PERIPHERAL NERVE LESIONS CAUSE PEPTIDERGIC ALTERATIONS IN THE SPINAL CORD

F. Borella, A.M. Di Giulio, A. Gorio[2], J.F. Hong[1], P. Mantegazza, M. Parenti, G. Vantini*[2] & R. Zanoni[2], Department of Pharmacology, Chemotherapy and Medical Toxicology, University of Milan, Milan, [1]Laboratory Behavioral & Neurological Toxicology NIEHS, Research Triangle Park, N.C.27709 and [2]Fidia Research Laboratories, Abano Terme (PD)

Neuronal deafferentation has often been correlated to the phantom limb sensation. The causes of such state of pain are unknown, however it is speculated that the presence of unmyelinated sprouts and the high firing rate of nerve may induce this condition. On the other hand peripheral nerve lesions are associated with intraspinal changes of peptides such as substance P and VIP and correlated with profound electrophysiological rearrangement. We have investigated whether these lesions alter also the metabolism of opioid peptides such as met-enkephalin and dynorphin which are present in the spinal cord and may play a role in nociception. Rat sciatic nerve was cut a few millimeters distally to the point where L_4 and L_5 form the sciatic nerve, then the proximal stump was either ligated or sutured intraperitoneally. The former way caused the formation of a small neuroma which conversely is very large in the latter case. The levels of substance P and met-enkephalin dropped simultaneously by 50% within 10 days from the lesion. Such a decrease was similar for both experimental groups in spite of the very different extent of aberrant neurite outgrowth. These changes were maintained even 30 days after lesioning. Immunocytochemistry showed that there is a decrease of both substance P and met-enkephalin in the dorsal horn ipsilaterally to the lesion. The extent of the damage and the time-course were similar if the lesions were performed bilaterally. On the other hand, the anatomical loss of both peptides was bilateral but less remarkable that in the case of monolateral lesion, as if the peptide decrease was intraspinally regulated. The loss of FRAP staining from the dorsal horn was, however, bilateral and of similar intensity of monolateral lesions, suggesting that a kind of information on the bilateral deafferentation has reached the spinal cord undistorted. This result would suggest that FRAP staining faithfully reflects nerve lesions, while peptidergic losses may be regulated by more sophisticated intraspinal mechanisms. We have also observed that dynorphin levels in the cord are decreased by 20%, 10 days after lesioning of the sciatic nerve. Both met-enkephalin and dynorphin are characterized by a powerful anti-nociceptive activity therefore it is feasible that pain sensation caused by peripheral nerve lesions may be due to their loss rather than to aberrant neurite outgrowth. We have recently observed a higher 5-HT turnover in the cord a few days after nerve lesioning, while at 10 days the 5-HT metabolism is normal. We are now investigating if this event is the trigger of the peptidergic loss in the cord.

CHARACTERIZATION OF ANTINOCICEPTIVE PROFILES OF INTRATHECAL DYNORPHIN AND CALCITONIN-RELATED PEPTIDES

S.Candeletti, E.Cavicchini, S.Ferri*, P.Romualdi, S.Spampinato & E.Speroni
Institute of Pharmacology, University of Bologna, Irnerio 48, 40126 Bologna,Italy.

The presence of several neuropeptides at spinal cord level suggests their involvement in spinal functions and interest has been focused on the modulation of nociceptive transmission. At this regard, we have previously reported that the opioid peptide dynorphin A (DYN A), ascertained in the spinal cord (Spampinato & Goldstein, 1983), causes distinguishable effects on nociception and motor function after intrathecal injection (Spampinato & Candeletti, 1985).

Since the dynorphin precursor contains, in addition to DYN A,several structurally related peptides, we investigated the antinociceptive and motor effects of different dynorphin-related peptides, injected directly into the spinal cord in freely moving rats bearing a chronic intrathecal catheter. In addition, a comparison has been carried out with the non opioid peptides salmon calcitonin(sCT, for which we have previously suggested a function in nociceptive transmission(Spampinato *et al.* 1984) and calcitonin gene-related peptide (CGRP) recently ascertained in the spinal cord of several animal species (Gibson *et al.* 1984). The antinociceptive tests used were: hot plate, tail flick and vocalization tests (electrical stimulation of the tail).

The intrathecal (i.t.) administration of 25 nmol DYN_{1-32} elicited an antinociceptive effect, assessed by the vocalization and tail flick tests, of the same intensity than that of an equimolar dose of DYN A but longer in duration (vocalization test: from 0.35 to 1.20 mA, $P < 0.05$; 60 min after i.t. administration). Higher DIN B doses (50 nmol) were necessary to elicit an antinociception similar to that induced by 25 nmol DYN A, while motor effects (flaccid hindlimb paralysis) were far below that of DYN A and DIN $_{1-32}$. The described alterations seem to be mediated by a κ-type receptor since the purported κ antagonist MR 1452 (30 nmol i.t.) was more effective than naloxone and the δ antagonist ICI 154,129 (120 nmol i.t.) in their prevention. Potency and duration of effects of dynorphin related peptides increase with molecular weight.

The i.t. administration of the peptide sCT (0.25-2.00 µg) produced a dose-related elevation of nociceptive threshold evidenced by the hot plate test only (2 µg sCT: MPE = 58±8 at 60 min); no appreciable effects were seen by tail flick and vocalization tests. Antinociceptive effect was duplicated by a dose of CGRP twenty-fold higher, approximately.sCT antinociception is uninfluenced by the narcotic antagonists naloxone, MR 1452 and ICI 154,129 (120 nmol i.t.).

Present findings indicate a different involvement of prodynorphin and calcitonin--gene derived peptides on the complex spinal circuits that modulate nociception.

Supported by the National Research Council (No. 84.02324.56), Rome, Italy.

Spampinato S. & Goldstein A. (1983) Neuropeptides 3, 193.
Spampinato S. & Candeletti S. (1985) Eur. J. Pharmacol. 110, 21.
Spampinato S. et al. (1984) Neurosci. Lett. 45, 135.
Gibson S.J. et al. (1984) J. Neurosci. 4, 3101.

Ca^{++}-CHANNEL INHIBITORS PREVENT MORPHINE WITHDRAWAL SYNDROME IN RATS

F.Bongianni, V.Carlà, F.Moroni* and D.Pellegrini. Department of Preclinical and Clinical Pharmacology. University of Florence. Viale G.B.Morgagni, 65,50134 Florence, Italy.

Physical dependence to morphine is associated with a selective increase in synaptosomal calcium in the brain of mice and rats; intracerebroventricular injections of lanthanum chloride antagonize morphine withdrawal symptoms in mice and the actions of several opioids are regulated by the brain Ca^{++} disposition (Chapman and Way,1980; Ben-Sreti et al., 1983). This investigation was performed to study the effects,on the morphine withdrawal syndrome in rats, of nimodipine (Hoffmeister et al.,1982) and verapamil, two Ca^{++}-channel inhibitors. Morphine pellets were subcutaneously implanted in rats (one pellet the first day, two pellets the third day). The withdrawal syndrome was challenged the fifth day by the administration of naloxone 1 mg/kg i.p. The syndrome was rated by continuously monitoring the following symptoms: diarrhea, exploring, jumping, wet dog shakes, writhing, grooming, teeth chattering, lacrimation and rhinorrhea. Body weight loss was also measured. One hour after naloxone administration the animals were decapitated and the parietal cortex, the hippocampus, the brainstem and the cerebellum were dissected. The content of norepinephrine (NE) was measured using HPLC and electrochemical detection; 3-methoxy-4-hydroxyphenethyleneglycol (MHPG) using GC/MS. During the evoked withdrawal syndrome the content of NE in the cortex, hippocampus and cerebellum decreased by approximately 20% ; in the same brain regions MHPG nearly doubled. Moreover, during this hour the rats lost 6.9 ± 0.5% of their body weight and clearly displayed most of the behavioral symptoms quoted. A dose dependent antagonism of the behavioral symptoms of the abstinence syndrome was obtained after the administration of nimodipine (1; 5; 10 mg/kg e.v.) or verapamil (10; 20; 30 mg/kg i.p.). Body weight loss was completely antagonized by 5 mg/kg e.v. of nimodipine and by 20 mg/kg i.p. of verapamil. The appearance of most of the behavioral manifestations was also prevented or the syndrome was greatly attenuated. In conclusion, our data support the hypothesis of changes in calcium disposition in the development of tolerance-dependence to opioids (Chapman and Way, 1980) and suggest that Ca^{++}-channel inhibitors could be useful in antagonizing withdrawal symptoms.

