992) *Am. Rev. Respir. Dis.* **145**, 1275-80.
Emonds Alt-X., Golliot F., Pointeau P. et al (1993). *Biochem.Biophys.Res.Commun.* **191**, 1172-7.
Joos G., Pauwels R. & Van Der Straeten M. (1987). *Thorax.* **42**, 779-783.

52P ALTERNATIVE SPLICING GENERATES TWO ISOFORMS OF THE β_3-ADRENOCEPTOR WHICH ARE DIFFERENTIALLY EXPRESSED IN MOUSE TISSUES

R.J. Summers, M. Papaioannou, S. Hamilton & B.A. Evans, Department of Pharmacology, Monash University, Clayton, Victoria 3168, Australia.

The β_3-adrenoceptor (AR) differs from the β_1- and β_2-AR's in that it contains three exons and two introns (Van Spronsen *et al.*, 1993; Granneman & Lahners, 1995) Previous studies in mice demonstrate that the use of alternate acceptor splice sites results in the generation of two β_3-AR transcripts which differ in their 3' untranslated regions (Van Spronsen *et al.*, 1993). Treatment of mice with the β_3-AR agonist BRL 26830 or incubation of 3T3-F442A adipocytes with (-) isoprenaline, dexamethasone or phorbol 12-myristate 13-acetate causes marked reduction of β_3-AR mRNA in adipose tissue but has no effect on the proportion of transcripts (Granneman & Lahners, 1995). Here we report that the use of alternate splicing sites generates two isoforms of the β_3-AR which have interesting differences in the C-terminus tail and are differentially expressed in mouse tissues.

RNA was extracted from mouse (male, 30-40 g, C57BL/6J) intrascapular brown fat (BAT), ileum smooth muscle and hypothalamus by homogenization in Trizol. The extracted RNA was checked for purity by gel electrophoresis and quantified by spectrophotometry. Aliquots (1μg) were reverse transcribed and PCR (20-30 cycles) conducted on 1/10th the cDNA using primers that spanned the first intron. β_3-cDNA was transferred to a nylon support, probed with a [^{33}P] labelled oligo complimentary to a central region of the expected product and quantified by phosphorimaging. RNA extracted from BAT, ileum and hypothalamus contained two transcripts of the β_3-AR. The proportions of the splice variant to the known cDNA sequence were for BAT, 7.6 ± 0.4% (n=12), ileum, 17.5 ± 0.5% (n=9) and hypothalamus, 44.6 ± 0.7% (n=9). Previous studies (Summers *et al.*, 1995) have demonstrated the presence of β_3-mRNA in several regions of rat brain including hypothalamus. RNA from mouse hypothalamus was subjected to RT/PCR and the PCR products excised from an agarose gel. The upper band corresponding to the novel transcript was subcloned into the PCR-Script vector, transformed into E.coli XL1-Blue cells and four different subclones sequenced using the chain-termination method. The β_3-AR encoded by the alternatively spliced cDNA has a C-terminus with 17 amino acids (SSLLREPRHLYTCLGYP) following the sequence coded by the first exon region compared to 13 (RFDGYEGARPFPT) in the known receptor. The splice variant C-terminal region has a cysteine residue which may represent an alternative palmitoylation site, 8 serine and threonine residues rather than 6 which may make the splice variant a better substrate for G-protein receptor kinase phosphorylation and a consensus site for PKC phosphorylation. These studies show that there is an additional novel β_3-AR which is differentially expressed in mouse tissues with the highest proportion of the splice variant present in hypothalamus. The novel receptor may differ from the known receptor in signalling and regulatory properties.

Supported by the National Health and Medical Research Council of Australia.

Granneman, J.G. & Lahners, K.N. (1995) *Am.J.Physiol.* 268, C1040-C1044.
Summers, R.J., Papaioannou, M., Harris, S., Evans, B. A. (1995) *Br. J. Pharmacol.* 116, 2547-2548.
Van Spronsen, A., Nahmias, C., Krief, S. *et al.*, (1993) *Eur.J.Biochem.*213, 1117-1124.

53P β-ADRENOCEPTOR MEDIATED VASODILATION IS TRANSIENTLY ENHANCED IN RAT AORTIC ARTERIES WITH INTIMAL HYPERPLASIA

F.J. Heijenbrok, M. Pfaffendorf & P.A. van Zwieten, Department of Pharmacotherapy, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

In previous studies we have shown that the α_1-adrenoceptor mediated contractile response as well as the endothelium-dependent vasodilation with methacholine become severely impaired as a consequence of balloon denudation of the rat carotid artery with subsequent neointima formation. However, both functions recover in time (Heijenbrok *et al*, 1997). In this study we investigated the vasoconstrictor response of rat carotid arteries with intimal hyperplasia to the thromboxane A_2 (TxA_2) agonist, U46619, since thromboxane is known to play a role in the development of restenosis after angioplasty. As an endothelium-independent vasodilator we studied the β-adrenoceptor agonist isoprenaline.
After balloon denudation of the carotid artery in male Wistar rats (250-300g), the animals were sacrificed after 0, 14, 56 and 112 days, respectively. The contractile responses of the denuded left and control right carotid arteries to potassium chloride (K^+; 100mM), TxA_2 agonist (U46619; 0,1nM-0,3μM), and phenylephrine (PhE; 3μM) were evaluated together with the vasorelaxation induced by isoprenaline (ISO; 10μM) and methacholine (MCh; 3μM) in an isometric wire myograph (PSS, 37°C, pH7.4, gassed with 95% O_2/5% CO_2).
In previous morphological studies we have shown that the standardized surgical procedure induced significant intimal hyperplasia in the left rat carotid artery after 14 days. From this moment onwards, the neointimal surface increased with time, but reached equilibrium after 84 days (Heijenbrok *et al.*, 1997). The endothelium was removed by balloon denudation, but gradually reappeared after 84 days following surgery. The decrease in contractile responses to PhE after 14 days, and the direct loss of endothelium-dependent vasodilation with MCh are in agreement with earlier findings (see Table 1). Balloon denududation with subsequent neointima formation did not influence the maximal contractile responses (E_{max}) to K^+-depolarisation and neither to U46619, for all time points measured (see Table 1). Also, the sensitivity (pD_2) to U46619 was not different for all injured preparations when compared to their controls (directly measured after balloon denudation: pD_2=8.12±0.04 vs. pD_2=8.06±0.03; injured and control artery, respectively, n=6). The vasodilator responses to isoprenaline were significantly enhanced in the injured vessel preparations at 14 and 56 days after balloon denudation, with the effect being maximal at 14 days (see Table 1). At 112 days this vasodilator effect of isoprenaline was reduced to the same value as in the control vessels.
Both U46619- and phenylephrine-mediated contractile responses are mediated through activation of phospholipase C. However, the effect of the thromboxane agonist is uninfluenced by balloon denudation for all time points measured, whereas the α_1-adrenoceptor mediated response becomes severely impaired. This might indicate that this deterioration is due to a specific α-adrenoceptor related mechanism. The contractile responses of the injured vessels to potassium chloride and U46619 were comparable to the control preparations for all time points measured. From this, we conclude that the smooth muscle cells present in the neointima are unlikely to contribute to the contractile force development as measured with the isometric wire myograph technique. The transiently increased vasodilator response to isoprenaline in the injured vessels possibly indicates an increased expression of functional β-adrenoceptors in the balloon denuded vessels.

Heijenbrok, F.J. *et al*.(1997) Br.J.Pharmacol., 120, P33.

	direct (n=6)		2 weeks (n=7)		8 weeks (n=6)		16 weeks (n=7)	
Contraction (N/m)	injured	control	injured	control	injured	control	injured	control
K^+ (100mM)	3.6±0.1	3.6±0.1	3.7±0.2	3.6±0.3	3.7±0.2	3.6±0.2	4.2±0.3	4.3±0.2
U46619 (0,3μM)	3.5±0.1	3.7±0.1	3.0±0.3	2.9±0.2	3.9±0.2	4.5±0.3	3.4±0.2	3.7±0.2
PhE (3μM)	3.0±0.2	2.8±0.1	0.9±0.2*	2.2±0.3	1.7±0.1*	2.6±0.2	2.8±0.3	3.1±0.3
Relaxation (%)								
ISO (10μM)	6.5±1.1	9.2±0.7	46.5±2.5*	3.5±1.5	20.9±3.0*	7.5±1.7	2.3±1.1	2.7±1.1
MCh (3μM)	n.r.*	87.2±2.2	n.r.*	77.8±4.2	9.0±8.1*	71.8±7.4	60.5±7.2	58.7±3.7

Table 1: Experimental data. * = $p<0.05$ vs. control; n.r. = no relaxation

54P BLOOD PRESSURE ELEVATION IS ASSOCIATED WITH A DECREASE IN INTERFERON-Γ IN MICE AND RATS

S.J. De Kimpe, D. Van Heuven-Nolsen, V. Schijns[1] & F.P. Nijkamp, Dept of Pharmacology and Pathophsyiology, Utrecht Institute of Pharmaceutical Sciences, and [1]Dept of Virology, Faculty of Veterinary Sciences, Utrecht University, P.O.Box 80.082, 3508 TB Utrecht, The Netherlands.

Growing evidence indicates a strong association between changes in the immune system and the development of hypertension in humans and animal models (Fu, 1995). For instance, lymphocytes from patients with hypertension exhibit an enhanced reactivity to vascular antigens. Autoreactive T cells to arterial antigens have also been isolated from spontaneously hypertensive rats (SHR) (Ofosu-Appiah *et al*, 1997). The present study investigated the role of the T cell cytokine interferon (IFN)-γ in blood pressure regulation.

Blood was obtained from 5 month old SHR under anaesthesia (pentobarbitone 100 mg/kg). Peripheral blood mononuclear leukoctyes (PBMC) were isolated by centrifugation using a Percoll gradient (1.081 mg/ml). Subsequently PBMC (10^6 ml^{-1}) were stimulated by concanavaline A (20 μg ml^{-1}). After 20 h, cells were collected and total RNA was isolated using RNAzol B (Pharmacia, Sweden). Expression of mRNA for IFN-γ was determined by polymerase chain reaction following reverse transcription (RT-PCR) and presented as percentage of the expression of glyceraldehyde-3-phosphate dehydrogenase.
IFN-γ receptor knock out and wild type control mice were anaesthetised using pentobarbitone (100 mg/kg). The carotid artery was cannulated for the measurement of blood pressure and the jugular vein for the administration of noradrenaline (0.6 μg/kg) and acetylcholine (0.2 μg/kg). After the heamodynamic measurements, small mesenteric arteries were isolated and mounted in a wire myograph. The reactivity to noradrenaline (10^{-8}-3×10^{-5} M) was determined. Results are expressed as mean ± s.e.mean and statistically evaluated using a student's *t*-test.

The expression of IFN-γ mRNA in activated PBMC was impaired in 5 month old SHR compared to age-matched normotensive WKY rats (16 ± 6 % for SHR vs 41 ± 5 % for WKY, n=6, $P<0.05$). A possible role of the decrease in IFN-γ on blood pressure was further investigated in IFN-γ receptor knock out mice (Schijns *et al*, 1996). In these mice, the mean arterial pressure was elevated compared to the wild type control mice (125 ± 6 mmHg vs. 88 ± 5 mmHg, n=6, $P<0.05$). The acetylcholine depressor response was increased in the IFN-γ receptor knock out mice (34 ± 3 mmHg vs. 20 ± 3 mmHg, n=6, $P<0.05$), but the pressor response to noradrenaline was not significantly influenced (31 ± 5 mmHg and 34 ± 2 mmHg, respectively, n=6). The elevated blood pressure in IFN-γ receptor knock out mice was associated with an increase in the maximal contraction to noradrenaline of isolated small mesenteric arteries compared to the wild type control mice (12.2 ± 0.8 mN vs 10.1 ± 0.5 mN, n=8, $P<0.05$).

Thus, a decrease in IFN-γ production or function is associated with an elevated blood pressure in mice and rats.

Fu, L.X. (1995) *J. Hypertension* 13: 1259-1265.
Ofosu-Appiah, W., Sfeir, G., Kennel, A. *et al* (1997) *Am. J. Hypertens.* 10: 535-40.
Schijns, V.E., Wierda, C.M., Van Hoeij, M. *et al* (1996) *J Immunol* 157, 815-821.

SJDK is a fellow of the Netherlands Heart Foundation.

55P EFFECTS OF BMS-181885 IN EXPERIMENTAL MODELS FOR MIGRAINE THERAPY

P.R. Saxena, P. De Vries, J.P.C. Heiligers, W.A. Bax, A. MaassenVanDenBrink and [1]F.D. Yocca, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands and [1]CNS Drug Discovery Division, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT, USA.

Several acutely acting antimigraine drugs have the ability to constrict porcine arteriovenous anastomoses (AVAs) as well as the human isolated coronary artery. These two experimental models seem to serve as indicators, respectively, for the therapeutic and coronary side-effect potential of the compounds (Saxena & Ferrari, 1996). Using these models (for details, see Saxena *et al.*, 1997), we studied the effects of a 5-$HT_{1B/1D}$ receptor ligand BMS-181885 (Fig. 1), which has a slow dissociation rate from the receptors and, unlike sumatriptan and other such drugs, does not constrict isolated canine saphenous vein or guinea pig iliac artery, demonstrating competitive antagonism at these vessels (Yocca *et al.*, 1997). In 7 pentobarbital anaesthetized pigs (12-14 kg, ♀), i.v. BMS-181885 caused a dose-dependent decrease in the total carotid blood flow from a baseline of 116±9 ml min^{-1} to 87±10 ml min^{-1} at the highest dose (300 µg kg^{-1}). This decrease was exclusively at the expense of the arteriovenous anastomotic (AVA) fraction, which decreased from a baseline of 84±6 ml min^{-1} by 52±6% at the highest dose (Fig. 2, *left panel*). When carotid haemodynamic changes after a single 100 µg kg^{-1} dose of BMS-181885 or sumatriptan (n=6 each) were studied at different time-points, BMS-188185 decreased AVA flow for the observation period of 120 min; the decrease with sumatriptan was only for 60 min (Fig. 2, *right panel*). Human isolated coronary artery segments (n=4) contracted to BMS-181885 (pD_2: 8.0±0.1; E_{max}: 6±2% of the contraction to 100 mM K^+) as well as sumatriptan (pD_2: 6.4±0.1; E_{max}: 28±13% of the contraction to 100 mM K^+). The above results suggest that (i) the longer-lasting vasoconstrictor action of BMS-181885 on porcine carotid AVAs may be related to its reported slow dissociation from 5-$HT_{1B/1D}$ receptor and (ii) BMS-181885 should be able to abort migraine headaches in patients, but may exhibit less sumatriptan-like effects on coronary arteries. Initial clinical studies have demonstrated the therapeutic action of the drug in acute migraine.