Acknowledgements: supported by CNR (Progetto Finalizzato Tossicodipendenze). Nimodipine was supplied by Dr. Hoffmeister, Merck, Basel.

Ben-Sreti M.M., Gonzalez J.P. and Sewell R.D.E. (1983) Europ.J.Pharmacol. 90, 385-391.
Chapman D.B. and Way E.L. (1980) Ann.Rev.Pharmacol.Toxicol. 20, 553-579.
Hoffmeister F;, Benz V., Heise A., Krause H.P. and Nauser V. (1982) Arzneim. Forsch. / Drug Res. 32, 347-360.

530P

DISSOCIATION OF TOLERANCE AND DEPENDENCE TO MORPHINE: A POSSIBLE ROLE FOR CHOLECYSTOKININ

P.Mantegazza, A.E.Panerai, L.C.Rovati, P.Sacerdote, Dept. Pharmacology, Chemotherapy and Medical Toxicology, School of Medicine, Univ. of Milano, 20129, Milano, Italy.

Proglumide, a cholecystokinin receptor antagonist (Hahne, 1981), potentiates morphine induced analgesia, and reverses tolerance to morphine experimentally induced in the rat by means of short term (14 hrs/6 days) parenteral treatment with the opiate (Tang, 1984; Watkins, 1984).
In the present study the effects of the concomitant administration of morphine and proglumide on the development of tolerance during long term administration of the opiate were calculated in an experimental setting similar to what happens in clinical practice.
Moreover, the effects of proglumide on the development of dependence were observed. Drugs were administered either orally dissolved in drinking water, or parenterally.
Under all experimental conditions the concomitant administration of morphine and proglumide inhibited the development of tolerance to the analgesic effect of the opiate.
However, proglumide did not prevent the withdrawal syndrome induced by the administration of naloxone as evaluated by either graded (e.g. wet dog shakes, self stimulation, writhing), or quantal (e.g. diarrhea, teeth chattering, irritability to touch) signs, and body weight decrease.
The data obtained induce to confirm the hypotheses that a cholecycstokinin like peptide might play a role in the development of tolerance to morphine and that proglumide might induce the effects through an inhibition of cholecystokinin.
In order to further evaluate these possibilities, studies are in progress with other substances that show in in vivo and in vitro studies a cholecystokinin antagonistic activity 500/600 folds that of proglumide.

Acknowledgements

Rotta Labs., Monza is acknowledged for proglumide and the other anticholecystokinin compounds.

Hahne,W.F., Jensen,R.T., Lamp,G.F. and Gardner,J.D. Proc.Natl.Acad.Sci., USA, 78, 6304-6307, 1981
Tang,J., Chou,J., Idarola,M., Yang,H-Y.T., and Costa,E. Neuropharmacology, 23, 715-718, 1984
Watkins,L.R., Kinscheck,I.B., and Mayer,D.J. Science, 224, 395-397, 1984.

EFFECTS OF ACETYLSALICYLATE ON SEROTONIN AND OPIATES IN RAT CNS: CORRELATION TO DRUG'S ANTINOCICEPTION

G.Biella[1], P.C.Braga, A.Groppetti*, M.Parenti, Department of Pharmacology "Emilio Trabucchi", Via Vanvitelli 32 and [1]Department of Physiopathology of Pain, Via della Commenda 12, University of Milan, Milano, Italy.

There is much evidence for suggesting that serotoninergic neurons are involved in nociceptive responses as well as in the pain inhibition induced by morphine. Administration of endogenous or exogenous opiates has been reported to stimulate the synthesis of brain serotonin (5-HT).

Recently we have found that in some brain areas, the interaction between opiates and 5-HT neurons may be reciprocal. In fact in striatum and hypothalamus, alterations of 5-HT neuronal activity are often reflected by changes in the content of [Met^5]enkephalin-like immunoreactive material (ME-IR). It is therefore feasible that the antinociceptive properties of non-narcotic drugs, such as salicylates, known to affect brain 5-HT, are, at least in part, related to their interactions with 5-HT and/or opiate systems.

In order to verify this hypothesis we have studied the effects of salicylates on 5-HT and opiate-peptides in the CNS and we have evaluated whether the integrity of these neuronal systems is essential for the antinociceptive activity of these drugs.

Among the methods used for the nociception-antinociception analysis before and after drug administration, electrophysiological recording of the neuronal single-unit activity was the method of choice for this study.

An electrode has been inserted in the posterior group of thalamic nuclei, involved in the area of the somatotopical organized projections from the posterior contralateral limb of anaesthetized rats. A mechanical stimulation, known to elicit nocifensive behavioural response in waking animals, was produced. The neuronal response was manifested as dramatic increases of frequency of the basal discharge of the neuron in the meanwhile period of the stimulation.

We have found that like morphine the acetyl salicylate of lysine (50 mg/kg i.v.) was able to abate the evoked activity by noxious stimulation, suppressing the increase in frequency due to the stimulation. The effects of the two drugs were significantly reduced by p-chlorophenylalanine (100 mg/kg x 4 s.c.) or metergoline (3 mg/kg s.c.) pretreatment, while naloxone (1 mg/kg i.p.) antagonized only morphine responses.

Moreover, as expected, we have found that after administration of acetyl salicylate of lysine (50-100 mg/kg i.v.), 5-hydroxyindoleacetic acid concentrations were increased while those of ME-IR were decreased in several areas of CNS.

The time-course of the biochemical events was parallel to the antinociceptive efficacy of the drug.

It is concluded that 5-HT, but not naloxone sensitive opiate mechanisms may be relevant for salicylates-mediated antinociception.

Acknowledgments: This work was supported by grants from Consiglio Nazionale Ricerche and Ministero Pubblica Istruzione.

OPPOSITE EFFECTS OF μ AND κ OPIATE AGONISTS ON DOPAMINE RELEASE IN THE NUCLEUS ACCUMBENS AND CAUDATE OF FREELY-MOVING RATS

A. Imperato & G. Di Chiara, Institute of Experimental Pharmacology and Toxicology, University of Cagliari, Cagliari, Italy.

The opiate analgesics are known to produce a variety of pharmacological and behavioural syndromes such as analgesia, physical and psychological dependence, euphoria and sedation. μ-Receptors agonists, like morphine, show addicting properties in animals, while pure k-agonists are apparently devoid of this property. Moreover, k-agonists, elicit sedation, while μ-agonists in low doses produce behavioural excitation. Brain dopamine has been related both to the reinforcing and the motor stimulating properties of opiates, but the evidence supporting this, is essentially indirect. Recently we have developed the technique of trans-cerebral dialysis for estimating the release and metabolism of DA in awake, freely-moving rats (1,2). Using this procedure we have studied the effect of morphine, μ-agonist, and bremazocine and U 50,488, k-agonists, on the in vivo release and metabolism of DA and we have correlated it, to the spontaneous behaviour of the animals. Systemic administration of a low dose of morphine (1.0 mg/kg s.c.) significantly stimulated DA-release in the nucleus accumbens but not in the caudate. DA-release in the accumbens was maximally stimulated (30% basal values) already 20 minutes and up to 80 min after administration. This dose of morphine elicited behavioural stimulation characterized by episodes of grooming alternating with hypermotility and rearing. Administration of a low dose of naloxone (0.1 mg/kg s.c. 15 min before morphine) completely prevented the stimulation of DA-release as well as the stimulation of behaviour. Higher doses of morphine (5 mg/kg s.c.) produced frozen postures and immobility for about one hour, followed by behavioural stimulation for the next 2hr 20 min. DA-release increased maximally by about 80% in the accumbens and by about 50% in the caudate. After this dose of morphine, DA-release stimulation was initially associated with behavioural inhibition but out lasted it, and continued for the whole duration of the behavioural stimulation. In contrast to morphine, a dose of 5.0 mg/kg s.c. of the highly selective k-agonist U 50,488, produced a rapid and pronounced reduction of DA release in the accumbens and in the caudate. This dose of U 50,488, in agreement with the literature, reduced motility. Both the behavioural and the biochemical effects of U 50,488 were unaffected by low doses of naloxone (0.5 mg/kg s.c.) while rather large doses of naltrexone (5 mg/kg s.c.), a pure opiate antagonist were able to reverse the inhibitory effects of U 50,488 on behaviour and on DA-release in the accumbens and in the caudate. The present results provide evidence for the idea that the differential effects of μ and k opiate agonists on behaviour are dependent upon a differential effect on mesolimbic and mesostriatal DA-system. In particular our results suggest that the lack of behavioural stimulant properties and the low abuse liability of k-opiate agonists is related to the lack of stimulation of the mesolimbic DA system.