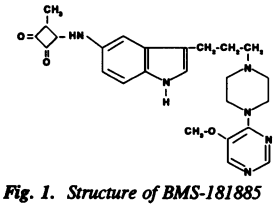

Fig. 1. Structure of BMS-181885

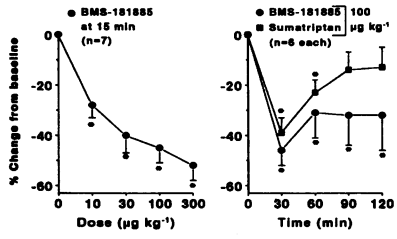

*Fig. 2. Effect of BMS-181885 and sumatriptan on carotid AVA blood flow. *, P<0.05 vs baseline.*

Saxena, P.R. & Ferrari, M.D. (1996). *Exp. Opin. Invest. Drugs* **5**, 581-593.

Yocca, F.D. *et al.* (1997). *Cephalagia* **17**, 404.

Saxena, P.R. *et al.* (1997). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **355**, 295-302.

56P PORCINE CAROTID HAEMODYNAMIC EFFECTS OF ALNIDITAN, A SELECTIVE 5-$HT_{1B/D}$ RECEPTOR LIGAND

P. De Vries, C.M. Villalón, J.P.C. Heiligers & P.R. Saxena, Dept. of Pharmacology, Erasmus University, Rotterdam.

A property shared in common by acute acting antimigraine drugs is their ability to constrict porcine carotid arteriovenous anastomoses (AVAs) via 5-$HT_{1B/1D}$ receptors and this seems to be of therapeutic relevance (Saxena & Ferrari, 1996). In the present study we investigated the carotid vascular effects by alniditan, a non-indole, potent 5-$HT_{1B/D}$ receptor ligand, virtually devoid of 5-ht_{1F} receptor interaction (Fig. 1; Leysen *et al.*, 1996; Van de Water *et al.*, 1996). For this purpose we used pentobarbital anaesthetized, bilaterally vagosympathectomized female pigs (12-14 kg). Total carotid blood flow was measured using an electromagnetic flow probe, whereas its AVA and nutrient fractions were calculated using the radioactive microsphere method (De Vries *et al.*, 1996). The animals were divided into two groups (n=6 each), which were pretreated with either saline (control) or 0.5 mg kg^{-1}, iv of GR127935, which abolishes the carotid haemodynamic effects of sumatriptan (De Vries *et al.*, 1996). Baseline values of heart rate (beats min^{-1}), mean blood pressure (mmHg), total carotid, AVA and nutrient conductance (ml min^{-1} $mmHg^{-1}$) were 95±4, 95±3, 1.44±0.13, 1.08±0.16 and 0.37±0.03 in control animals, and 99±3, 92±2, 1.44±0.18, 0.88±0.14 and 0.56±0.10 in the GR127935 treated group. Alniditan (3-100 µg kg^{-1}, iv) decreased heart rate and mean blood pressure by up to 4±1 and 23±5%, respectively; these responses were unaffected by GR127935. As shown in Fig. 2, alniditan produced a dose-dependent decrease in carotid vascular conductance, exclusively caused by a potent constrictive action on the AVAs; nutrient conductance increased. The dose of alniditan that decreased AVA conductance by 50% (ED_{50}) was 24±7 µg kg^{-1} (66±21 nmol kg^{-1}); the ED_{50} for sumatriptan was 65±22 µg kg^{-1} (156±54 nmol kg^{-1}; De Vries *et al.*, 1996). Treatment with GR127935 potently reduced all alniditan-induced carotid vascular responses, although the blockade was not significant in the nutrient fraction (Fig. 2). In conclusion, the results show that alniditan potently constricts AVAs, mainly via 5-$HT_{1B/D}$ receptors, and should, therefore, be able to abort migraine headache. Indeed, phase II studies confirm the antimigraine property of alniditan (Goldstein, 1996).

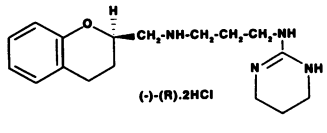

Fig.1 Structure of alniditan (Janssen Research Foundation, Belgium)

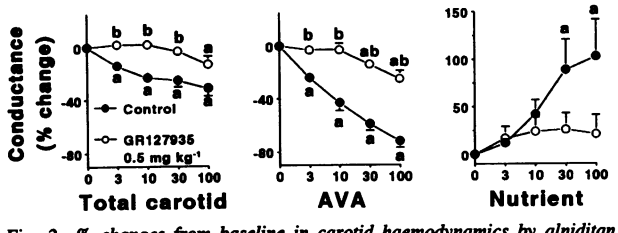

Fig. 2 % changes from baseline in carotid haemodynamics by alniditan (µg kg^{-1}, iv). a, P<0.05 vs baseline; b, P<0.05 vs control.

De Vries, P. *et al.* (1996). *Br. J. Pharmacol.*, 118, 85-92.

Leysen, J.E. *et al.* (1996). *Mol. Pharmacol.*, 50, 1567-1580.

Saxena, P.R. & Ferrari, M.D. (1996). *Exp. Opin. Invest. Drugs*, 5, 581-593.

Van de Water, A. *et al.* (1996). *Eur. J. Pharmacol.*, 299, 127-137

Goldstein, J. *et al.* (1996). *Cephalalgia.*, 16, 497-502.

57P REGIONAL CARDIAC OUTPUT DISTRIBUTION DURING 5-HT$_7$ RECEPTOR-MEDIATED HYPOTENSION IN RATS

P. De Vries, C.M. Villalón, J.P.C. Heiligers, P.A. De Visser & P.R. Saxena, Dept. of Pharmacology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

We have previously shown that the 5-HT-induced late depressor response in rats is mediated by 5-HT$_7$ receptors, blockable with R(+)lisuride, but not with GR127935 (De Vries *et al.*, 1997). The present study further characterizes this response by localizing vascular beds responsible for the decrease in total peripheral resistance (TPR), using intracardiac administered radioactive microspheres (see Saxena *et al.*, 1980). The effects of consecutive 10-min infusions of 5-HT (1, 3 and 10 μg kg^{-1} min^{-1}, iv at 15 min interval) on mean arterial blood pressure (MAP), heart rate (HR) and cardiac output (CO) and its distribution were analyzed in vagosympathectomized, pentobarbital anaesthetized rats (male Wistar, 300-330 g), pretreated with iv doses of either saline (0.5 ml), R(+)lisuride (100 μg kg^{-1}), S(-)lisuride (30 μg kg^{-1}) or GR127935 (300 μg kg^{-1}); n=5 each. Each animal received ritanserin (50 μg kg^{-1}, iv) to block 5-HT$_2$ receptor-mediated hypertensive response. 5-HT decreased MAP (baseline 76±3 mmHg) by up to 46±3% ($P<0.05$), without affecting baseline values of CO (105±6 ml min^{-1}); consequently, TPR (baseline: 730±50 mmHg min l^{-1}) decreased by up to 44±6%. The highest dose decreased HR (baseline 243±6 beats min^{-1}) by 16±6% ($P<0.05$). The 5-HT-induced decrease in TPR was caused by increases in muscle, carcass (muscle residue, bone, urogenital tract, fat and tail) and mesentery/pancreas vascular conductances. No changes were observed in the gastrointestinal tract, skin, kidneys, brain, heart, adrenals, spleen and liver. Treatment with R(+)lisuride abolished the 5-HT-induced hypotension (max change: +7±2%) and vasodilatations in muscle, carcass and mesentery/pancreas. S(-)lisuride or GR127935 did not affect MAP (max decreases: 34±6 and 41±4%, respectively) or tissue conductance responses (Fig. 1). It is concluded that the 5-HT-induced decrease in MAP in rats does not affect perfusion to vital organs and is mainly caused by dilatation in skeletal muscle vasculature. The potent and stereoselective blockade by R(+)lisuride and the lack of effect of GR127935 suggests the involvement of 5-HT$_7$ receptors. The lack of effect of 5-HT on cardiac output and on blood flow distribution to vital organs, implicates that development of selective 5-HT$_7$ receptor agonists may turn out to be a useful strategy in the treatment of hypertension.

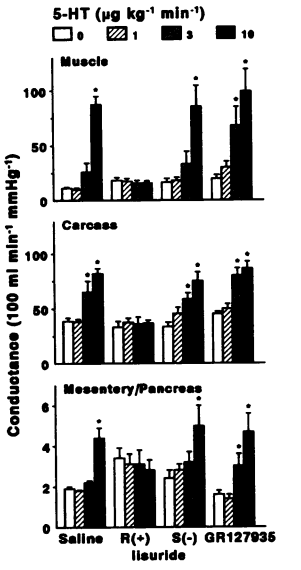

*Fig. 1. Effect of 5-HT on tissue conductance. *, $P<0.05$ vs baseline.*

Saxena, P.R. *et al.* (1980). *Comput. Prog. Biomed*, 12, 63-84.
De Vries, P. *et al.* (1997). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 356, 90-99.

58P DELAYED MATURATION OF CARDIAC CALCIUM CHANNELS AFTER AORTIC CONSTRICTION IN NEWBORN RATS

V. M. N. Cunha, F. Kolar*, T. Godfraind & M. Wibo
Laboratoire de Pharmacologie, Université Catholique de Louvain, B-1200 Bruxelles, Belgium and *Institute of Physiology, Academy of Sciences, CZ-142 20 Prague, Czech Rep.

Postnatal growth of mammalian heart is accompanied by marked changes in Ca handling, the sarcoplasmic reticulum (SR) progressively assuming a prominent role in Ca transients. SR Ca release through ryanodine-sensitive channels (RYRs) is triggered by Ca entry via dihydropyridine-sensitive channels of the sarcolemma (DHPRs). During maturation of ventricular tissue, most DHPRs concentrate into specialised domains of developing T-tubules, at the level of dyadic couplings with SR membranes containing the RYRs, and this process is delayed by hypothyroidism (Wibo *et al.*, 1995, 1997). The aim of this study was to analyse the effect of pressure overload on early postnatal maturation of Ca channels in ventricular tissue.

After constriction of abdominal aorta in 2-day-old Wistar rats (Zheng *et al.*, 1996), hearts were collected at 10 days. Control (SHAM) and severly hypertrophied hearts (BANDED) were grouped in 4 samples, each containing 3-6 ventricles; the weight of left ventricle (plus septum) was 83.5 mg ± 1.6 (s.e.m.) for SHAM, and 160 mg ± 11.1 for BANDED. Total membranes prepared from each sample were analysed for ^{3}H-PN200-110 binding (DHPR), ^{3}H-ryanodine binding (RYR) and ^{3}H-CGP12177 binding (ß$_1$-adrenoceptor, ßAR) in saturation experiments (Zheng et al., 1996). Membranes were also subfractionated by isopycnic density gradient centrifugation (Wibo *et al.*, 1995) to investigate the subsarcolemmal localisation of DHPRs.

The number of DHPRs and RYRs per g tissue decreased by 25-30% after aortic constriction, while ßARs decreased by 45-50% (Table 1, $P<0.02$).

Table 1. Tissue density (pmol / g tissue) of receptors from binding studies on total membranes. Results are given as mean ± s.e.m. (n=4).

	DHPR	RYR	ßAR
SHAM	14.0 ± 0.4	67.0 ± 3.1	3.70 ± 0.07
BANDED	10.1 ± 0.6	49.2 ± 3.8	1.95 ± 0.08

After equilibration of membranes in sucrose gradient, ßARs and RYRs were mainly recovered in low- and high-density subfractions, respectively, and distribution patterns were not modified after aortic banding. DHPRs showed broad density distributions, which differed significantly between SHAM and BANDED ($P<0.02$, n=3): the percentages of DHPRs recovered at low ($1.13<d<1.17$) and high densities ($1.17<d<1.21$) were 44.7 ± 1.3 and 39.5 ± 2.4, respectively, for BANDED, as compared to 34.6 ± 2.0 and 51.1 ± 1.3 for SHAM. The distribution pattern of DHPRs after equilibration in sucrose gradient of membranes from 10-day old banded rats resembled that observed previously with 1-day-old untreated rats (Wibo *et al.*, 1997).

We conclude that pressure overload, leading to severe hypertrophy, delays maturation of DHPRs in left ventricular tissue, as regards both their tissue density and, possibly, their association with dyadic couplings in nascent T-tubules.

Wibo, M., Feron, O., Zheng, L. *et al.* (1997) *Cardiovasc. Res.* (1997) in press.
Wibo, M., Kolar, F., Zheng, L. *et al.* (1995) *J. Cell. Mol. Cardiol.* 27, 1731-1743.
Zheng, L., Wibo, M., Kolar, F. *et al.* (1996) *Mol. Cell. Biochem.* 163/164, 23-29.

59P EVIDENCE THAT ATP AND UTP REGULATE SPONTANEOUSLY HYPERSENSITIVE RAT AORTA SMOOTH MUSCLE CELL PROLIFERATION BY ACTING ON $P2Y_4$ RECEPTORS

S. Harper, S.J. Charlton, L.L. Ng & M.R. Boarder, Dept. of Cell Physiology and Pharmacology and Dept. of Medicine, University of Leicester, P.O. Box 138, Medical Sciences Building, Leicester LE1 9HN, UK.

The P2Y receptor responsible for the stimulation of smooth muscle proliferation by ATP and UTP released from platelets is not known. It has been proposed to be a $P2Y_2$ and/or $P2Y_6$ (Chang et al, 1996). Here we have investigated the identity of the receptor in vascular smooth muscle cell cultures. We studied cells from the spontaneously hypersensitive rat (SHR), since these give a larger phospholipase C (PLC) response and a clear increase in DNA synthesis in response to stimulation with nucleotides alone.

Methods are as described in Wilkie et al (1996). Cells were grown from aortic explants of SHR origin and used at passages 6-12. Cells were serum free for 24h prior to use. [^{3}H]thymidine incorporation 24h after addition of agonist was used as an index of mitogenesis, accumulation of [^{3}H]inositol phosphates ([^{3}H]$InsP_x$) in the presence of LiCl as an index of PLC, and Western blot specific for phosphorylated forms of p42 and p44 MAPK as an index of MAPK activation. Transfected $P2Y_4$ 1321N1 cells were as described previously (Charlton et al, 1996).

The SHR cells gave a larger maximal [^{3}H]$InsP_x$ PLC response to UTP than to ATP (UTP, 24.8 ± 7.4, ATP 13.3 ± 5.6 ($P<0.05$); data are fold over basal from 3 experiments). The EC_{50} for ATP was the same as that for UTP (log EC_{50} -5.18 ± 0.19 and -5.21 ± 0.15 respectively). ADP, 2-methythioATP and α,β-methylene ATP were essentially ineffective. UDP gave a maximal response which was 63.6 ± 26.4% of that of UTP. Hexokinase substantially reduced the response to UDP, indicating this is due to the presence of UTP. When increasing concentrations of ATP (30-300 μM) were present with a maximally effective concentration of UTP (30 μM), the size of the [^{3}H]$InsP_x$ response diminished in a concentration dependent manner (effect of ATP significantly at $P<0.05$ by ANOVA and Dunnet's post test.) This is consistent with UTP and ATP acting at a single population of receptors, with ATP a partial agonist. A very similar agonist profile was obtained with 1321N1 cells transfected with $P2Y_4$ receptors. Stimulation of SHR derived cells with UTP enhanced the tyrosine phosphorylation of both p42 and p44 MAPK. ATP and UTP (300μM) also enhanced incorporation of [^{3}H]thymidine into DNA by 2.9 ± 0.7 and 3.1 ± 1.1 fold respectively. This response was diminished in the presence of PD98059, an inhibitor of activation of MAPK ($P<0.01$).

These results are inconsistent with a response at either a $P2Y_2$ or a $P2Y_6$ receptor alone, but they are consistent with a regulation of SHR smooth muscle proliferation by a $P2Y_4$ receptor at which UTP is a full agonist and ATP is a partial agonist.

Chang, K Hanaoka, K. Kamada, M. & Takuwa, Y. (1995) *J. Biol. Chem.* 270, 26152-26158.
Charlton, S.J., Brown,C.A. Weisman, G.A. et al. (1996) *Br. J. Pharm.* 119, 1301-1303.
Wilkie, N., Morton, C., Ng, L.L. & Boarder, M.R. (1996) *J. Biol. Chem.* 271, 32447-32453.

60P CARDIAC ENDOTHELIN-RECEPTOR FUNCTION IN THREE ANIMAL MODELS OF LEFT VENTRICULAR HYPERTROPHY

K. Becker, I. Heinroth-Hoffmann, P. Röhnert, M. Preiss, T. Weisselberg, C. Werner & O.-E. Brodde. Institute of Pharmacology, University of Halle-Wittenberg, D-06097 Halle (Saale), Germany

Endothelin (ET) is a potent vasoconstricting agent but exerts also cardiac effects including positive inotropic and growth-promoting effects (Sugden & Bogoyevitch, 1996). To study the role of ET-1 in cardiac hypertrophy, we assessed ventricular ET-receptor (R) density and functional responsiveness in 3 models of left ventricular hypertrophy: a) left ventricles from 12-14 weeks old male spontaneously hypertensive rats (SHR) vs. age-matched normotensive Wistar-Kyoto (WKY) rats, b) left ventricles from 4 weeks old male rats subtotally nephrectomized (SNX, according to Amann et al., 1993) 8 weeks after SNX vs. sham-operated rats and c) left ventricles from 4 weeks old male rats supra-renal aortic-banded (AOB, band-diameter 1.0 mm, according to Linz & Schölkens, 1992) 8 weeks after AOB vs. sham-operated rats. ET-R density was assessed by [^{125}I]-ET-1 binding, ET-1-induced inositol phosphate (IP) formation as accumulation of total [^{3}H]-IP's in [^{3}H]-myo-inositol labelled ventricular slices during a 45 min incubation at 37°C in Krebs-Henseleit solution that contained 10 mM LiCl, as detailled elsewhere (Becker et al., 1996). All data are means ± SEM of n experiments.

Results: 1) SHR: In the SHR the heart/body weight (H/BW) ratio (4.2±0.06 mg/g, n=30) was significantly higher ($p<0.01$) than in WKY-rats (3.3±0.05 mg/g, n=26). ET-1 (10^{-10}-10^{-6}M) concentration-dependently increased IP-formation in ventricular slices: maximal increase (E_{max}), however, was in SHR (172 ± 15% above basal, n=8) significantly ($p<0.01$) higher than in WKY-rats (112±12%, n=10). On the other hand, ET-R density did not differ significantly between SHR and WKY-rats (238±63 vs. 256±25 fmol/mg protein, n=7 and 5, resp.). 2) SNX-rats: Eight weeks after subtotal nephrectomy the H/BW ratio was in SNX-rats (4.02±0.14 mg/g, n=32) significantly higher ($p<0.01$) than in sham-operated (control) rats (3.4±0.12 mg/g, n=25). ET-1 increased IP-formation significantly more in SNX-rat ventricular slices (E_{max}: 129±14% above basal, n=9) than in control (E_{max}: 79 ± 8%, $p < 0.02$, n=7); in addition, ET-R density was in SNX-rats (232±40 fmol/mg protein, n=8) significantly ($p<0.05$) higher than in control (144±7 fmol/mg protein, n=10). 3) AOB-rats: Eight weeks after aortic-banding the H/BW ratio was in AOB-rats (4.4±0.05 mg/g, n=25) significantly higher ($p<0.01$) than in sham-operated (control) rats (3.3±0.05 mg/g, n=19). ET-1 increased IP-formation in ventricular slices from AOB-rats (E_{max}: 174±23% above basal, n=10) significantly more than in control (E_{max}: 108±11%, n=7, $p < 0.05$).

Conclusion: From the fact that in 3 models of left ventricular hypertrophy a similar degree of hypertrophy is accompanied by enhanced ET-1-induced IP-formation we conclude that ET-1 might play an important role for the development of cardiac hypertrophy.

Amann, K., Irzyniec, T., Mall. G. & Ritz, E. (1993) *J. Hypertens.* 11, 969-975.
Becker, K., Erdbrügger, W., Heinroth-Hoffmann, I., Michel, M.C. & Brodde, O.-E. (1996) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 354, 572-578.
Linz, A. & Schölkens, B.A. (1992) *Br. J. Pharmacol.* 105, 771-772.
Sugden, P.H. & Bogoyevitch, M.A. (1996) *Trends Cardiovasc. Med.* 6, 87-94.

61P YC-1 IS NOT A PURE AGONIST OF THE SOLUBLE GUANYLATE CYCLASE

H. Ruetten, M. Keil, B.A. Schoelkens & P.A. Martorana, Hoechst Marion Roussel, 65926 Frankfurt/M, Germany.

YC-1 (3-(5'-hydroxymethyl-2'furyl)-1-benzyl indazole has been reported to be a novel direct activator of soluble guanylate cyclase (sGC) with antiaggregatory and anti-thrombotic activities (Ko *et al.*, 1994; Teng *et al* 1997). However, the vasodilatatory effects of this compound have not been investigated in detail. In the present study, the effects of YC-1 were investigated on aortic rings from control rabbits as well as from rabbits with dietary and genetically-induced hyper-cholesterolaemia. The presence of the atherosclerotic lesions was assessed by means of light microscopy (LM) and scanning electron microscopy (SEM).

Three groups of male rabbits (2.5-3.5 kg) were studied. Group 1: New Zealand White (NZW) rabbits (C; n=6; standard control diet); group 2: NZW rabbits (H; n=8; 0.25% cholesterol and 3% coconut oil for 12 weeks); group 3: Froxfield Heritable Hypercholesterolaemic rabbits (FFH; n=8; standard control diet). All rabbits were sacrified at 25 weeks of age. The aorta was removed and endothelium-intact vessel rings of 3-4mm were suspended in an organ chamber. Rings were precontracted with phenylephrine (PE; 1μM) and the relaxant response to acetylcholine (Ach; 10^{-9} -$3x10^{-6}$M), as well as in the presence of L-NAME (10^{-5}M) to YC-1 ($3x10^{-7}$-$3x10^{-4}$M) and SIN-1 (10^{-8}-$3x10^{-5}$M) was assessed. In addition, samples of aorta from rabbits of all groups were fixed either in formalin or in glutaraldehyde and processed LM or SEM, respectively. Statistics: one-way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni *t* test to compare means between groups. $P<0.05$ were considered statistically significant. Data are expressed as mean±s.e.mean.

At LM the aortae of C rabbits were normal. On the other hand, 4/8 H and 8/8 FFH showed atheromatous changes. At SEM the C showed no lesions, while 1/8 H and 6/8 FFH rabbits had plaques. The maximum relaxation to ACh was significantly reduced in both H (68±4%) and FFH (55±4%) rabbits (both $P<0.05$), when compared to C rabbits (98±8%), indicating endothelial dysfunction. YC-1 as well as SIN-1 produced, at the highest concentration used, almost complete relaxation of PE-precontracted aortic rings (YC-1: 91±4%; SIN-1: 98±3%;) in C rabbits. YC-1-induced maximal relaxation was similar in C, FFH and H rabbits (91±4%; 84±7%; 81±4%). In contrast, for SIN-1, the maximum relaxation was significantly reduced in FFH (75±7; $P<0.05$), but not in H (83±3%) rabbits, when compared to C rabbits. ODQ (10^{-5}M; a selective inhibitor of sGC; Garthwaite *et al.*, 1995) inhibited only partially the relaxation to YC-1 (C: -59±4%; H: -62±6%; FFH: -61±4%). On the other hand, ODQ almost completely reversed the relaxation response to SIN-1 in all groups (C: -85±8%; H: -83±4%; FFH: -90±4%).

In summary, YC-1 is capable of inducing a concentration-dependent relaxation of aortic rings which is not endothelium-dependent, and which is only in part due to direct activation of the sGC. Thus, mechanisms of action other than a direct stimulation of sGC, may contribute to the vasorelaxant activity of YC-1.

Ko, F.N., Wu, C.C., Kuo, S.C. (1994) *Blood* 84:4226-4233.
Teng, C.M. *et al.* (1997) *Eur. J. Pharmacol.* 320:161-166.
Garthwaite, J. *et al.* (1995) *Mol. Pharmacol.* 48:184-188.

62P POSSIBLE MECHANISM OF THE NEGATIVE INOTROPIC EFFECTS OF α_1-ADRENOCEPTOR AGONISTS IN RAT ISOLATED LEFT ATRIA AFTER EXPOSURE TO FREE RADICALS

S.L.M. Peters, H.D. Batink, *M.C. Michel, M. Pfaffendorf and P.A. van Zwieten. Dept. Pharmacotherapy, Academic Medical center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. * Dept. Medicine, Uni-klinikum Essen, Hufelandstraße 55, 45147 Essen, Germany.

In a previous study we have shown that free radicals (generated via electrolysis of the organ bath fluid) reduce responses to a variety of inotropic stimuli in isolated rat left atria (Peters *et al.*, 1997). Additionally, we reported that the α_1-adrenoceptor agonists methoxamine, cirazoline and ST 587 (2-chloro-5-trifluoro-methyl-phenylamino-2-imidazolidine) all induce negative inotropic actions in isolated atria exposed to free radicals. The present study was designed to investigate the underlying mechanism(s) of the latter phenomenon in more detail. The initial force of contraction prior to application of electrolysis amounted to 9.4 ± 0.3 mN (n=10). Whereas the force of contraction remained stable in control preparations, a gradual decrease in contractile force was observed in atria subjected to electrolysis. Accordingly, the basal force of contraction determined 30 min. after electrolysis was 3.6 ± 0.5 mN (approximately 40% of initial force of contraction) vs. 8.6 ± 0.4 mN (approximately 90% of initial force of contraction) in control preparations. Methoxamine (300 μM, added 30 min. after the begin of the experiment) increased contractile force by 1.6 ± 0.2 mN in control atria but decreased contractile force in electrolysis-treated atria by 2.0 ± 0.1 mN (n=4-6, $P<0.05$), as determined 10 min after methoxamine addition. In contrast, the positive inotropic effects of endothelin-1 (30 nM) and isoprenaline (10 μM) were reduced from 2.6 ± 0.3 to 1.3 ± 0.1 mN and from 2.6 ± 0.3 to 1.7 ± 0.2 mN, respectively, by electrolysis treatment (n=4-6, $P<0.05$), but these agents did not cause any reduction of contractile force. In an inositol phosphate assay we observed that the stimulation of phospholipase C by methoxamine was attenuated by electrolysis when the (electrolysed) medium from the organ bath was used (136 ± 19% over basal in control vs. 68 ± 15% over basal in electrolysis treated atria). However, the phospholipase C responses were restored by the use of fresh medium. Medium refreshment did not counteract the negative inotropic effect of methoxamine. Accordingly, the negative inotropic effect of methoxamine is not directly related to the impaired phospholipase C responses seen in atria subjected to electrolysis. Ouabain (10 μM) and the protein kinase C inhibitor calphostin C (50 nM), when added 15 min. prior the addition of methoxamine, completely prevented the negative inotropic effect of 300 μM methoxamine in electrolysis treated atria, indicating a possible involvement of the Na^+/K^+ ATPase and/or PKC in the negative inotropic effects of methoxamine after oxidative stress. Measurement of the Na^+/K^+ ATPase activity by determination of the K^+-dependent *p*-nitrophenyl phosphatase activity, revealed that in control atria α_1-adrenoceptor stimulation with 300 μM methoxamine, *de*creased the Na^+/K^+ ATPase activity by 14.4 ± 7.7%. In contrast, methoxamine *in*creased the Na^+/K^+ ATPase activity by 48.8 ± 8.9% (n=6, $P<0.05$) in electrolysis treated atria. Interestingly this increase in Na^+/K^+ ATPase activity was completely suppressed by calphostin C (1.4 ± 0.1% over basal). These results indicate that the negative inotropic effects of α_1-adrenoceptor agonists, observed in isolated rat left atria exposed to free radicals, may be the result of a protein kinase C mediated phosphorylation and subsequent activation of the Na^+/K^+ ATPase.