1) Imperato and Di Chiara. The Journal of Neuroscience 4, 966-977; 1984.
2) Imperato and Di Chiara. The Journal of Neuroscience 5, 297-306; 1985.

RELEASE OF ^{3}H-DOPAMINE FROM RABBIT RETINA BY DIFFERENT DEPOLARIZING STIMULI: EFFECT OF MELATONIN

M.L. Dubocovich*, J.T. Eells & J.G. Hensler , Department of Pharmacology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611, USA.

Dopamine is the predominant catecholamine of the retina and fulfills most of the criteria for a functional neurotransmitter in this tissue. The hormone melatonin, at picomolar concentrations, inhibits the release of ^{3}H-dopamine elicited by field stimulation from the rabbit retina through activation of putative melatonin receptor sites (Dubocovich, 1983, 1984). The aim of the present investigation was to study the inhibitory effect of melatonin on the release of ^{3}H-dopamine elicited by potassium and field stimulation from the rabbit retina .

Retinas from albino rabbits were labelled with (7,8-^{3}H)-dopamine (0.1 uM, S.A. 41 Ci/mmol) in Krebs' solution (1.3 mM calcium) and experiments were conducted as previously described (Dubocovich & Weiner, 1981). The release of tritium was elicited either by electrical field stimulation (3 Hz, 20 mA, 2 msec), or by exposure to potassium (25 and 40 mM).

Potassium and field stimulation-evoked release of dopamine from the rabbit retina was characterized. The percent of the total tissue tritium released above spontaneous levels following the first period of field stimulation (S_1) at 3 Hz (180 pulses) was 1.49$\pm$ 0.21 % (n=8) and following the first exposure to potassium (40 mM, 2 min) was 2.01 $\pm$ 0.16 % (n=4). Tritium overflow evoked by either potassium or field stimulation was completly calcium-dependent. Only the field stimulation-evoked release of tritium was abolished by tetrodotoxin (100 nM), which blocks voltage-operated sodium channels. These results suggest that the potassium-evoked depolarization does not involve changes in sodium permeability.

The inhibitory effect of melatonin (1pM - 1nM) was more pronounced when tritium overflow was elicited by 180 pulses (IC_{50} = 9 pM) than when elicited by 360 pulses (IC_{50} = 46 pM). When tritium release was elicited by 360 pulses, but the calcium concentration in the Krebs solution was reduced to 0.65 mM, the inhibitory potency of melatonin increased (IC_{50} = 8 pM). Tritium overflow evoked by exposure to 40 mM potassium (2 min) was not affected by melatonin (10 - 100 nM) added before the second stimulation (S_2/S_1 ratio : 0.75 $\pm$ 0.12, n=4, for controls; 0.95 $\pm$ 0.10, n=4, for melatonin 10 nM; and 0.95 $\pm$ 0.13, n=3, for melatonin 100 nM). In contrast , when tritium overflow (S_1 = 2.11 $\pm$ 0.04 %, n=3) was elicited by a lower potassium concentration (25 mM, 10 min) in the presence of nomifensine (1uM) , melatonin (10 nM) completly abolished release. These results indicate that decreases in the external calcium concentration, the duration of field stimulation or the concentration of potassium enhance the inhibitory actions of melatonin on dopamine release from the rabbit retina.

In summary, these results suggest that melatonin inhibits dopamine release elicited by depolarizing stimuli involving either changes in the sodium permeability (i.e., field stimulation) or the potassium equilibrium potential of the synaptic membrane (i.e., potassium). We conclude that the potency of melatonin to inhibit ^{3}H-dopamine release from rabbit retina is more pronounced the lower the availability of external calcium for the secretory process.

Supported by USPHS grant EY 04788 (MLD), ES 07124 (JTE) and MH 09215 (JGH)

Dubocovich, M. L. (1983) Nature 306, 782
Dubocovich, M. L. (1984) Eur. J. Pharmac. 105, 193
Dubocovich, M. L. & Weiner, N. (1981) J. Pharmac. Exp. Ther. 219, 701

NALOXONE INCREASES ELECTRICALLY EVOKED DOPAMINE RELEASE PREFERENTIALLY IN THE NEURAL LOBE OF THE RAT NEUROINTERMEDIATE LOBE

Elisabeth Böhm, E. Muscholl & K. Racké,* Department of Pharmacology, University of Mainz, Obere Zahlbacher Str. 67, D-6500 Mainz, F.R.G.

There is good evidence that endogenous dopamine (DA) is involved in the modulation of hormone secretion from the neural lobe (NL) and intermediate lobe (IL) of the pituitary gland (see Holzbauer et al, 1983). The NL and IL contain also opioid peptides (see Höllt et al, 1981) which may modulated DA release. In the present experiments the effect of naloxone on the electrically evoked release of DA from the combined NIL or isolated NL was studied.

Isolated NILs or NLs with their stalk held in a platinum wire electrode were incubated in 80 µl Krebs-HEPES solution which contained also the DA uptake inhibitor GBR 12921 (200 nM, van der Zee et al, 1980). The medium was changed every 10 min. DA released into the medium was measured by HPLC with electrochemical detection (Muscholl & Racké, 1985). The pituitary stalk was stimulated twice after 50 min (S1) and 90 min (S2) of incubation with pulses of 0.2 ms, 10 V, 3, 7 or 15 Hz, 3 times 1 min at intervals of 1 min.

Table: Effect of naloxone (1 µM) on the electriaclly evoked DA release

Stimulation frequency	No drug fmol / S1	No drug S2/S1	Naloxone (30 min before S2) S2/S1
		NIL	NIL
3 Hz	178 ± 18 (11)	1.09 ± 0.06 (6)	1.11 ± 0.11 (5)
7 Hz	528 ± 42 (9)	1.12 ± 0.03 (5)	1.59 ± 0.12 (4) *
15 Hz	881 ± 53 (11)	0.98 ± 0.05 (7)	1.33 ± 0.06 (4) *
		NL	NL
15 Hz	48 16 (10)	0.92 0.12 (5)	3.15 0.45 (5) **

Means ± S.E.M. Significance of difference from No drug * $P < 0.05$; ** $P < 0.002$. Number of experiments in brackets.

The DA tissue contents at the end of the experiments were 19.0 ± 0.89 pmol/NIL (n= 31) and 9.1 ± 1.23 pmol/NL (n=10). Thus, the fractional DA release evoked by S1 (15 Hz) was substantially higher from the NIL than from the NL (3.72 ± 0.30 and 0.56 ± 0.13 % of the respective tissue DA content).

In conclusion, endogenous opioids likely to be released by electrical stimulation (7 and 15 Hz) inhibit the evoked DA release preferentially in the NL, compared with the IL. Since ir-dynorphin and Leu-enkephalin in the NL are concomitantly depleted with vasopressin during endocrine manipulations (Höllt et al, 1981), the present experiments may also explain the observation that dehydration of rats causes an increase of evoked in vitro release of DA preferentially from the NL compared with the IL (Holzbauer et al, 1985).