Peters, S.L.M., Pfaffendorf, M. & van Zwieten, P.A. (1997). *Naunyn Schmiedeberg's Arch. Pharmacol.*, 355:390-397.

63P INFLUENCE OF H_2O_2 ON INOTROPIC RESPONSES TO ADRENOCEPTOR AGONISTS IN RAT ISOLATED HEARTS

S.L.M. Peters, M-J. Mathy, H.D. Batink, M.Pfaffendorf and P.A. van Zwieten. Academic Medical Center, University of Amsterdam, Dept. Pharmacotherapy, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

In a previous study we have shown that free radicals (generated via electrolysis of the organbath fluid) reduce responses to a variety of inotropic stimuli in isolated rat left atria. Additionally we have found that the α_1-adrenoceptor agonists methoxamine, cirazoline and ST-587 all induce negative inotropic actions in isolated rat left atria subjected to oxidative stress (Peters et al., 1997). This negative inotropic effect is most likely the result of a protein kinase C-mediated activation of the Na^+/K^+ ATPase (Peters et al., 1997b). The present study was undertaken to investigate the influence of oxidative stress on adrenoceptor agonist mediated inotropic responses in isolated heart preparations. Hearts of male Wistar rats (weighing 300-350 g) were perfused with a constant flow of 10 ml/min., according to the method of Langendorff. Left ventricular end diastolic pressure (LVEDP) was set at 10 mmHg via a latex balloon inserted into the left ventricle. Hydrogen peroxide and drugs were infused via a side arm just above the aortic cannula. The final concentration of H_2O_2 was 600 μM, and it was infused for 9 min., followed by a 30 min. washout period. After H_2O_2-infusion a rapid increase in left ventricular pressure (LVP) was seen, which reached a stable maximum (59.3 ± 6.7 mmHg) 30 min. after the end of the infusion period. In control preparations the LVP remained stable (40.0 ± 1.7 mmHg). There occured no significant change in LVEDP due to H_2O_2 infusion. In hearts perfused with H_2O_2 the inotropic response to 30 nM isoprenaline (infused after the 30 min. washout period) was reduced (45.1 ± 8.2 mmHg) when compared to control preparations (78.0 ± 4.5 mmHg) (n=4, $P<0.05$). Methoxamine (30 μM) increased LVP by 38.1 ± 3.7 mmHg in control hearts but decreased LVP in H_2O_2-perfused hearts by 3.0 ± 3.3 mmHg (n=6-9, $P<0.05$), determined after 15 min. of agonist infusion. Adrenoceptor binding studies showed a significant reduction in binding affinity (K_d) for the α_1-adrenoceptor (0.19 ± 0.04 for control vs. 0.08 ± 0.03 nmol/l for H_2O_2 perfused hearts, n=3, $P<0.05$). No differences were found in the K_d of β-adrenoceptors nor in B_{max} values for α- and β-adrenoceptors. In the presence of the protein kinase C inhibitor calphostin C (10 nM), 30 μM methoxamine induced a significant positive inotropic response (10.9 ± 5.1 mmHg) in H_2O_2-perfused hearts (n=6-9, $P<0.05$). In contrast to our findings in atrial preparations, ouabain (10 μM) proved ineffective in this respect in isolated hearts; we observed no significant positive inotropic effect of methoxamine in the pressence of ouabain (4.7 ± 5.7 mmHg, n=6-8). Indeed, measurement of the Na^+/K^+ ATPase activity 15 min. after methoxamine stimulation showed that there were no significant differences between control- and H_2O_2-perfused hearts (67.0 ± 31.6% vs. 102.3 ± 31.4% over basal, respectively). From these results we conclude that oxidative stress reduces inotropic responses to adrenoceptor stimulation in isolated hearts in a similar fashion as in isolated left atria, although the negative inotropic actions of methoxamine are less pronounced in perfused hearts. It is unlikely that the reduced responses to methoxamine are caused by a protein kinase C mediated activation of the Na^+/K^+ ATPase, because ouabain and calphostin C had no or little effect on the methoxamine induced inotropic actions. The diminished response to methoxamine may be the result of a reduced K_d of the α_1-adrenoceptor or caused by an injury of the contractile apparatus. This difference with the findings in isolated atria might be caused by the presence of ventricular tissues, or by the fact that H_2O_2 and electrolysis generate different types of free radicals.

Peters et al. (1997). *Naunyn Schmiedeberg's Arch. Pharmacol.*, 355:390-397.
Peters et al. (1997b). *This Meeting.*

64P EVIDENCE AGAINST ANANDAMIDE AS AN EDHF MEDIATING THE VASODILATOR ACTION OF BRADYKININ (BK) IN THE RAT HEART

John Quilley and David Fulton. Dept. Pharmacology, New York Medical College, Valhalla, NY 10595, USA. (Introduced by M. Carroll).

In the isolated heart of the rat, BK elicits endothelium-dependent vasodilation that is independent of NO but susceptible to inhibitors of cytochrome P450 (P450), phospholipases and Ca^{2+}-activated K^+ channels, implicating a P450-derived metabolite of arachidonic acid (AA) as an EDHF (Fulton et al., 1994, 1995, 1996). Recently, anandamide (arachidonylethanolamide) was proposed as a hyperpolarising factor in the rat mesentery (Randall et al., 1996). Consequently, we addressed the possibility of anandamide as a mediator of the NO-independent coronary vasodilator effect of BK using pharmacological interventions shown to inhibit coronary responses to BK. We also tested the effects of the cannabinoid receptor antagonist, SR141716A, on vasodilator responses to BK and anandamide.

Using the rat isolated perfused heart treated with nitroarginine (50μM) and indomethacin (2.8μM) to isolate P450-dependent responses to BK and elevate perfusion pressure (PP) from approx. 30-40 mmHg to 130-140 mmHg, we compared vasodilator responses to anandamide and AA in the presence of nifedipine (5nM), charybdotoxin (10nM; CTX) and SR141716A (2μM) to inhibit voltage-dependent Ca^{2+} channels, Ca^{2+}-activated K^+ channels and cannabinoid receptors (CB1), respectively. Nifedipine and SR141716A reduced PP which was restored to its original level with U46619. In vehicle control preparations (n=8), 1, 3 and 10μg anandamide dose-dependently reduced coronary PP by 11±2, 24±3 and 40±3mmHg, respectively. AA, at the same doses, reduced PP by 10±1, 19±2 and 45±3 mmHg, respectively. CTX (n=5), at a concentration shown to markedly reduce vasodilator responses to BK (Fulton et al., 1994) was without effect on vasodilator responses to anandamide but reduced the responses to 1, 3 and 10μg AA to 4±1, 9±1 and 22±4 mmHg, respectively. In the presence of nifedipine (n=4), 1, 3 and 10μg anandamide reduced PP by 12+1, 17+2 and 24+6 mmHg, respectively, whereas AA decreased PP by 8±1, 11±2 and 23±4 mmHg, respectively.

Inhibition of CB1 receptors with SR141716A (n=6) reduced the vasodilator responses to the lower doses (1 and 3μg) of anadamide (6±1 and 15±2 mmHg) but was without effect on the response to the highest dose (10μg). Responses to AA were unaffected. Similarly, vasodilator responses to BK were not affected by SR141716A (n=4). Thus, in vehicle control preparations (n=4), 10, 100 and 1000ng BK decreased PP by 8±2, 46±6 and 63±4 mmHg, respectively, compared to 7±1, 42±4 and 57±4 mmHg, respectively, in the presence of SR141716A. Collectively, these results provide strong evidence against anandamide as a mediator of the NO-independent vasodilator effect of BK in the isolated heart of the rat.

Fulton et al., 1994.Br. J. Pharmacol. 113: 954-948.
Fulton et al., 1995. Br. J. Pharmacol. 114: 99-102.
Fulton et al., 1996. J. Pharmacol. Exp. Ther. 278: 518-526.
Randall et al, 1996. Biochem. Biophys. Res. Commun. 229: 114-120.

65P STUDIES ON THE EFFECTS OF CANNABINOID RECEPTOR LIGANDS IN THE SMALL MESENTERIC ARTERY OF THE RAT

Richard White & C. Robin Hiley. Department of Pharmacology, University of Cambridge, Cambridge CB2 1QJ

Randall *et al.* (1996) proposed that anandamide, an endogenous cannabinoid, might be endothelium-derived hyperpolarising factor (EDHF). In part, this conclusion was based on the sensitivity of vasorelaxation to both EDHF and anandamide to the cannabinoid receptor antagonist, SR 141716A. In view of the importance of cannabinoid receptor ligands to their conclusion, we report here further studies on their vasorelaxant actions.

Two mm long segments of third generation mesenteric arteries from male Wistar rats (250-300g) were mounted in a myograph under a normalised tension (Mulvany & Halpern, 1977) in Krebs buffer (mM: NaCl 115.3, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5, glucose 5.5) equilibrated with 5% CO_2 in O_2 at 37°C. If required, endothelium was removed by rubbing the intima with a hair. Vessels were precontracted with a submaximal concentration of methoxamine (10 μM) and exposed to 10 μM carbachol (CCh); >90% or <5% relaxation, respectively, was taken as showing the presence or absence of endothelium. Cumulative concentration/relaxation curves to cannabinoid agents were determined in methoxamine- or KCl- (60 mM) preconstricted vessels. When used as an antagonist, vessels were equilibrated with SR 141716A for 30 min before determining concentration/response data. Data were fitted to a logistic equation (McCulloch *et al.*, 1997) except where a clear maximum response was not obtained when the potency is given as the negative logarithm of the concentration giving 50% relaxation of induced tone ($pEC_{50\%}$). Statistical comparisons were by analysis of variance.

Palmitoylethanolamide, a putative endocannabinoid acting at CB_2 receptors, gave concentration-dependent, but endothelium-independent, relaxations ($pEC_{50\%}$ = 4.37±0.09, R_{max} [maximum response] = 63.8±8.3% reduction of induced tone, n = 6). Precontracting vessels with KCl instead of methoxamine reduced responses to palmitoylethanolamide (*e.g.* at 100 μM to 19.5±2.5%; $P < 0.001$; n = 4) but the responses were not affected by the presence of 1 μM apamin plus 300 nM charybdotoxin.

The cannabinoid receptor agonists HU-210 and WIN 55,212-2 also gave endothelium-independent relaxations. HU-210 had a pEC_{50} of 5.73±0.10 and an R_{max} of 77.7±3.9% (n = 4). WIN 55,212-2 had a $pEC_{50\%}$ of 4.20±0.02 (n = 4) and a response at the highest concentration that could be used (100 μM) of 66.8±3.7%. Precontraction with KCl reduced the potency of HU-210 (pEC_{50} = 5.08±0.17, $P < 0.01$, n = 4) but had no effect on the R_{max} (77.9±7.8%). For WIN 55,212-2, precontraction with KCl had no significant effect on the response at 0.1 mM (69.6±4.3%, n = 4) or on the $pEC_{50\%}$ (4.23±0.05).

The cannabinoid antagonist, SR 141716A (1 μM) had no effect on relaxations to CCh, levcromakalim or the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP). However 10 μM SR 141716A inhibited relaxations to CCh and levcromakalim but not to SNAP. SR 141716A alone caused endothelium-independent relaxation ($pEC_{50\%}$ = 4.40±0.07; response at 100 μM SR 141716A = 72.8±6.2; n = 4) which was not signficantly affected by precontracting with KCl instead of methoxamine ($pEC_{50\%}$ = 4.33±0.16; response at 100 μM SR 141716A = 58.5±7.6; n = 6). The relaxant potency of SR 141716A was increased by the phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine (IBMX; 50 μM: control, $pEC_{50\%}$ = 4.31±0.04; n = 8; IBMX, $pEC_{50\%}$ = 4.55±0.11; n = 7; $P < 0.05$) but the R_{max} was unchanged (control, 64.0±4.2; IBMX, 71.0±6.5).

These results show that the relaxant mechanisms of some cannabinoid receptor ligands are different to those of EDHF. Furthermore, although SR 141716A blocks the actions of EDHF, this effect may not be due to antagonism of cannabinoid receptors.

RW is an A.J. Clark student of the British Pharmacological Society.

McCulloch, A.I. *et al.* (1997). *Br. J. Pharmacol.*, 120, 1431-1438.
Mulvany, M.J. & Halpern, W. (1977). *Circ. Res.*, 41, 19-26
Randall, M.D. *et al.* (1996) *Biochem. Biophys. Res. Commun.*, 229, 114-120.

66P THE CANNABINOID RECEPTOR ANTAGONIST SR141716A REDUCES L-DOPA-INDUCED DYSKINESIA IN THE MPTP-TREATED PRIMATE MODEL OF PARKINSON'S DISEASE

J.M. Brotchie, S.Fox, B. Henry, C. Hille C, Y. Maneuf, S. McGuire, D. Peggs, A.R. Crossman. School of Biological Sciences, University of Manchester, UK.

Dopamine-replacement therapy, primarily using the dopamine precursor L-dihydroxyphenylalanine (L-DOPA) represents the mainstay of current pharmacological treatments for Parkinson's disease. However, long term dopaminergic therapy is invariably compromised by several side effects including involuntary movements characterised by chorea and dystonia i.e. L-DOPA-induced dyskinesia. L-DOPA-induced dyskinesia can become so severe as to severely compromise the anti-parkinsonian utility of dopamine replacement therapy. The neural mechanisms underlying L-DOPA-induced dyskinesia include abnormalities of GABAergic transmission within the globus pallidus and substantia nigra pars reticulata. We have recently demonstrated that cannabinoid receptors play a crucial role in regulating synaptic GABA levels in these regions. In the present study we have investigated whether reducing endogenous cannabinoid tone with the cannabinoid receptor antagonist SR141716A (Rinaldi-Carmona et al., 1995) can reduce L-DOPA-induced dyskinesia in the MPTP-treated primate.