Supported by the Deutsche Forschungsgemeinschaft

Höllt, V., Haarmann, I., Seizinger, B.R. & Herz, A. (1981) Neuroendocrinology, 33, 333 - 339.

Holzbauer, M., Muscholl, E., Racké, K. & Sharman, D.F. (1983) Prog. Brain Res. 60, 357 - 364.

Holzbauer, M., Racké, K. & Sharman, D.F. (1985) J. Physiol. (Lond), abstr. in press

Muscholl, E. & Racké, K. (1985) Brit. J. Pharmac. 84, 3P.

Van der Zee, P., Koger, H.S., Gootjes, J. & Hespe, W. (1980) Europ. J. Chem. Chim. Ther. 15, 363 - 370.

CORTICAL ACETYLCHOLINE RELEASE IS INCREASED AND GABA OUTFLOW IS REDUCED DURING MORPHINE WITHDRAWAL

T. Antonelli, L. Beani*,C.Bianchi,S.Rando,M.Simonato & S.Tanganelli,Department of Pharmacology,University of Ferrara,Ferrara,Italy.

The firing rate of the noradrenergic neurons increases during the morphine withdrawal syndrome, as a consequence of the lack of the exogenous inhibitory control (Redmond & Krystal,1984). The stimulation of Locus Coeruleus or i.c.v. injection of norepinephrine (NE) decreases the release of Acetylcholine (ACh) while increasing that of GABA from the guinea-pig cortex (Bianchi et al.,1979;Moroni et al.,1982). In order to study the differences between noradrenergic receptor activation in normal conditions and during the morphine abstinence syndrome, the cortical release of ACh and GABA was studied in dependent guinea-pigs,after naloxone administration.

Morphine pellets (75 mg) were implanted in animals of either sex according to the following schedule: 1 pellet the day 1st, two pellets the day 4th.The 5th day the animals were submitted to the implantation of one epidural cup (Beani et al.,1978) and the abstinence syndrome was challenged the 7th day,by injecting 3 mg/kg naloxone i.p. The release of ACh and GABA was measured in two different groups of animals,by collecting the Krebs solution added to the cup every 30 min. ACh and GABA were evaluated as previously described using bioassay and GC-MS.

During the morphine abstinence syndrome the guinea-pigs displayed jumping, defecation,wet dog shakes and agitation. The release of ACh more than doubled, reaching the maximum in 1 hr and lasting at least two hrs after naloxone challenge. On the contrary, the output of GABA from the cerebral cortex decreased from 1.08 ± 0.1 to 0.85 ± 0.07 nmol/cm^2/30 min (5expts, $P < 0.01$).In control animals Tetrodotoxin 0.5 $\mu\bar{M}$,locally applied,decreased the release of this aminoacid by 50%,thus suggesting that neuronal GABA release is a consistent fraction of the total GABA output. Assuming that in our experimental conditions only the release of the neuronal GABA changes, a large decrease in synaptic GABA release during the abstinence syndrome seems to ensue.

In conclusion, these data indicate that the neurochemical pattern associated to morphine withdrawal is the opposite of that induced by an increased NE signal in normal animals. It is,therefore,possible that the increased firing rate of Locus Coeruleus neurones is only an epiphenomenon of morphine withdrawal.Other events seem able to change the cortical release of both ACh and GABA.

This research was supported by C.N.R. Grant.

Beani,L.,Bianchi,C;Giacomelli,A. & Tamberi, F. (1978) Eur.J.Pharmacol. 48,179-193.
Bianchi,C,Spidalieri,G.,Guandalini,P.,Tanganelli,S. & Beani,L.(1979) Neurosci. Letters, 14, 97-100.
Moroni,F.,Bianchi,C.,Moneti,G.,Tanganelli,S,Spidalieri,G.,Guandalini,P. & Beani,L. (1982) Brain Res. 232, 216-221.
Redmond,DE. & Krystal,JU. (1984) Ann.Rev.Neurosci. 7, 443-478.

CONTINUOUS RELEASE IN VIVO OF GABA AND OTHER AMINO ACIDS FROM RAT HIPPOCAMPUS INTO PUSH-PULL PERFUSATES

L.P. Hudson & R.J. Reiffenstein*, Department of Pharmacology, Faculty of Medicine, University of Alberta, Edmonton, AB, Canada T6G 2H7

Recent electrophysiological evidence that ACh and septal nucleus regulates GABA release in rat hippocampus (Krnjević et al, 1981; Ropert & Krnjević, 1981) points to a role for GABA, and an aternative therapeutic approach, in Alzheimer's disease. We have begun a study of this hypothesis by examining the release of GABA from hippocampus. A previous study has shown that muscarinic stimulation inhibits GABA release from cerebral cortex (Reiffenstein, 1979).

Perfusion-dialysis techniques (Lerma et al, 1984) have demonstrated very low levels of extracellular recovery of GABA in rat hippocampus; such low levels are thought to reflect a precise regulation of inhibitory neural transmission in this brain region (Ben-Ari et al, 1981). In vivo estimation of taurine, however, is much higher, and may point to a neuromodulatory role for this compound (Lehmann et al, 1984).

We have studied changes in the release of GABA, glutamate, taurine, aspartate and glutamine using the local perfusion method described by Errington et al (1983). Male Sprague-Dawley rats were anaesthetized with urethane (1.25-1.5 g/kg) and a bipolar stimulating electrode stereotaxically placed in entorhinal cortex. A stainless steel push-pull cannula with attached recording microelectrode was placed in CA1 or CA3. Localization of the tip of the assembly was determined by the amplitude of the positive field potential evoked by low frequency perforant path stimulation. Local tissue perfusion was done at 20 ul/min; 10 min samples were collected and stored at -80°C. Amino acid analysis was performed using reverse-phase fluorometric HPLC, with pre-column OPA derivitization.

In the presence of 5 mM nipecotic acid in the perfusate we have demonstrated a substantial increase in GABA and taurine recovery. Although perforant path stimulation evoked an increase in recovery of taurine, glutamate, and aspartate under these conditions, a corresponding increase in GABA recovery was not observed; there was a continuous slow decline in GABA recovery. Pretreatment of animals with the irreversible GABA transaminase inhibitor ethanolamine-O-sulphate (EOS) (Fletcher and Fowler, 1980) caused a further elevation of GABA recovery, and a small additional release of GABA in response to perforant path stimulation. Nipecotic acid or EOS did not change glutamate or aspartate recovery, but nipecotic acid did increase glutamine. These findings suggest that suppression of mechanisms responsible for removal and metabolism of GABA is necessary for realistic appraisal of GABA release under changing physiological conditions.

Research supported by the MRC of Canada. LPH holds a Studentship of the Alberta Heritage Foundation for Medical Research.

Ben-Ari, Y. et al (1981) Neuroscience 6, 2445-2463
Errington, M.L. et al (1983) J.Neurosci.Meth. 7, 353-357
Fletcher, A. & Fowler, L. (1980) Biochem.Pharmacol. 29, 1451-1454
Krnjević, K. et al (1981) Neuroscience 6, 2465-2474
Lehmann, A. et al (1984) Neurosci.Lett. 52, 341-346
Lerma, J. et al (1984) Neuropharmacology 23, 595-598
Reiffenstein, R.J. (1979). Can.J.Physiol.Pharmac. 57, 798-803
Ropert, N. & Krnjević, K. (1981) J.Physiol. 319, 111P-112P

EFFECTS OF GLYCINE ON SYNAPTIC TRANSMISSION IN THE FROG OPTIC TECTUM IN VITRO

A. Nistri & Lucia Sivilotti*[1], Department of Pharmacology, St. Bartholomew,s Hospital Med. Coll., University of London, Charterhouse Square, London EC1M 6BQ.

Glycine is an important inhibitory neurotransmitter in the spinal cord (Young & Macdonald, 1983). In some brain areas a transmitter function of glycine is also likely (Pycock & Kerwin, 1981), particularly in the first relay station of the visual system (the optic tectum in lower vertebrates or the lateral geniculate body in mammals) (Hunt & Brecha, 1984). In the amphibian optic tectum uptake and release of glycine have been demonstrated (Davidoff & Adair, 1976) but there are few studies on the electrophysiological actions of this amino acid (Milson & Mitchell, 1977). In our laboratory we have developed an in vitro frog optic tectum preparation (Sivilotti, 1985) on which we studied the action of GABA (Nistri & Sivilotti, 1985). We therefore decided to use this preparation to assess the effects of glycine on tectal synaptic transmission.