Marmosets were rendered parkinsonian with MPTP (2 mg/kg s.c., on 5 consecutive days). Following stabilisation of the parkinsonian state (7 months post-MPTP), marmosets were administered L-DOPA/ benserazide (as Madopar) (12.5 mg/kg orally twice a day for 3 weeks). Within a week of commencing treatment, marmosets showed dyskinesias whilst on L-DOPA-treatment. By 21 days, stable dyskinesia, characterised by an idiosyncratic mix of chorea and dystonia was observed. The anti-parkinsonian actions of drug treatments were assessed using a parkinsonian disability rating scale. Mobility was assessed using a scale that assessed the range of movements performed in a given time period. Dyskinesia was assessed using a scale that assessed severity of chorea and dystonia over time. Drugs and/or vehicle were administered orally and behaviour was videotaped. Animals were not disturbed during the assessment period. Behavioural analysis was performed by a clinically-qualified observer blinded to the treatment. Analyses were carried out over the 90 minutes immediately following drug administration.

Following vehicle treatment, the median disability score was 17 (range 2-35, n=4), median mobility score was 12.5 (range 8-20, n=4), median dyskinesia score was 0 (n=4). Following L-DOPA-treatment (12.5 mg/kg) parkinsonian symptoms were reversed, median disability score 0 (n=4, P<0.05, non-parametric Page test) and median mobility score 26.5 (range 24-27, n=4). Dyskinesia commenced within 5 minutes of L-DOPA administration and was maintained throughout the period of assessment. Median dyskinesia score was 17.5 (range 3-21, n=4). Co-administration of SR141716A (0.3 mg/kg) with L-DOPA did not affect the anti-parkinsonian action of L-DOPA, median disability score 0 (range 0-6) median mobility score 25.5 (range 19-27) (both n=4, P>.05 compared to L-DOPA alone, Page test). In contrast dyskinesia was reduced, median dyskinesia score 3 (range 1-14, P<.05, Page test).

These data demonstrate that blockade of cannabinoid receptors can reduce L-DOPA-induced dyskinesia without affecting the anti-parkinsonian efficacy of L-DOPA. They suggest that endogenous cannabinoid systems play a key role in the generation of inappropriate movements following dopamine receptor stimulation in the parkinsonian primate.

Rinaldi-Carmona M., Barth F, Heaulme M, et al. (1995) Life Sci. 56: 1941-7.

67P ANTI-PARKINSONIAN EFFECT OF NR2B-SELECTIVE NMDA ANTAGONISTS: A STRIATAL MECHANISM OF ACTION?

J.E. Nash, A.R. Crossman, J.M. Brotchie.
School of Biological Sciences, University of Manchester, UK

The symptoms of Parkinson's disease are generated, in part at least, by overactive NMDA-transmission in the striatum of the basal ganglia (Greenamayre, 1993). However, due to the widespread distribution of NMDA receptors throughout the central nervous system, systemic administration of NMDA antagonists is generally associated with many side effects that would compromise any anti-parkinsonian action. Subtypes of NMDA receptors arise from distinct combinations of different subunits (NR1a-h, NR2A-D), each displaying differing degrees of modulation by ligands acting at regulatory sites on the receptor (Kutsuwanda et al., 1992). NR2B-containing NMDA receptors predominate in the striatum (Standaert et al., 1994), and display a high affinity for glycine (Sakurai, 1993) and polyamine site antagonists (Williams et al., 1993). We have previously shown that systemic administration of the polyamine antagonist ifenprodil alleviates parkinsonian symptoms in rats and primates, without producing any visible side-effects (Mitchell et al., 1995). The current work was carried out to determine the mechanisms underlying the anti-parkinsonian action of ifenprodil.

Male Sprague-Dawley rats (250-275g) were rendered hemi-parkinsonian using 6-hydroxydopamine (6-OHDA) (Understedt, 1968) (12.5 µg in 1 µl, injected into the medial forebrain bundle under general anaesthesia [pentobarbitone, 60mg/kg i.p.]). Twenty one days post operation, polyamine site (ifenprodil 10-100nmol in 0.5µl) or glycine site (7-chlorokynurenate 37nmol in 0.5µl; HA-966 44-400nmol in 0.5µl) NMDA antagonists/ partial agonists were injected into the dopamine-depleted striatum. Ifenprodil, HA-966 and 7-chlorokynurenate induced a dose-dependent rotational response contraversive to the injection site (e.g. ifenprodil (100nmol): 150±50 turns, compared to vehicle 0±4, ANOVA, post-hoc SNK test, $P<0.01$ n=10). Additionally, there was topographical variation in the effect of these drugs. Increased mobility was seen subsequent to intrastriatal injection into the rostral, but not caudal striatum. Neither glycine nor polyamine site antagonists had effect when injected into the unlesioned striatum ($P>0.05$). Competitive (D-APV 14nmol) and non-competitive (MK-801 24nmol) NMDA antagonists also induced contraversive rotational behaviour when injected into the rostral striatum, but their effect was less marked and was not selective for the lesioned striatum. Radioligand binding studies were carried out using glutamate/glycine stimulated [^{3}H]-MK-801 protocols to define NMDA channel function in a striatal membrane preparation (pH7.4; 25°C). In rat models of Parkinson's disease, it was found that the striatal NMDA receptor has a higher affinity for polyamine antagonists compared to normal rats. Thus, in membranes prepared from reserpine treated rats, ifenprodil was more potent in reducing MK-801 binding than in membranes from vehicle-treated animals, (IC_{50} 37µM (reserpine) compared to 143µM (vehicle) (RM-ANOVA, $P<0.01$; n=6)).

Selective blockade of NR2B-containing NMDA receptors in the rostral striatum may have potential as a novel therapeutic approach to the treatment of parkinsonian symptoms.

Greenamayre (1993) J. Neural. Transm. Gen. Sect. 91: 255-269
Kutsuwanda et al. (1992) Nature, 358: 36-41
Mitchell et al. (1995) Behav. Pharmacol. 6, 492-507.
Sakurai et al. (1993) J. Neurochem. 60, 1344-1353.
Ungerstedt (1968) Eur. J. Pharmacol. 5, 107-110.
Williams et al. (1991) Life Sci. 48, 469-498.

68P REPEATED i.c.v. ADMINISTRATION OF 5-HT$_7$ RECEPTOR ANTISENSE OLIGONUCLEOTIDE DOWN-REGULATES 5-HT$_7$ RECEPTOR BINDING IN THE RAT HYPOTHALAMUS WITHOUT ALTERING EXPLORATORY BEHAVIOUR

D.A. Clemett, M.I. Cockett[1], C.A. Marsden, & K.C.F. Fone.
School of Biomedical Sciences, Queen's Medical Centre, Nottingham NG7 2UH. [1]Wyeth-Ayerst Research, CN8000 Princeton, NJ 08536 USA.

5-HT$_7$ receptor activation mediates relaxation in both vascular and non-vascular smooth muscle (see review, Eglen *et al.*, 1997) and may account for the prolonged hypotensive effect of intravenous 5-HT in anaesthetised rats (De Vries *et al.*, 1997). Within the central nervous system 5-HT$_7$ receptor function is less clearly defined. 5-HT$_7$ receptor binding sites have been located by autoradiography in rat thalamus, hippocampus, cortex and hypothalamus (Gustafson *et al.*, 1996). As there are currently no selective ligands available to study the potential role of this receptor, we have used intracerebroventricular (i.c.v.) injections of antisense oligonucleotides to investigate the effects of 5-HT$_7$ receptor down-regulation on functions in which the hypothalamus is involved.

Adult male hooded Lister rats (205-255g) were implanted under halothane anaesthesia (2% v/v in nitrous oxide) with guide cannulae into the left lateral ventricle (A -0.9mm, L +1.4mm, D -1.8mm from bregma) 10 days prior to i.c.v. administration of oligonucleotides or vehicle (n=12, each group). A 19mer antisense oligonucleotide, (AO) complementary to bases 10 - 28 of the coding region, or mismatch (MO) control, end-capped at both ends by two phosphorothioate modifications, was injected i.c.v. twice daily (2µg in 2µl sterile water) for six days. On day 4 of treatment locomotor activity was monitored for 20min in computerised infra-red beam activity boxes. 20h after the final injection, behaviour was monitored for 5min on an elevated plus maze, immediately before decapitation and collection of trunk blood for plasma corticosterone radioimmunoassay (ImmunoDiagnostic Systems). Hypothalami were immediately dissected out for determination of 5-HT$_7$ receptor levels in pooled hypothalamic membranes using the [^{3}H]5-HT binding method of Sleight *et al.* (1995), modified by increasing the masking concentration of pindolol to 3µM. Values reported are mean±s.e.mean.

AO treatment significantly reduced hypothalamic 5-HT$_7$ receptor density (by 43%, $p<0.01$, Duncan's New Multiple Range following ANOVA; $F_{(2,7)}=69.4$, $p<0.0001$) from a B_{max} of 78.3±5.9 fmol mg protein^{-1} in vehicle-treated animals, whilst K_D values remained unchanged (AO, 2.3±0.3; MO, 2.4±0.1; and vehicle, 4.5±0.8nM). On the elevated plus maze, AO treatment had no effect either on % of total entries made into the open arms (AO, 38±3; MO, 45±3 and vehicle, 40±3%)or on the % of total time spent in the open arms (AO, 29±4; MO, 42±5 and vehicle, 33±5%). Similarly, there was no effect on ethological behaviours (rears, head-dips and stretch attends) observed on the maze. Analysis of data from computerised activity boxes showed that AO had no significant effect on locomotor activity (AO, 254±23; MO, 261±24 and vehicle, 255±23 beam breaks in 20min). Plasma corticosterone levels, following exposure to the elevated plus maze, were also unchanged by AO administration (AO, 66.9±10.8; MO, 60.6±5.5; vehicle 65.3±5.2ng ml^{-1}).

These data indicate that repeated i.c.v. administration of a 5-HT$_7$ antisense oligonucleotide attenuates expression of 5-HT$_7$ receptor binding sites in the rat hypothalamus. This decrease in receptor levels was not associated with any change in locomotor activity, anxiogenic/anxiolytic effects or alteration of plasma corticosterone levels. Nevertheless, this antisense strategy has potential as a means of elucidating a central function for the 5-HT$_7$ receptor.

DAC is the recipient of an MRC studentship in collaboration with Wyeth-Ayerst Research.

De Vries, P., Villalón, C.M., Heiligers, J.P.C. *et al.* (1997) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 356, 90-99.
Eglen, R.M., Jasper, J.R., Chang, D.J. *et al.* (1997) *Trends Pharmacol. Sci.* 18, 104-107.
Gustafson, E.L., Durkin, M.M., Bard, J.A. *et al.* (1996) *Brit. J. Pharmacol.* 117, 657-666.
Sleight, A.J., Carolo, C., Petit, N. *et al.* (1995) *Mol. Pharmacol.* 47, 99-103.

69P LAMOTRIGINE DECREASES ULTRASOUND-INDUCED DEFENCE BEHAVIOURS IN THE LISTER HOODED RAT

Neophytou SI, Aspley S[1], Marsden CA and Beckett SRG
Biomedical Sciences, Neuroscience division, Nottingham University Medical School, Nottingham, NG7 2UH, [1]Knoll Pharmaceuticals R&D, Pennyfoot Street, NG1 1GF

Rats produce ultrasonic vocalisation (20-30 kHz) in aversive situations such as predator exposure (Blanchard *et al.* 1991). Previous studies have shown that artificially generated 20 kHz ultrasound (US) produces defensive flight behaviour in the Lister hooded rat similar to the response seen following electrical or chemical stimulation of brain regions known to mediate aversive responses. In addition, we have immunohistochemical evidence that US-induced defence is associated with activation of brain nuclei (e.g. periaquiductal grey, central nucleus of the amygdala) which constitute the brain aversive system (Beckett & Marsden, 1995) and it is sensitive to pharmacological manipulation with both anxiolytic and anxiogenic compounds (Beckett *et al.*, 1996). The use-dependent sodium channel blocker lamotrigine is used to treat epilepsy and is known to be a functional glutamate receptor antagonist (Goa *et al.*, 1993). This study investigated the effects of lamotrigine (0.5, 1.25, 5 mg/kg, 2 hrs pre-treatment) on the expression of 20 kHz induced aversive behaviour in the rat. Male Lister hooded rats (250-350g, n=8 per group) were placed in an open field arena with a wall mounted speaker. After a 2 min period without stimulus, animals received 1 min of 20 kHz US (91, 98 or 101 dB intensity) followed by a further 2 mins without sound. Locomotor behaviour was analysed via a computer tracking system. US exposure produced intensity-related defence behaviour characterised by a significant increase in the maximum speed and distance travelled. Lamotrigine produced a significant, dose dependent decrease in maximum speed and distance travelled (Table 1) in the period of US exposure without any sedative effects during the pre-stimulus period. Lamotrigine reduces seizure activity in rats (Miller *et al.*, 1986). These results therefore suggest that intense flight defence behaviours and pre-seizure running may form part of a behavioural continuum. These findings also further support the view that glutamate receptors are involved in the regulation of PAG mediated behaviours (Shaikh *et al.*, 1994).

Beckett, S.R.G., Aspley, S., Graham, M. *et al.* (1996) *Psychopharm.* 127, 384-390.
Beckett, S.R.G. & Marsden C.A. (1995) *J. Neurosc. meth.* 58, 157-161.
Blanchard, R.J., Blanchard, D.C., Agullana, R., *et al.* (1991) *Physiol. Behav.* 50, 967-972.
Goa, K.L., Ross, S.R.& Chrisp P. (1993) *Drugs* 46, 152-176.
Miller, A.A., Wheatley, P., Sawyer, D.A. *et al.* (1986) *Epilepsia*, 27, 483-9.
Shaikh, M.B., Schubert, K., Siegel, A. (1994) *Brain Res.* 635, 187-195.

Table 1. Effects of Lamotrigine (ip) on locomotor activity induced by 101 dB ultrasound (20 kHz) in the rat (n=8). *=p<0.05, **=p<0.01. 2 way ANOVA followed by 1-way ANOVA and post hoc Duncan's NMR.

	Distance travelled (m)		Maximum speed (m/s)	
Treatment	1 min pre-stimulus	During stimulus	1 min pre-stimulus	During stimulus
Vehicle (0.25%Methyl Cellulose)	3.4±0.6	5.2±0.9	0.7±0.1	1.5±0.2
Lamotrigine 0.5 mg/kg	3.1±0.4	5.8±0.9	0.6±0.1	1.5±0.2
Lamotrigine 1.25 mg/kg	3.3±0.4	4.4±0.5	0.4±0.1	1.0±0.2*
Lamotrigine 5.0 mg/kg	3.1±0.6	3.6±0.5*	0.5±0.1	0.6±0.1**

70P CHARACTERISATION OF THE PURINOCEPTOR SUBTYPE WHICH MEDIATES RESPONSES TO αβmeADP ON RAT LOCUS COERULEUS NEURONES

A.J. Sansum, I.P. Chessell and P.P.A. Humphrey, Glaxo Institute of Applied Pharmacology, University of Cambridge, Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1QJ.

The purine nucleotide αβmeADP has high affinity for [^{3}H]αβmeATP binding sites in the brain postulated to be P2X purinoceptors (Michel *et al.*, 1994). Functional studies have demonstrated that αβmeADP elicits excitatory responses in the locus coeruleus (LC) (Trezise *et al.*, 1995) and the medial vestibular nuclei (Chessell *et al.* 1997). In these studies it was not clear which type of purinoceptor was activated by αβmeADP since P2X and P2Y purinoceptors have been reported to co-localise in some neurones (Fröhlich *et al.*, 1996; Chessell *et al.*, 1997). The aim of the present study was to characterise the purinoceptor type which mediates the increase in the firing rate of rat locus coeruleus neurones elicited by αβmeADP.

Brain slices containing the locus coeruleus, from male Sprague-Dawley rats (100-150g), were placed in a recording chamber maintained at 32°C and perfused at 2ml min^{-1} with ACSF gassed with 95% O_2/ 5% CO_2. Extracellular recordings were made with glass microelectrodes filled with 2M NaCl. LC neurones were characterised by their regular spontaneous firing (0.5-4Hz) and sensitivity to noradrenaline. Spikes were filtered and amplified using standard neurolog units, digitised and discriminated online using an analogue-digital converter and Spike 2 software. Agonist responses were measured as the increase in the spontaneous firing frequency.

Applications of αβmeADP, αβmeATP and ADPβS (1-300μM) all produced concentration-dependent increases in the firing rate of LC neurones, although there were qualitative and quantitative differences in the responses. Applications of αβmeADP and αβmeATP had no effect upon the spike shape or amplitude and there was no fade of the response in the continued presence of these agonists. At 300μM the increases produced were 1.56 ± 0.19 Hz and 1.53 ± 0.03 Hz, respectively. Estimated EC_{50} values were 27 (18-40)μM (n=5) and 73 (37-145)μM (n=4), respectively. Conversely responses elicited by ADPβS faded in the continued presence of the agonist and at higher concentrations caused a decrease in the spike amplitude. At 300μM the increase produced by ADPβS was 2.3 ± 0.3 Hz. Concentration-effect curves for ADPβS yielded an EC_{50} value of 16.5 (9-30) μM (n=4).

Additive studies were performed in the continued presence of αβmeATP since the excitatory effects were sustained for this agonist. In the presence of 300μM αβmeATP, an additional application of ADPβS elicited a further and significant increase in the spontaneous firing rate of 1.39 ± 0.06 Hz, (n=4), whilst in similar experiments neither αβmeADP nor αβmeATP produced any further increase in the firing frequency of the LC neurones.

Responses to αβmeATP (100μM), αβmeADP (100μM) and ADPβS (30μM) were reduced following a 30min incubation with the P2 purinoceptor antagonist suramin (100μM) to 8.6 ± 5.1, 37.0 ± 5.4, 43.6 ± 11.9% of the control responses, respectively, and in the presence of PPADS the responses were reduced to 44.0 ± 9.8, 42.1 ± 17.6, 41.4 ± 6.1%, respectively. The inhibitory effect of these antagonists were fully reversible (n=4-6).

In conclusion these data suggest that two distinct P2 purinoceptor subtypes are present on rat LC neurones which can mediate increases in the spontaneous firing rate elicited by purine nucleotides. The ability of αβmeATP to produce excitatory responses suggests the involvement of ionotropic P2X purinoceptors which are sensitive to suramin and PPADS. The similarity between the responses elicited by αβmeADP and αβmeATP and the inability of αβmeADP to elicit further excitatory responses in the continued presence of αβmeATP suggests that they both act at the same receptor site, a P2X purinoceptor. The responses elicited by ADPβS were qualitatively and quantitatively different to those elicited by αβmeATP and this suggests that metabotropic P2Y purinoceptors mediating excitatory responses, may also be present in the LC. Since transcripts for the known αβmeATP sensitive P2X purinoceptor types are sparse or absent in the brain, including the LC, the molecular nature of the putative P2X purinoceptor identified in this study, which is also activated by αβmeADP, remains to be determined.

Chessell I.P., Michel A.D. *et al.*, 1996. Neuroscience 77(3), 783-791.
Fröhlich R., Boehm S. *et al.*, 1996. Eur. J. Pharmac. 315, 255-261.
Michel A.D., McGuire C. *et al.*, 1994. Br. J. Pharmac. 112, 513P.
Trezise D.J., Black M.D. *et al.*, 1996. Br. J. Pharmac. 117, 319P.

71P PARAVENTRICULAR NUCLEUS NEURONAL FIRING RATE AND PATTERN ARE RELATED TO HIPPOCAMPAL THETA ACTIVITY IN THE RAT

J. W. Smythe and B. Costall. Dept. of Pharmacology, Univ. of Bradford, Bradford, BD7 1DP

All mammals exhibit a rapid, adaptive response to acute stress characterized by activation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress activates cells in the paraventricular nucleus (PVN) of the hypothalamus, which secrete corticotrophin-releasing hormone (CRH) into the median eminence (Saphier, 1993). CRH stimulates the release of pituitary adrenocorticotrophin (ACTH) into the systemic circulation. ACTH promotes the rapid synthesis and efflux of corticosterone (CORT) from the adrenal gland into the plasma, by which it is carried to most body tissues and organs (Dallman *et al.*, 1995). Once the stressor has ended, a negative-feedback sytem in the brain and pituitary, sensitive to plasma CORT concentrations, acts to dampen further CRH/ACTH release. One of the principal sites involved in suppressing HPA axis activity is the hippocampus. Hippocampal theta activity is a rhythmic, sinusoidal waveform with a frequency range of 3-10 Hz that occurs in alert, immobile rats presented with threatening stimuli (Bland, 1986). This type of theta is abolished by cholinergic receptor antagonists and is reliably modelled using urethane-anaesthetized rats (Smythe *et al.*, 1992). In the present study, we have recorded hippocampal theta and large, irregular activity (LIA), while monitoring PVN cell responses to determine if on-going hippocampal electrical field activities signal alterations of PVN neuronal discharges.

Adult male, Lister hooded rats (350-550g) served as subjects. Under isoflurane anesthesia, rats were implanted with jugular cannulae for delivery of i.v. urethane (0.8g/ml) anesthesia. A hippocampal recording electrode (tungsten;impedance of 0.2-0.5 MΩ) was positioned in the stratum moleculare (A-P -3.3; M-L +2.5; D-V 2.5mm) to recordtheta and LIA activities, and PVN cells (A-P -2.1; M-L + 0.6; D-V 8-9mm) were recorded with glass micropipettes (1-3 MΩ) backfilled with 2M sodium acetate/direct blue dye. Theta and LIA states were discriminated using a computer-based FFT calculation, and discharge rates (mean±SD), and patterns (rhythmic, phasic, tonic) during each condition were assessed by auto-correlation and cross-correlation functions. Independent t-tests were used to compare discharge rates during theta with those during LIA applying a randomization process (Edgington, 1987). At the conclusion of each experiment a 0.25mA D.C. current was passed through the cell electrode to eject dye and localize placements.

A total of 18 cells were recorded from 11 rats, 17 of which were localized to the PVN region. 7/17 cells (41%) were either significantly more active during theta compared with LIA, or exhibited phasic-firing during theta, but no rhythmicity during LIA (Theta-On) (θ-5.57±1.88; LIA-3.39±1.14; $p<.05$). 2/17 cells (12%) were less active during theta compared to LIA (Theta-Off) (θ-0.43±0.18; LIA-1.67±1.0; $p<.05$). 8/17 cells (47%) were unrelated to theta (θ-3.87±1.29; LIA-3.47±1.1; ns), and 4 of these cells were rhythmic during theta and LIA.

These data show that some PVN cells are related to hippocampal field activity, and suggest that each region receives a common input or that PVN activity follows that occurring in the hippocampus. Changes in theta or LIA may be a transduction mechanism that signals alterations in PVN neuronal activity and HPA axis control.

Bland, B.H. (1986) *Prog. Neurobiol.* 54, 1-54.
Dallman, M.F. et al. (1995) *J. Neuroendo.*, 4: 519-526.
Edgington, E. Randomization Tests. Academic Press, N.Y. (1987)
Saphier, D. (1993) *Am. J. Physiology*, 264:891-902.
Smythe, J.W.(1992) *Neurosci.BioBehav. Rev.*16, 289-308.

72P THE ROLE OF THE HIPPOCAMPUS IN CONTROL OF THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS: AN *IN VITRO* APPROACH

D.N. McKenzie, R.E.J. Dyball* & J.C. Buckingham, Dept of Neuroendocrinology, Imperial College School of Medicine, Charing Cross Hospital, London W6 8RF. *Department of Anatomy, University of Cambridge, Cambridge CB2 3DY.

The hippocampus is well known to influence the neurones in the hypothalamic paraventricular nucleus (PVN) that secrete corticotrophin releasing hormone or vasopressin particularly at times of emotional or homeostatic stress and thereby promote the release of corticosterone. With the aim of developing an *in vitro* model in which to examine hippocampus-hypothalamus interactions, we have developed a protocol for producing organotypic hippocampus-hypothalamus co-cultures (McKenzie *et al.* 1997).

Slices of hypothalamus, containing the paraventricular nucleus (PVN), were obtained *post-mortem* from 5-7 day old rats and co-cultured with hippocampal slices (collected simultanaeously) at 36°C in a 5% CO_2-enriched atmosphere with a serum-containing medium. After at least 6 days, when they were found to have normal synaptic morphology under electron microscopy, the co-cultures were used for conventional electrophysiological recording (McKenzie *et al.* 1997). A stimulating electrode was placed on the hippocampal part of the co-culture (in either the dentate gyrus or CA2 region, with the electrode position verified after each experiment) and a recording electrode (0.5 M sodium acetate filled micropipette) was lowered into the parvocellular PVN.

Recordings were made from 14 PVN neurones. In each case, stimuli (1 ms x 1 ms paired bipolar, 1 mA) were applied at 1 Hz, and the incidence of action potentials before and after each stimulus recorded for 300-600 consecutive stimuli. Eight of these cells showed a reduced probability of firing action potentials during the 200 ms following hippocampal stimulation (typically from 0.276±0.042 pre-stimulus to 0.094±0.024 post-stimulus, mean±s.e.mean, $P<0.01$, Student's *t*-test). One cell showed an excitation (the probability of seeing an action potential was increased from 0.0368±0.0041 pre-stimulus to 0.241±0.056 post-stimulus, $P<0.01$, Student's *t*-test). Two cells showed complex effects (which involved both excitation and inhibition) and three were unaffected by hippocampal stimulation. When the stimulating electrode was localised to the dentate gyrus, then the only responses that could be recorded were inhibitory, whilst stimulation of the CA2 region evoked inhibitory, excitatory and complex responses. This pattern of connections is broadly in agreement with the findings of Dunn & Orr (1984) who found that electrical stimulation of the dentate gyrus reduced the plasma corticosterone concentration whilst stimulating the CA2 region increased the plasma corticosterone concentration. These results demonstrate the development of functional connections between hippocampal and hypothalamic slices and suggest that the co-culture system may offer a useful model of hippocampal regulation of the hypothalamo-pituitary axis.

The authors would like to thank Chemodyne SA for supporting this project.

Dunn J.D. & Orr S.E. (1984) *Exp. Brain Res.* 54, pp 1-9.
Mckenzie D.N.; Dyball R.E.J. & Buckingham J.C. (1997) *J. Physiol. proceedings* in press.

73P P2 RECEPTOR AGONISTS HAVE A MODULATORY EFFECT ON EVOKED GLUTAMATE RELEASE FROM RAT CORTICAL SLICES

G.C. Bennett & M.R. Boarder, Department of Cell Physiology and Pharmacology, University of Leicester, P.O. Box 138, University Road, Leicester LE1 9HN.

Adenine nucleosides (e.g. adenosine) and nucleotides (e.g. ATP) have been shown to inhibit electrically evoked noradrenaline (NA) release in rat cortical slices (von Kügelgen *et al.*, 1994). In this study we have investigated whether nucleosides and nucleotides can also be implicated in modulation of glutamate release from rat cortex.

Initial studies involved the measurement of total [^{3}H]-inositol phosphate accumulation in rat cross chopped cortices. Slices were loaded with [^{3}H]-inositol for 1 hr and stimulated in the presence of LiCl. Subsequent studies investigated glutamate release from superfused cortical slices using two depolarising pulses of 46 mM KCl, applied 30 mins apart. The first pulse (S_1) was always without agonists while in some cases the agonist (300 μM) was introduced 2 min before and during the second KCl pulse (S_2). Glutamate determination in the perfusate was by a fluorimetric technique involving the conversion of NADP to NADPH in the presence of glutamate dehydrogenase. Effect of agonists was determined by comparison of test S_2/S_1 ratios to control ratios.

Re-uptake of glutamate into the slices was investigated using the uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC), inclusion of which considerably increased the concentration of evoked glutamate observed.

ATPγS (300 μM, 5 min) gave a small but significant increase in total [^{3}H]-inositol phosphate accumulation (control, 3322 ± 88, ATPγS 4596 ± 287, $p < 0.05$, n = 4-5) and also had an inhibitory effect on evoked glutamate release (Table 1). The evoked glutamate release was significantly reduced by ATPγS ($p < 0.05$), ATP ($p < 0.01$) and adenosine ($p < 0.05$) whilst UTP exerted no effect.

Table 1: S_2/S_1 ratios showing the effect of compound treatment on KCl evoked glutamate release from rat superfused cortical slices.