Experiments were done on in vitro brain preparations of the frog (R. temporaria) during fast superfusion with oxygenated Ringer at 7°C (Sivilotti, 1985). A microelectrode was placed in the superficial layers of the optic tectum to record extracellular field potentials elicited by electrical stimulation of the controlateral optic nerve. Responses were amplified, digitized and played back on a pen recorder. All drugs were given through precooled flowlines.

In 32 preparations 1 mM glycine nearly doubled the amplitude of monosynaptic excitatory potential waveforms (U_1 and U_2; Chung et al., 1974). Dose response curves showed that this effect had a threshold dose of 50 μM and an apparent ED_{50} value of 300 μM for the U_1 and U_2 waves. In most preparations the enhancing action of glycine was followed by a depression of these waves below their control amplitude. Strychnine (0.2 - 1 μM) reduced the enhancing and depressing actions of glycine but did not significantly change control U_1 and U_2 waves. Bicuculline (20-100 μM) or picrotoxin (50-100 μM) did not block the enhancing effect of glycine although with picrotoxin a small reduction in the glycine depressant effect was sometimes noted. With the recording microelectrode located in the optic tract the fibre volley response had reduced time to peak (87%) and smaller amplitude (47%) in the presence of 1-2 mM glycine.

Our results show that glycine had strychnine-sensitive biphasic actions on excitatory synaptic transmission in the optic tectum. The initial enhancement of postsynaptic waves might be partly due to a presynaptic facilitation of transmitter release followed by a depression. These data suggest that glycine can modulate visual inputs through a mechanism distinct from the one operated by GABA (Nistri & Sivilotti, 1985).

[1]Fellow of the European Molecular Biology Organization (EMBO).

Chung, S.H., Bliss, T.V.P. & Keating, M.J. (1974) Proc. Roy. Soc. Lond. B 187, 421-447
Davidoff, R.A. & Adair, R. (1976) Brain Res. 118, 403-415
Freeman, J.A. & Norden, J.J. (1984) in Comparative Neurology of the Optic Tectum, H. Vanegas, ed., Plenum, New York, 469-545
Hunt, S.P. & Brecha, N. (1984) ibid., 619-647
Milson, J.A. & Mitchell, J.F. (1977) Br. J. Pharmac., 59, 484P
Nistri, A. & Sivilotti, L. (1985) Br. J. Pharmac. in press
Pycock, C.J. & Kerwin, R.W. (1981) Life Sci., 28, 2679-2686
Sivilotti, L. (1985) Br. J. Pharmac., 84, 102P
Young, A.B. & Macdonald, R.L. (1983) Hand. Spinal Cord, Dekker, New York, 1-43

N-METHYLASPARTATE ANTAGONISTS BLOCK EPILEPTIFORM DISCHARGES IN RAT CORTICAL SLICES MAINTAINED IN MAGNESIUM-FREE MEDIUM

By J.A. Aram* and D. Lodge, Department of Physiology, Royal Veterinary College, London NW1 OTU.

Croucher et al. (1982) have shown that antagonists of N-Methylaspartate, such as 2-amino-5-phosphonovalerate (AP5), are anticonvulsant in a number of animal models. Recently it has become clear that, beside the glutamate analogues such as AP5, dissociative anaesthetics such as ketamine and sigma opiates such as (+) cyclazocine are also selective N-methylaspartate antagonists (Berry et al., 1984; Lodge et al., 1985) and that such substances also block certain synaptic excitations in the cerebral cortex (Thomson et al., 1985). We have recently been examining some of the effects of dissociative anaesthetics and sigma opiates on synaptic activity in a cortical slice preparation.

Using techniques similar to those of Thomson et al. (1985), 400 μm thick slices of rat cerebral cortex were maintained near 27°C at the interface between an artificial CSF and humidified 5% CO2 in O2. Stimulating electrodes were placed in the corpus callosum and extracellular field potentials were recorded from the grey matter with tungsten microelectrodes. Removal of the magnesium from the superfusion resulted in the appearance of spontaneous epileptiform potentials which were essentially identical in form to those evoked from stimulation of the callosum. A burst of up to 12 afterpotentials were superimposed on the decay phase of the early negative going potential. Replacement of the magnesium led to a dose-dependent reduction in the number of afterpotentials, this effect being apparent with 50 μM magnesium and maximal at about 400 μM.

In magnesium-free medium we tested the effects of D-AP5, (±) ketamine and (+) cyclazocine on the epileptiform activity. All three substances reduced the duration of the burst discharge in a dose-dependent manner. Near maximal effects were achieved with concentrations between 5 and 20 μM which is known to be in the range that selectively blocks NMA in vitro (Harrison & Simmonds, 1985; Lodge et al., 1985). There were only small differences in the potency of the three drugs but recovery from the effects of D-APV was faster than of (+) ketamine which was in turn faster than that of (+) cyclazocine.

Our results add to the body of evidence suggesting that NMA antagonists have anticonvulsant properties and support the idea that at least part of this action may be exerted at the level of the cerebral cortex.

Berry, S.C. et al. (1984) Br. J. Pharm. 83, 179-185
Harrison, N.L. & Simmonds, M.A. (1985) Br. J. Pharm. 84, 381-391
Lodge, D. et al. (1985) Neuropeptides 5, 245-248
Croucher, M. et al. (1982) Science 216, 899-901
Thomson, A.M. et al. (1985) Nature 313, 479-481

Supported by the MRC. J.A.A. is an MRC Scholar. (+) Cyclazocine and (±) ketamine were kindly donated by NIDA and Warner-Lambert, UK, respectively.

HALOPERIDOL AND THE ACTIONS OF N-METHYLASPARTATE, KETAMINE AND (+) SKF 10,047 ON FROG SPINAL CORD

D. Lodge, D. Martin* and C.T. O'Shaughnessy, Department of Physiology, Royal Veterinary College, London NW1 OTU.

Dissociative anaesthetics, such as phencyclidine and ketamine, and sigma opiates such as N-allylnormetazocine (SKF 10,047) share a common binding site (Zukin & Zukin, 1981) and common behavioural properties (Shannon, 1983). There are, however, some reports which suggest that the dopamine (D2) antagonist, haloperidol, displaces the binding of (+) SKF 10,047 but not that of PCP from CNS membranes (Tam, 1982; Su, 1982; Tam & Cook, 1984). Since we have recently shown that ketamine, like 2-amino-5-phosphonovalerate and magnesium, is a selective antagonist of N-methylaspartate on the isolated hemisected spinal cord of the frog (Martin & Lodge, 1985), we decided to investigate the effects of (+) SKF 10,047 and haloperidol in this preparation.

Haloperidol (1-100 μM) caused a small sustained depolarisation of frog motoneurones but no such effects were seen with ketamine and (+) SKF 10,047. Control dose-response curves of the depolarising effect of potassium chloride and the excitatory amino acids, N-methyl-D-aspartate (NMDA), kainate and quisqualate were constructed and the effects of drugs on these were examined. (+) SKF 10,047 (3.16-31.6 μM) and (±) ketamine (1-10 μM) selectively reduced the action of NMDA in a dose-dependent manner whereas haloperidol (1-100 μM) had a small non-selective action on all excitants.

When the cords were constantly superfused with 100 μM haloperidol, both ketamine and (+) SKF 10,047 remained potent antagonists of NMDA. The dose ratios for NMDA with 10 μM ketamine were 4.1 ± 0.4 and 3.2 ± 0.2 with and without 100 μM haloperidol respectively. Equivalent figures for (+) SKF 10,047 in initial studies were 10.6 ± 0.7 and 5.9 ± 0.7.

Thus from these studies it appears that, as with mammalian neurones in vivo (Berry et al., 1984), the dissociative anaesthetic, ketamine, and the sigma opiate, (+) SKF 10,047, are selective NMDA antagonists, whereas haloperidol has no clear selective effect on the depolarising actions of amino acids on frog motoneurones. Furthermore, judging from the ketamine results and the doses of haloperidol required to influence the action of (+) SKF 10,047, it seems unlikely that the haloperidol-sensitive (+) SKF 10,047 binding site is related to the phencyclidine/sigma receptor which appears to mediate NMDA antagonism.

Berry, S.C. et al. (1984) Br. J. Pharm. 83, 179-185
Martin, D. & Lodge, D. (1985) Neuropharmacology In press
Shannon, H.E. (1983) J. Pharm. exp. Ther. 225, 144-152
Su, T-P. (1982) J. Pharmacol. exp. Ther. 223, 284-290
Tam, S.W. (1983) Proc. natl. Acad. Sci. 80, 6703-6707
Tam, S.W. & Cook, L. (1984) Proc. natl. Acad. Sci. 81, 5618-5621
Zukin, R.S. & Zukin, S.R. (1981) Molec. Pharmac. 20, 246-254

Supported by the MRC. (+) SKF 10,047 was kindly donated by NIDA.

SODIUM VALPROATE: EFFECTS ON DEVELOPMENT, SOCIAL BEHAVIOUR AND SEIZURE SUSCEPTIBILITY IN THE OFFSPRING OF GERBILS

J.B. Chapman & M.G. Cutler, Department of Biological Sciences, Glasgow College of Technology, Glasgow, G4 0BA.

Anticonvulsants are among the drugs that can adversely affect brain development, learning and behaviour following prenatal or neonatal exposure. There is evidence that the newer anticonvulsant, sodium valproate, may not be any safer than the previous drugs in this respect (Diaz & Shields, 1978), although such effects depend upon dosage and age at exposure. We observed no evidence of retarded development or reduction of brain weight in mice exposed in utero and throughout postnatal life to a dose within the lower range of anticonvulsant activity (Chapman & Cutler, 1984), although behavioural change did occur. In the present studies, effects in the gerbil have been examined as an alternative species that is susceptible to convulsions.

Sodium valproate was given in drinking fluid (600 mg/l) during pregnancy and lactation to Mongolian gerbils and to their offspring after weaning. This treatment did not adversely affect breeding performance. The only evidence in the pups of developmental retardation was a delay in self-righting, seen at a mean age of 8 days in treated pups and 5 days in controls ($P < 0.05$). The average daily intake of valproate by the offspring ranged from 82 - 111 mg/kg body weight, depending on age and sex.

Gerbil pups after weaning were housed in single sex groups of 4-7 animals. Behaviour was examined by ethological procedures at 6 weeks and at 20 weeks for 10 min and 5 min periods respectively using procedures described by Mackintosh et al (1977). Behaviour in encounters between unfamiliar animals of the same sex and treatment group was examined in a neutral cage during the early phase of the 24 hr light-dark cycle. Acts and postures shown by the animals were recorded by two observers, one for each animal.

At 6 weeks of age, treated males showed a decreased frequency of social investigation ($P < 0.05$) and exploration and scanning ($P < 0.05$) while the duration of other non-social activity was increased ($P < 0.05$). Treated females showed no behavioural change. At 20 weeks, however, treated females showed an increased frequency and duration of exploration and scanning ($P < 0.05$) and a reduced duration of the other elements of non-social activity ($P < 0.05$), whereas behaviour of treated males resembled that of controls. Behavioural effects therefore changed with time and differed between the sexes.

Susceptibility of valproate-treated gerbil offspring to handling-induced seizures increased progressively with age and duration of treatment. A small increase of seizure susceptibility was evident in treated animals at 6 and 20 weeks of age. At 50 weeks, seizure susceptibility of animals of both sexes became significantly greater than in controls ($P < 0.01$).

These experiments thus indicate that sodium valproate at a dosage within the lower range of anticonvulsant activity produced in the gerbil not only behavioural change and a minor degree of developmental retardation but also increased seizure susceptibility via an as yet unknown mechanism.

Chapman, J.B. & Cutler, M.G. (1984) Psychopharmacology 83, 390-396.
Diaz J. & Shields, W.D. (1978) Ann. Neurol. 4, 198.
Mackintosh, J.H. et al (1977) in Handbook of Psychopharmacology, Iversen, L.L. eds., Plenum, London, vol. 7, 3 - 35.

GABA SYNTHESIS RATE AND RELEASE IN RAT BRAIN FOLLOWING REPEATED ELECTROCONVULSIVE SHOCK

A.R. Green and N.D. Vincent*, MRC Clinical Pharmacology Unit, Radcliffe Infirmary, Oxford OX2 6HE

Five min following the administration to rats of a single electroconvulsive shock (ECS) the rate of GABA synthesis is almost totally inhibited in cortex, hippocampus and striatum (Green et al, 1985a). An inhibition of K^+-evoked release of endogenous GABA from slices prepared from these brain regions has also been demonstrated at this time (Green et al, 1985b). Both synthesis rate and release return to normal values 2 h after the ECS and it has been proposed that the inhibition of release might initiate the inhibition of GABA synthesis (Green et al, 1985a). The effect of repeated ECS on these changes in neurotransmitter function has now been investigated.

Rats were handled or given repeated ECS (125V, 1s, through ear-clip electrodes, 5 times over 10 days) and killed 30 min or 24 h after the final treatments. Estimation of K^+-evoked release of GABA from brain slices was performed as described previously (Green et al, 1985b) with subsequent analysis of GABA by a fluorimentric assay (Bowdler & Green, 1982). There was a significant decrease in K^+-evoked release of endogenous GABA 30 min following the final ECS from slices prepared from both hippocampus and striatum (approximately 20% and 30% reduction respectively in K^+-evoked over spontaneous release compared with controls). However, in contrast to the data with a single ECS no change in K^+-evoked release was seen from cortical slices prepared from rats given repeated ECS. Twenty-four hours after chronic ECS treatment GABA release was still significantly reduced in the striatum (20% reduction in K^+-evoked release).

For estimation of the synthesis rate for GABA in these areas, the rate of GABA accumulation following administration of amino oxyacetic acid (AOAA) was measured, as described by others (e.g. Bernasconi et al, 1982). Twenty-four hours following repeated ECS, rats were injected with AOAA (10 mg kg^{-1} i.p.) and killed 20, 40 or 60 min later by a focussed high intensity microwave beam (70W/cm^2 for 4 sec), brain regions dissected and GABA concentration measured fluorimetrically. In confirmation of a previous report (Bowdler et al, 1983) 24 h following chronic ECS, GABA levels were raised in the striatum but unchanged in cortex and hippocampus. This elevation persisted for up to 72 h.

Using a mass fragmentographic technique the rate of GABA synthesis has previously been observed to be inhibited in the striatum following repeated ECS (Green et al, 1978). This was confirmed using the present approach (control: 5.2, ECS: 3.6 μmoles g^{-1} tissue h^{-1}, n = 6) and in addition a significant inhibition was also observed in hippocampus (control: 4.85, ECS: 3.0 μmoles g^{-1} tissue h^{-1}, n = 6). In contrast, the GABA synthesis rate was unchanged in the cortex following repeated ECS (control: 4.05, ECS: 4.15 μmoles g^{-1} tissue h^{-1}, n = 6).

Following ECS, therefore, GABA release is inhibited in the hippocampus and striatum and this may lead to the inhibition of the synthesis rate. The prolonged inhibition of release observed in the striatum may additionally produce the rise in GABA concentration seen in that region.

NDV holds a MRC Studentship.

Bernasconi, R. et al (1982) J. Neurochem. **38**, 57-66.
Bowdler, J.M. & Green, A.R. (1982) Br. J. Pharmac. **76**, 291-298.
Bowdler, J.M. et al (1983) J. Neural Transm. **56**, 3-12.
Green, A.R. et al (1978) J. Neurochem. **31**, 607-611.
Green, A.R. et al (1985a) Br. J. Pharmac. **84**, 88P.
Green, A.R. et al (1985b) Br. J. Pharmac. **84**, 89P.