	S_2/S_1 Ratio	
	Control	Treatment
ATPγS (n=8)	0.86 ± 0.15	0.54 ± 0.08
ATP (n=6)	0.73 ± 0.07	0.42 ± 0.07
Adenosine (n= 9)	0.79 ± 0.08	0.56 ± 0.07
UTP (n=6)	0.81 ± 0.13	0.95 ±0.09

These results are similar to those obtained in the NA release studies and would indicate that either 1) there is a P2 receptor and a P1 receptor present on rat brain cortex which both mediate glutamate release in an inhibitory manner, or 2) there is a common receptor which can be activated by adenine nucleosides and nucleotides to lead to inhibition of release. More work must be done to elucidate the mechanism of action and characterise the receptor(s) present by utilising specific antagonists.

von Kügelgen, I., Späth, L. & Starke, K. (1994). *Br. J. Pharmacol.*, **113**, 815-822.

74P RELEASE OF NEUTROPHIL ACTIVATING CYTOKINES BY HUMAN ARTERIAL AND VENOUS SMOOTH MUSCLE CELLS

[1]J.A. Mitchell, D. Bishop-Bailey, [1]T.W. Evans, [2]M.A. Giembycz, M.G. [2,4]Belvisi, [3]S. Chivers, [3]T.J. Williams and [1]J.R. Pepper
[1]*Unit of Critical Care Medicine, Royal Brompton Hospital, Sydney Street, London SW36NP:* [2]*Thoracic Medicine/*[3]*Applied Pharmacology, NHLI at Imperial College of Science and Technology, Dovehouse Street, London SW36LY*

Recruitment of neutrophils to damaged vessels is an early event in vascular dysfunction. Interleukin (IL)-8 is a powerful and specific chemoattractant for this cell type and as such is likely to be important in the initiation and development of vascular disease. Once present in the vessel wall, neutrophils do not differentiate and rapidly die. However, the survival of neutrophils (Lee *et al.*, 1995) can be potentiated by the cytokine, GMCSF. Activated leukocytes and/or endothelial cells are thought to be a major source of IL-8 and GMCSF. However, here we show that human venous and arterial smooth muscle cells are capable of releasing both GMCSF and IL-8 after stimulation with cytokines. We have previously shown, in the same cells, that cytokines induce cyclo-oxygenase-2 (COX-2) activity (Bishop-Bailey *et al.*, 1997). Thus, we have investigated the effects of COX-2 on IL-8 and GMCSF release by these cells.

Segments of human internal mammary artery (IMA) and saphenous vein (SV) were collected, dissected and cultured as described previously (Bishop-Bailey *et al.*, 1997). Venous and arterial cells were then plated onto 96 well culture plates and grown to confluence. Culture medium was replaced with fresh medium containing cytokines and/or indomethacin at a where COX-1 and COX-2 are inhibited in these cells (10μM; Bishop-Bailey *et al.*, 1997). After 24h, medium was removed and GMCSF measured by ELISA (Saunders *et al.*, 1996) and IL-8 by RIA (Ivey *et al.*,1995). Under control culture conditions, both arterial and venous cells released GMCSF and IL-8 (figure 1). This release was increased significantly ($p<0.05$; one-way ANOVA) by IL-1β (10ng/ml) or TNFα (10ng/ml) but not by endotoxin (LPS; 10μg/ml; figure 1). In the case of IL-8, arterial and venous cells released similar levels of chemokine, whereas arterial cells released greater amounts of GMCSF than equivalently treated venous cells (figure 1). In the same experiments, the release of IL-8 was unaffected by inhibition of COX-1/COX-2 with indomethacin (for SV; basal, 1.5±0.6; plus IL-1β, 7.9±3.2; plus TNFα, 4.2±1.6: for IMA; basal, 1.8±0.1; plus IL-1β, 5.8±0.5; plus TNFα, 4.2±1.0). By contrast the release of GMCSF was increased in both cell types by indomethacin (for SV, basal, 558±166; plus IL-1β, 1728±393; plus TNFα, 1056±302: for IMA, basal, 954±238; plus IL-1β, 3360±564; plus TNFα, 945±194).

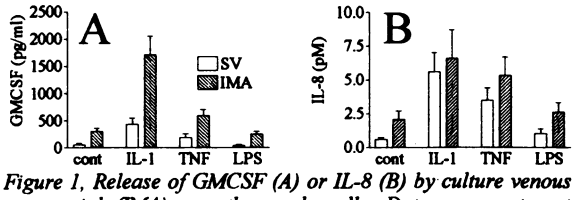

Figure 1, Release of GMCSF (A) or IL-8 (B) by culture venous (SV) or arterial (IMA) smooth muscle cells. Data represents cytokine release from n=9 experiments from 3 separate patients.

These observations implicate a role for vascular smooth muscle cells in the release of inflammatory mediators, particularly those involved with neutrophil recruitment and survival. Moreover, our data show that COX-2 activity strongly regulates GMCSF, but not IL-8 release. Since smooth muscle cells are the major component of arteries and veins, the finding that these cells are able to secrete inflammatory mediators may have important implications for vascular homeostasis.

This work funded by grants from the British Heart Foundation and the Wellcome Trust

Bishop-Bailey, D., Pepper, *et al.*, (1997). *Br. J. Pharmacol.* **120**, 73P.
Chan, C-C., Boyce, S., *et al.*, (1995). *J. Pharmacol. Exp. Ther.* **274**, 1531-1537.
Ivey, C.L., Williams, F.M., *et al.*, (1995) *J. C. Invest.*, **95**, 270-2728
Lee, A., Whyte, M.K. *et al.*, (1993). *J. Leukoc Biol.*, **54**, 283-288
Saunders, M., Mitchell, J.A., *et al.*, (1997) *Br. J. Pharmacol.*, **120**, 545-546

75P PARADOXICAL INFLAMMATORY RESPONSE TO CAPSAICIN IN A RAT STRAIN WITH INCREASED TISSUE CONTENT OF SUBSTANCE P

Y. S. Bakhle, C. Bell* and Juliet D. Brogan*, Applied Pharmacology, NHLI, Imperial College School of Medicine, London SW3 6LY and *Department of Physiology, Trinity College, Dublin.

The neuropeptide, substance P (SP), may play important roles in airway inflammation (Joos et al, 1994). One of the major effects of SP is to increase microvascular permeability causing airway oedema, experimentally quantifiable by extravasation of intravascular Evans Blue dye. The genetically hypertensive Otago Wistar rat (GH) has more SP-containing neurones in cervical dorsal root ganglia and more immunoreactive SP in the trachea than their normotensive controls (Bakhle & Bell, 1994). We have therefore measured the extravasation of Evans Blue in trachea and main bronchus, following either liberation of SP by capsaicin (Germonpre et al, 1995) or exogenous SP, in GH and normotensive Wistar rats.

Rats of either sex (200-300g body weight) were anaesthetized with sodium pentobarbitone (60mg.kg^{-1} i.p.) and the jugular vein was cannulated for i.v injections. Each animal received Evans Blue (30mg.kg^{-1} in isotonic saline) and 90s later capsaicin (75μg.kg^{-1}), SP (3.3nmol.kg^{-1}) or an equal volume of saline. After a further 5 min the thorax was opened and the animal was perfused transcardially with 300ml of saline at 100mmHg pressure to flush out intravascular dye. The trachea and main bronchus were then dissected, blotted dry, weighed and the dye extracted with formamide (1.5ml) at 37° overnight. Dye in the supernatant solution was assayed by absorption at 620 nm against a standard curve.

Extravasation of Evans Blue, as ng dye.(mg wet weight of tissue)$^{-1}$ was less in airways from GH than those from control rats, after capsaicin. This difference was more striking in the main bronchus (165$\pm$25, normal vs. 93$\pm$12, GH; n=8, $p<0.05$, Welch test) than in the trachea (90$\pm$10, normal vs. 63$\pm$10, GH; n=8). Extravasation induced by exogenous SP was less in the trachea from GH rats (95$\pm$15) than from normals (133$\pm$+6; n=5). In both strains, pretreatment (5min before the Evans Blue) with the selective NK-1 receptor antagonist SR140333 (360nmol.kg^{-1}; Emonds-Alt et al, 1993) abolished the extravasation response to capsaicin.

The reduced inflammatory responses to capsaicin and SP in GH rats could reflect reduced receptor number, altered receptor behaviour or increased clearance of SP. It is tempting to suggest that this difference is a developmental consequence of the increased density of SP innervation known to exist in GH airways However further experiments will be needed to resolve this issue.

We thank the HRB (Ireland), the British Council, the Royal Irish Academy and Trinity College Dublin for support and the Wellcome Trust for a Summer Studentship to JDB. We are grateful to Dr J-C Brelière (Sanofi) for a gift of SR 140333.

Bakhle, Y.S. & Bell, C. (1994) *Neuropeptides* 27, 169-174
Emonds-Alt, X. et al (1993) *Eur J Pharmacol.* 250, 403-413
Germonpre, P.R et al (1995) *Am J Respir Crit Care Med*, 152 1796-1804
Joos, G.F. et al (1994) *Eur Respir J.* 7, 1161-1171

76P NEUROGENIC CUTANEOUS VASODILATATION AND PLASMA EXTRAVASATION IN DIABETIC RATS: EFFECT OF INSULIN AND NERVE GROWTH FACTOR

Gavin S. Bennett[2], Neil. E. Garrett[1], Lara T. Diemel[1], Susan D. Brain[2] and David R. Tomlinson[1]
[1] Department of Pharmacology, Queen Mary and Westfield College, Mile End Road, London, E1 4NS
[2] Pharmacology Group and Vascular Biology Research Centre, King's College, Manresa Road, London, SW3 6LX, UK.

Neuropathy is a major complication of diabetes mellitus, characterized by hypoaesthesia and impaired axon-reflex vasodilatation. This is associated with a loss of neuropeptides from sensory nerves, which may be secondary to a reduction in peripheral nerve growth factor (NGF) (Fernyhough *et al*, 1994). We have investigated neurogenic vasoactive responses in rat skin following 8 week streptozotocin-induced diabetes. Male Wistar rats (300g) were made diabetic with a single injection of streptozotocin (50mg/kg, i.p.). Diabetic animals were divided into three groups: (1) untreated; (2) insulin (4IU/day weeks 4-8); (3) NGF (0.2 mg/kg three times weekly, weeks 4-8). A fourth group of rats served as a non-diabetic control. Electrical stimulation of the saphenous nerve (10 V, 2 Hz, 1 ms) for 30 s produced an increase in blood flow in the ipsilateral paw skin (Escott & Brain, 1993). The peak increase in blood flow was similar between groups, but the time taken for flow to return to baseline was significantly reduced in untreated diabetic rats, but not in insulin- or NGF-treated diabetic groups. A further stimulation of the saphenous nerve (10 V, 2 Hz, 1 ms) for 5 min produced plasma extravasation in the skin which was significantly attenuated in the untreated diabetic group, but not the insulin-treated group, compared to non-diabetic controls. The response was present, though reduced, in the NGF-treated group. Plasma extravasation in response to intradermal substance P was similar in all groups, implicating impaired release in diabetes-associated defcicts. This suggests that NGF and insulin are both able to restore the neurogenic vasoactive deficits seen in diabetes.

We would like to thank the BHF (GSB), the BDA (NEG) and the MRC (LTD).

Fernyhough, P. *et al*, (1994). Neurosci., **62** 337-344.
Escott, K.J. & Brain, S.D., (1993). Br. J. Pharmacol., **110** 772-776

Table 1. Results are mean ± s.e.mean, n=8-10. * p<0.05, ** p<0.01, ***p<0.001 compared with non-diabetic control. † p<0.05, ††† p<0.001 compared with sham paw. ‡‡‡ p<0.001, compared with Tyrode (vehicle)-injected sites

	Non-diabetic Control	Untreated diabetic	Insulin-treated diabetic	NGF-treated diabetic
(a) Saphenous Nerve experiments				
Peak blood flow after stimulation (flux, arbitrary units)	65.5 ± 12.1	50.8 ± 13.6	58.0 ± 16.2	44.8 ± 14.4
Time for blood flow to return to baseline (s)	46.1± 7.9	24.0 ± 3.7 *	47.0 ± 7.4	44.7± 7.7
Plasma extravasation (µl/100mg tissue)	17.7 ± 2.2†††	6.8 ± 1.7***	21.0 ± 1.8†††	10.7 ± 2.7*†
(b) Skin reactivity experiments (plasma extravasation, µl/site)				
Substance P (30 pmol/site)	79.0 ± 9.6‡‡‡	53.0 ± 7.1‡‡‡	55.2 ± 4.3‡‡‡	50.5 ± 6.7‡‡‡
Substance P (30 pmol/site) + CGRP (20 pmol/site)	111.8 ± 10.5‡‡‡	76.7 ± 7.8‡‡‡	90.7 ± 12.2‡‡‡	84.4 ± 11.4‡‡‡

77P EFFECT OF TREATMENT OF RATS WITH NERVE GROWTH FACTOR ON THERMAL NOCICEPTION AND ON CALCITONIN GENE RELATED PEPTIDE CONTENT OF PRIMARY AFFERENT NEURONS

R. Schuligoi, B.A. Peskar & R. Amann, Dept of Exp & Clin Pharmacology, University of Graz, Univ.-Platz 4, A-8010 Graz, Austria