542P

CENTRAL NORADRENERGIC NEUROTRANSMISSION AND ADRENOCORTICAL ACTIVATION BY HYDROXYUREA - A NEW α2-BLOCKING AGENT?

Carmignani M., Cerrito F., Del Carmine R., Martire M., Preziosi P.* & Vacca M.
Department of Pharmacology, School of Medicine, Catholic University, Rome, Italy.

Anticancer drug hydroxyurea (HYD) given to male adult rats by oral route at doses (800 mg/kg) 10 times higher than those employed in humans strongly stimulates the hypothalamic-hypophyseal-adrenal axis (HHAA), as shown by a 3-4 fold increase in plasma corticosterone (plasma B) 2-4 hrs after administration, a finding lacking in hypophysectomized animals and without tolerance after a 5-day treatment (Vacca & Preziosi, 1984). Changes in catecholamine content of the hypothalamus after single treatment with 800 mg/kg HYD do not seem to be responsible for HHAA activation (a tonic noradrenergic control on the above axis is well established in the rat, Scapagnini & Preziosi, 1973). On the contrary, a reduction in hypothalamic noradrenaline (NA) and adrenaline (A) content of up to 40 and 35% respectively was observed 2-4 hrs after the 5th daily dose. The discrepancy between the lack of effect after single as compared with repeated HYD treatment was investigated on the basis either of possible drug interference on NA synthesis through an inhibitor effect on hypothalamic tyrosine hydroxylase (TH) or of an α-blocking agent profile. TH was not modified by HYD (10^{-3} M) added *in vitro* to hypothalamic synaptosomes nor in the same synaptosomes taken by 5-day HYD-treated rats (dose as above) two hours after the last HYD administration. With regard to receptorial study, HYD (1 μM) partially counteracted the clonidine (0.1 μM) inhibition of [^{3}H]-NA overflow elicited by high K^+ (15 mM) in hypothalamic synaptosomes. HYD also antagonized the [^{3}H]-*p*-aminoclonidine ([^{3}H]-PAC) binding to hypothalamic membranes (K_i = 1000). The Scatchard analysis of data obtained in binding studies indicates an increased binding of [^{3}H]-PAC to membranes from the P_2-synaptosomal fraction prepared from 5-day HYD-treated animals 48 hrs after the last drug treatment, due to an apparent increase in the number of binding sites (+47% Bmax) without K_D changes, a possible marker of an antagonistic HYD action also at the postsynaptic α2-receptors. An α2-blocking activity, followed by an enhanced NA release by the presynaptic terminals, may explain, in chronically HYD-treated rats, the further (~20%) 5th-dose-induced reduction of the already-lowered NA and A hypothalamic content by α-methyl-*p*-tyrosine (250mg/kg) given i.p. 30 min before. Such a reduction is lacking in rats treated over 4 days with HYD,but receiving saline as the 5th administration. An α2-blocking activity of HYD was also observed in *in vivo* experiments on rat BP, in which HYD (800 mg/kg) partially antagonizes the hypotensive effect of i.v. clonidine infusion (5 μg/kg/min over 5 min) 2 hrs later. Thus the HYD-induced increase in plasma B may be due to a central α2-antagonistic activity of the drug, with a subsequent reduction of noradrenergic tone in the brain. Let us remember that phentolamine, an α1-α2-blocking agent,that does not cross the blood-brain barrier as easily as HYD, given i.p. (1-2 mg/kg) elicits a remarkable increase in plasma B (Scapagnini & Preziosi, 1973).

Supported by the Italian National Research Council, Special Project "Oncology" contract number 84.00747.44 and Ministero Pubblica Istruzione 40%-84.

Scapagnini, U., Preziosi, P. (1973) Neuropharmacol. 12, 57-62.
Vacca, M., Preziosi, P. (1984) Arch. Toxicol. suppl. 7, 98-102.

CHARACTERIZATION OF NEUROTENSIN RECEPTORS ASSOCIATED WITH CALCIUM CHANNELS AND PROLACTIN RELEASE IN RAT PITUITARY

M. Memo*, L. Castelletti, A. Valerio, C. Missale, and P.F. Spano, Inst. of Pharmacology and Experimental Therapeutics, University of Brescia, Via Valsabbina 19, 25100 Brescia, Italy.

Neurotensin (NT) is now reasonably well established as a neurotransmitter or neuromodulator candidate in the central nervous system.
In the present study, we characterized the NT receptors in dispersed cells from the anterior lobe of rat pituitary and investigated the involvement of cyclic AMP or calcium in the release of prolactin induced by NT receptor stimulation.
The ^{3}H-NT binding to membranes from anterior pituitary dispersed cells was found saturable and stereospecific. Scatchard analysis of the data gave a straight line indicating a B_{max} value of 121 fmoles/mg protein and a K_{d} value of 2.6 nM. The calculated IC_{50} for ^{3}H-NT binding were 5.8 nM, 7.8 nM and 3000 nM for NT, L-Phe-NT and the pharmacologically inactive form D-Phe-NT, respectively.
NT, up to the concentration of 1 uM, did not affect the cyclic AMP generating system in homogenates of anterior pituitary from male or lactating female rat. The same pattern of results was obtained measuring cyclic AMP formation in intact cells.
NT and its analogs stereospecifically enhanced the influx of calcium into dispersed cells from rat anterior pituitary. The effect was time and dose-dependent. It appeard to be associated with neurotransmitter-operated calcium channels since: i) preincubation of the cells with tetrodotoxin did not affect the increase of calcium influx induced by NT, ii) concentrations of verapamil which counteract the influx of calcium induced by potassium were unable to modify the influx of calcium induced by NT, iii) NT lost its ability to release prolactin in absence of extracellular calcium.
The significant correlation of the effective concentrations of NT and its analogs in binding and biological activities indicate that occupancy of NT receptors of anterior pituitary modifies calcium channel permeability of the cells membranes resulting in an accumulation of intracellular calcium which initiates the cascade of events that are ultimately expressed as an enhanced release of prolactin.

HUMAN AND RAT CALCITONIN GENE RELATED PEPTIDES (CGRP) ARE VASODILATORS IN CORONARY AND MESENTERIC VASCULATURE

S.J. Al-Kazwini, R.K. Craig[1], J.J. Holman & I.Marshall*, Department of Pharmacology & Therapeutics, and [1]Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W1P 6DB.

Rat and human CGRP have been identified by gene sequencing studies (Rosenfeld et al,1983; Edbrooke et al, 1985). In anaesthetised rats the peptides, given i.v., lower mean arterial pressure and increase heart rate (Adams et al, unpublished observations). These effects have been investigated further by comparing the effect of the two CGRPs (differing in 4 out of 37 amino acids) with sodium nitroprusside in the rat and rabbit heart and rat mesentery.

Isolated hearts from male Sprague-Dawley rats (260-360 g) or male New Zealand White rabbits (3.4-3.7 kg) were perfused via the aorta at either 7.8 or 12.0 ml. min^{-1} respectively. Changes in coronary perfusion pressure, heart rate and force of contraction were measured. Arginine vasopressin, 10^{-6} M, raised the perfusion pressure of the rat heart from 83±6 (mean±s.e.mean) to 117±4 mm Hg and of the rabbit heart from 31±2 to 111±6 mm Hg. Resting heart rate (258±16 $b.min^{-1}$ and 131±6 $b.min^{-1}$ in the rat and rabbit respectively) was not significantly altered.

In the presence of vasopressin, rat and human CGRP (3×10^{-11}-3×10^{-9} moles) were of equal potency in evoking dose-dependent falls in perfusion pressure in the isolated rat heart (eg falls of 43±3 and 56±10 mm Hg by rat and human CGRP, 10^{-9} moles, respectively). The peptides were about 10 times as potent as sodium nitroprusside (3×10^{-10}-3×10^{-8} moles). The two CGRPs, unlike nitroprusside, produced dose-related increases in heart rate (eg increases of 75±23 and 40±22 $b.min^{-1}$ by rat and human CGRP, 10^{-9} moles, respectively). None of the three drugs elicited dose-dependent changes in the force of contraction of the rat heart.