Treatment of rats with nerve growth factor (NGF) is known to stimulate the synthesis of substance P (SP) and calcitonin gene-related peptide (CGRP) in dorsal root ganglia, and it has been shown to increase basal and evoked release of CGRP (at higher NGF doses also release of SP) from spinal terminals of primary afferent neurons (Malcangio *et al.*, 1997). Therefore, the aim of the present study was to determine to which extent treatment with NGF with doses sufficient to cause an increase of afferent neuron CGRP, influences thermal nociceptive threshold and inflammatory response in rats. Male Sprague Dawley rats (150-200 g) received one s.c. injection of 0.1 mg/kg human recombinant NGF (1 mg/ml dissolved in 0.9 % NaCl) or vehicle on days one, three, five, and seven. The thermal nociceptive threshold was determined measuring paw withdrawal latencies (PWL) according to Hargreaves *et al.* (1988) on day one at different intervals after the first NGF administration, and on day eight, before rats were injected with a lethal dose of sodium pentobarbitone. Tissue samples were dissected and processed for radio immunological determination of CGRP as described previously (Amann *et al.*, 1996). In a different set of experiments, rats were treated with NGF or vehicle as described above. On day eight, they received an injection of complete Freund's adjuvant (CFA, 0.15 ml) into the plantar side of one hindpaw. 24 h later, paw edema and PWL were determined. The results were expressed as means ± s.e.mean, and evaluated using t-test or, when appropriate, one way analysis of variance followed by Dunnet's test. A single s.c. injection of NGF caused a reduction of PWL that was maximal 4 h after administration and lasted less than 23 h. Treatment of rats with indomethacin (ind; 2 mg/kg s.c., 0.5 h before NGF) did not significantly reduce this effect of NGF (Table 1). One day after the last of four NGF injections, there was no detectable difference of PWL between NGF and control group (Table 1). NGF treatment caused, however, a significant ($P < 0.05$) increase of the concentration of immunoreactive (IR) CGRP in the sciatic nerves (control: 661.1 ± 30.8, NGF: 878.4 ± 48.0 fmol/mg protein; n = 19 each) and paw skin (control: 3.16 ± 0.39, NGF: 4.64 ± 0.48 fmol/mg wet weight; n = 15 each), but had no significant effect on CGRP-IR in the stomach (control: 7.31 ± 0.73, NGF: 8.28 ± 0.61 fmol/mg wet weight; n = 19 each) or ureter (control: 126.7 ± 10.6, NGF: 141.7 ± 7.4 fmol/mg wet weight; n = 19 each). A separate set of experiments showed that intraplantar injection of CFA in NGF- treated rats caused a decrease of PWL (Table 1) and an increase of paw volume (CFA: 3.54 ± 0.18 ml; control: 1.66 ± 0.09 ml; n = 8 each) that was not significantly different from values obtained in the control group (CFA: 3.77 ± 0.16 ml; control: 1.67 ± 0.06 ml; n = 8 each). The present results show that s.c. NGF injection was followed by an indomethacin-resistant thermal hyperalgesia. However, one day after completion of the NGF treatment protocol that was sufficient to stimulate CGRP synthesis in somatosensory neurons, we found no evidence for altered thermal nociceptive threshold, or aggravation of CFA-induced edema and CFA-induced thermal hyperalgesia.

Amann, R., Sirinathsinghji, D.J.S, Donnerer, J. *et al.* (1996) *Neurosci. Lett.* 203, 171-174.
Hargeaves, K., Dubner, R., Brown, F. *et al.* (1988) *Pain* 32, 77-88.
Malcangio, M., Garrett, N.E., Tomlinson, D.R. (1997) *Eur. J. Neurosci.* 9, 1101-1104.

Table 1.

Treatment	time after 1st NGF	PWL (s)	n
vehicle	4 h	9.63 ± 0.47	8
NGF	4 h	6.89 ± 0.20*	7
vehicle & ind	4 h	9.08 ± 0.55	8
NGF & ind	4 h	6.79 ± 0.66*	6
vehicle	23 h	9.78 ± 0.67	5
NGF	23 h	9.98 ± 1.10	6
vehicle	8 d	11.4 ± 0.76	7
NGF	8 d	11.0 ± 0.74	7
vehicle & CFA	9 d	4.13 ± 0.56	8
NGF & CFA	9 d	4.00 ± 0.30	8

* $P < 0.05$ as compared to corresponding vehicle group

78P INHIBITION OF SAPHENOUS NERVE-INDUCED NEUROGENIC OEDEMA FORMATION BY NOCICEPTIN

P.K.Towler & S.D.Brain Pharmacology Group and Vascular Biology Research Centre, Kings College London, Manresa Road, London. SW3 6LX.

Nocicieptin/orphanin FQ is a novel opioid peptide which is active at the ORL_1 receptor (Meunier *et al.*, 1995, Reinscheid *et al.*, 1995). It inhibits tachykinin release from the guinea pig renal pelvis *in vitro* (Giuliani & Maggi, 1996), and has been suggested to inhibit saphenous nerve-induced oedema in the rat following i.p. administration (Helyes, *et al.*, 1997). We have now investigated the activity of nociceptin in the rat saphenous nerve at two stimulation parameters. We found nociceptin to be ineffective when given as a single i.v. or i.p. bolus injection, and have therefore used a model of continuous infusion for this study, where the drug is infused i.v. at an initial dose of 1 mg kg^{-1} and maintained at a dose of 30 μg kg^{-1} min^{-1}.

Male Wistar rats (200-250g), anaesthetised with sodium pentobarbitone (50mg kg^{-1}, i.p.), were prepared for electrical stimulation of the saphenous nerve of one hind leg, with the other serving as sham control. Drug was administered from 5 min prior to nerve stimulation until the end of the experiment. The nerve was stimulated at 10V, 1ms, 2Hz for 0-5 min with oedema measured 0-30 min, or at 25V, 2ms, 10Hz for 0-15 min, with oedema measured 0-15 min. Plasma extravasation was assessed in skin by the extravascular accumulation of i.v. ^{125}I-albumin.

The results (Table 1) show that nociceptin significantly inhibited plasma extravasation induced by saphenous nerve stimulation, at both parameters tested. Laser Doppler blood flow monitoring showed nociceptin, at this dose, to have no significant effect on cutaneous blood flow measured in the sham paw skin, with the change in flux from 5 min before to 5 min after drug administration being 47.7 ± 18.2 flux units for control rats, and 20.5 ± 12.3 units for drug-treated rats (mean ±s.e.mean, n=4). Therefore, nociceptin, via continuous intravenous infusion, significantly inhibits plasma extravasation in the rat, with no significant effects on cutaneous blood flow. Nociceptin is believed to inhibit tachykinin release in the periphery via a presynaptic inhibitory mechanism (Giuliani & Maggi, 1996), and thus has the potential to act in an anti-inflammatory manner.

P.K.Towler is the recipient of an MRC/Pfizer studentship.

Giuliani, S. & Maggi, C.A. (1996). *Br. J. Pharmacol.*, **118**, 1567-1569.
Helyes, Z., Nemeth, J., Pinter, E., *et al.*, (1997). *Br. J. Pharmacol.*, **121**, 613-615.
Meunier, J-C., Mollereau, C., Toll, L. *et al.*, (1995). *Nature*, **377**, 532-535.
Reinscheid, R.K., Nothacker, H-P., Bourson, A., *et al.*, (1995). *Science*, **270**, 792-794.

Table 1. Plasma extravasation induced by electrical stimulation of the saphenous nerve. Results are expressed as μl plasma extravasated per 100mg tissue. Mean ± s.e.mean., n = 4, ***P<0.001 & *P<0.05 compared to vehicles of the same group, using Bonferroni's modified t-test.

		10V, 1ms, 2Hz	25V, 2ms, 10Hz
Vehicle (saline, i.v. + infusion)	Stimulated paw	42.0 ± 1.6	37.2 ± 7.9
	Sham control paw	4.5 ± 0.8	3.4 ± 0.8
Nociceptin (1 mg kg^{-1}, i.v. + 30 μg kg^{-1} min^{-1})	Stimulated paw	16.9 ± 4.3 ***	13.1 ± 4.2*
	Sham control paw	3.3 ± 0.4	2.3 ± 0.5

79P THE EFFECT OF THE NOVEL SODIUM CHANNEL BLOCKER 4030W92 IN MODELS OF ACUTE AND CHRONIC INFLAMMATORY PAIN IN THE RAT

N.M Clayton, S.D. Collins, R. Sargent, T Brown, M. Nobbs, and C Bountra. (D.J. Trezise). Neuroscience Unit, Glaxo Wellcome Research & Development Ltd., Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY

We have identified a novel sodium channel blocker 4030W92 (Trezise D.J & Xie X., 1997, this meeting), [R (-) 2,.4-Diamino-5-(2,3-diclorophenyl)-6-fluoromethyl)-pyrimidine] which we have evaluated in models of acute and chronic inflammatory pain, (Formalin, Carrageenan and adjuvant) and nociceptive pain. Male Random hooded rats (180-220g) were fasted overnight. 4030W92 was administered 30mins before either 100µl, 2.5% of formalin, 100µl of 2% carrageenan or 100µl of 1mg/ml Freunds Complete Adjuvant into the left hind paw. In a model of chronic inflammatory pain 4030W92 was administered 6 days after adjuvant. In the formalin test the total time spent licking was recorded for the first 5 mins (early phase) and then 15 to 30mins (late phase). The effect of 4030W92 on carrageenan and adjuvant induced decrease in weight bearing on the inflamed left hind paw (dual channel weight averager. Clayton et al., 1997) was determined 3 and 6 hours respectively after the inflammatory insult, or in the model of chronic inflammatory pain 6 hours after 4030W92. For carrageenan the effect of 4030W92 on carrageenan induced decrease in mechanical paw withdrawal threshold (Randall & Selitto, 1957) was also determined 3 hours post carrageenan. The effect of 4030W92 on carrageenan and adjuvant induced paw oedema was assessed using a pethysmometer. Anti-nociceptive activity was determined by investigating the effect of 4030W92 on normal mechanical and thermal tail flick latency. Statistical analysis was carried out to determine whether there was a significant difference between the vehicle treated group and the drug treated group using unpaired student t test ($p<0.05$). 4030W92 inhibited the second phase of the formalin paw lick (ED_{50} 19mgkg^{-1} p.o.) and the carrageenan induced decrease in weight bearing (ED_{50} 4.1mgkg^{-1} p.o.), and mechanical withdrawal threshold (ED_{50} 10.6mgkg^{-1}) and also reduced the associated paw oedema (ED_{50} 10.6mgkg^{-1} p.o.). Similarly 4030W92 produced a dose related inhibition of the adjuvant induced decrease in weight bearing after 6 hours (ED_{50} 4.2mgkg^{-1} p.o.) or in a model of chronic inflammatory hyperalgesia after 6 days (ED_{50} 7.9mgkg^{-1} p.o.). 4030W92 (15mgkg^{-1} p.o) had no effect on the normal mechanical and thermal thresholds. In conclusion, 4030W92 inhibits acute and reverses chronic inflammatory pain without affecting normal baseline responses. 4030W92 also exhibited significant anti-inflammatory activity in the model of acute inflammation (carrageenan). The anti-hypersensitivity action of 4030W92 therefore, offers considerable potential for its clinical utility in acute and chronic inflammatory pain states.

Clayton, N.M. et al., (1997) Br. J. Pharmacol., 120, 219P.
Randall, L.O. & Selitto, J.J. (1957). Arch. Int. Pharmacodyn., 61 409-419.

80P EFFECT OF THE NON-PEPTIDE NEUROPROTECTIVE COMPOUND SR 57746A ON NGF AND BDNF SYNTHESIS IN PRIMARY ASTROCYTES IN CULTURE

C. Labie, C. Lafon, J. Fournier, P.E. Keane, G. Le Fur, J. P. Maffrand & P. Soubrié, Sanofi Recherche, 195 Route d'Espagne, 31036 Toulouse Cedex, France.

The non-peptide neuroprotective compound, SR 57746A (1- [2-(naphth-2-yl)-ethyl]-4-(3-trifluoromethylphenyl)-1,2,5,6 tetrahydropyridine, hydrochloride), has been found to possess a number of neurotrophic properties *in vitro* and *in vivo* (Fournier *et al.*, 1992, 1993). In a variety of experimental models, this compound elicits activities similar to those of neurotrophic factors, and particularly of nerve growth factor (NGF) (see Fournier *et al.*, 1993). *In vivo*, SR 57746A (2.5-10 mg/kg p.o. once daily for 11 days) reverses the reduction of hippocampal choline acetyl transferase activity induced by an intraseptal injection of vincristine, and antagonizes the effects of an ibotenic acid-induced lesion of the nucleus basalis magnocellularis (Fournier *et al.*, 1997).

The neurotrophic factor promoting activity of SR 57746A was evaluated in rat primary astrocyte cultures by studying the synthesis of various neurotrophins. As assessed by quantitative S1 analysis, a concentration-dependent (10^{-8} - 10^{-6} M) and time-dependent (3 - 9 h) increase of NGF mRNA was induced by SR 57746A in cortical astrocytes, with a maximum of 284 ± 17 % of control levels ($P<0.01$, n=3) reached after 6 h of incubation at 10^{-6} M. In these cells, brain derived neurotrophic factor (BDNF) mRNA contents were increased to a significant but smaller extent (175 ± 15 % of control; $P<0.01$, n=3), whereas β-actin mRNA showed no variation. When studies of *de novo* neurotrophin translation were performed by immuno-precipitation with specific antibodies following [^{35}S]-methionin incorporation, an increase in NGF translation was induced by incubation with SR 57746A. In contrast, in hippocampal astrocytes, SR 57746A (10^{-9} to 10^{-6} M) induced a specific increase of BDNF mRNA levels after 6 h and 9 h of incubation (to a maximum of 274 ± 39 % of control values, $P<0.01$, n=4). This effect was also associated with an increase of *de novo* BDNF translation. In these cells, SR 57746A incubation did not modify NGF, neurotrophin 3 or β-actin mRNA levels.

In cells from each brain region incubated with SR 57746A (10^{-8} M), the effects on neurotrophin synthesis were preceded by a transient two-fold augmentation ($P<0.01$, n=4) of c-fos and c-jun mRNA contents. In addition, an increase of junB mRNA (241 ± 52 % of control; $P<0.01$, n=4) was seen, but only in cortical astrocytes. The increases of AP-1 family mRNA were associated with increases in nuclear AP-1 binding activity, as evidenced by electro-mobility shift assay. These results suggest that SR 57746A enhances the synthesis of neurotrophins in astrocytes. In addition, the effects of the compound differ according to the region of origin of the cells.

Fournier, J., Gauthier, T., Keane, P. E., Coudé, F. X., Guzzi, U. *et al.*, (1992). *Br. J. Pharmacol.* 106, 120P.
Fournier, J., Steinberg, R., Gauthier, T., Keane, P. E., Guzzi, U. *et al*, (1993). *Neuroscience* 55, 629.
Fournier, J., Keane, P. E., Ferrara, P. & Soubrié, P. (1997). *CNS Drug Reviews*, 3, 148-167.