In the isolated heart of the rabbit, dose-dependent falls in coronary perfusion pressure were evoked by rat and human CGRP (3×10^{-11}-10^{-9} moles). Here the two peptides were approximately 30 times as potent as sodium nitroprusside. The peptides did not increase either heart rate or force.

The mesenteric artery of pentobarbitone anaesthetised rats was cannulated, the mesentery isolated and the ileum removed. The mesenteric vasculature was perfused at 5.8 $ml.min^{-1}$ through the mesenteric artery. The perfusion pressure was increased to around 125 mm Hg by noradrenaline, 10^{-5} M. In this preparation rat CGRP (3×10^{-11}-3×10^{-9} moles) was about 10 times as potent as human CGRP or sodium nitroprusside at eliciting falls in perfusion pressure. Rat CGRP, 10^{-9} moles, gave a fall of 85±11 mm Hg.

These results show firstly, relative potency differences between the two peptides because as vasodilators they were equipotent in the rat heart but rat CGRP was 10 times more potent than human CGRP in the rat mesentery. Secondly, there is a species difference as both peptides had a positive chronotropic effect in the rat heart but not in the rabbit heart.

We thank the Medical Research Council and Celltech for support.

Edbrooke, M.R. et al (1985) EMBO J. 4, 715-724
Rosenfeld, M.G. et al (1983) Nature 304, 129-135

DISSOCIATION OF CYCLIC GMP FORMATION AND INOSITOL PHOSPHOLIPID HYDROLYSIS IN RAT BRAIN SLICES

D.A. Kendall* & S.R. Nahorski, Department of Pharmacology and Therapeutics, Medical Sciences Building, University of Leicester, University Road, Leicester, LE1 7RH.

Stimulation of several receptors leads to hydrolysis of inositol phospholipids which can be accompanied by an accumulation of cyclic GMP in some tissues. It has been suggested that guanylate cyclase may be activated by metabolites of arachidonic acid released from the phospholipids (see Berridge, 1984). In the present study, we have examined the effects of various stimuli on cyclic GMP accumulation and inositol phospholipid hydrolysis in rat brain slices, to determine whether the two responses share common origins.

Rat brains were dissected and 350 x 350 µM slices were prepared, preincubated with ^{3}H-inositol in the presence of 5 mM LiCl and water soluble ^{3}H-inositol phosphates (^{3}H-IP) separated by anion-exchange chromatography (Brown et al. 1984). In parallel experiments, cyclic GMP was extracted under acid conditions and measured using a radioimmunoassay with a detection limit of 5 fmoles cyclic GMP.

The accumulation of ^{3}H-IP was enhanced by the muscarinic agonist carbachol (1 mM for 45 min) by 7, 4 and 1.5 fold in the cortex, striatum and cerebellum, respectively. Cyclic GMP was unaffected by any concentration of carbachol (up to 3 mM) at any time point examined (15 s to 30 min). The basal levels of cyclic GMP were 1.3 ± 0.09, 1.2 ± 0.06 and 13.1 ± 0.2 pmole/50 µl slices in cortex, striatum and cerebellum, respectively.

Elevated extracellular K^{+} in the incubation medium enhanced both ^{3}H-IP and cyclic GMP accumulation in cortical slices in a concentration-dependent manner. In the presence of 31 mM K^{+}, cyclic GMP was increased by almost 3-fold within 90 s. The cyclic GMP response to K^{+} was inhibited by 62% after preincubation with BW755C (30 µM), an inhibitor of lipoxygenase and cyclo-oxygenase (Higgs et al. 1979), and 20% by indomethacin (30 µM), a cyclo-oxygenase inhibitor. The 3-fold increase in ^{3}H-IP accumulation in the presence of 31 mM K^{+} was unaltered by indomethacin or BW755C.

As we have previously reported (Kendall & Nahorski, 1985), ^{3}H-IP accumulation in the presence of 18 mM K^{+} was enhanced by the dihydropyridine Ca^{2+} channel 'activator' BAY-K-8644 (1 µM) and antagonised by the dihydropyridine antagonist PN-200-110 (1 µM). Increased cyclic GMP (2-fold) in cortical slices evoked by 18 mM K^{+} was unaffected by either BAY-K-8644 or PN-200-110. In the absence of extracellular Ca^{2+}, 31 mM K^{+} evoked ^{3}H-IP accumulation was reduced by 60% and the cyclic GMP response under the same conditions was totally abolished. The inorganic Ca^{2+} channel antagonist Ca^{2+} blocked both responses with an IC_{50} of about 150 µM.

The data presented support the suggestion that cyclic GMP formation may be stimulated by arachidonic acid metabolites, but there is no support for the concept that guanylate cyclase activation inevitably accompanies inositol phospholipid breakdown.

We would like to thank the S.E.R.C. for financial support.

Brown, E. et al (1984) J.Neurochem. 42, 1379
Berridge, M.J. (1984) Biochem.J. 220, 345
Higgs, G.A. et al (1979) Biochem.Pharmacol. 28, 1959
Kendall, D.A. & Nahorski, S.R. (1985) Eur.J.Pharmacol. (in press)

REVERSAL OF AGONIST-INDUCED PHOSPHOINOSITIDE HYDROLYSIS IN HUMAN PLATELETS BY cAMP, cGMP AND 1,2-DIACYLGLYCEROL

A. McNicol* & D.E. MacIntyre, Dept. of Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland

Platelet reactivity is governed by the actions of second messenger molecules: cytosolic free Ca^{2+} ($[Ca^{2+}]i$) and 1,2-Diacylglycerol (DAG), formed as a consequence of receptor-mediated phosphoinositide metabolism, are considered to be stimulatory modulators whereas cAMP and cGMP are considered to be inhibitory modulators (Nishizuka, 1984; Feinstein et al, 1983; Haslam et al, 1980). As platelet activation can be reversible, the possibility exists that such second messengers may play a role in terminating or limiting platelet reactivity. Indeed, pretreatment of platelets with agents that stimulate the synthesis of cAMP (e.g. PGI_2, PGD_2) or cGMP (e.g. Na-nitroprusside, NaNP), or that mimic the effects of DAG (e.g. phorbol-12-myristate-13-acetate, PMA) inhibit agonist-induced phosphoinositide metabolism and Ca^{2+} flux (Lapetina, 1984; Bushfield et al, 1985; Takai et al, 1981; MacIntyre et al, 1985a). Moreover we have shown that PGD_2, NaNP or PMA added to platelets following agonist exposure results in acceleration of the reversal of the agonist-induced elevation of $[Ca^{2+}]i$ (MacIntyre et al, 1985b). In the present study we used PGD_2, NaNP and PMA to investigate the effects of cAMP, cGMP and DAG on the reversal of agonist-induced phosphoinositide metabolism.

All studies were performed at 37°C using plasma-free suspensions of human platelets pre-labelled with $[^{32}P]$-PO_4. Phosphoinositide metabolism, monitored as $[^{32}P]$-phosphatidate (-PtdA) formation (Pollock et al, 1984), was initiated by exposure of platelets to the thromboxane A_2 mimetic, EP171 (Jones et al, 1985) for 30s, before the addition of PGD_2, NaNP, PMA or appropriate vehicle for a further 60s.

PGD_2 (<1μM), NaNP (<1μM) and PMA (<1μM) alone exerted no significant effect on platelet $[^{32}P]$-PtdA. EP171 (10nM) augmented $[^{32}P]$-PtdA to 2-4 fold over basal within 30s, and this increased to 4-8 fold over basal at 90s after agonist addition. The increase in $[^{32}P]$-PtdA that occured within 30-90s of exposure to EP171 was inhibited by addition, at 30s, of PGD_2, NaNP and PMA (<1μM). NaNP and PMA suppressed EP171-induced $[^{32}P]$-PtdA formation to a greater extent than did PGD_2.

These results indicate that cAMP-, cGMP- and DAG-dependent processes can act to reverse agonist-induced phosphoinositide metabolism. As DAG and cGMP, but not cAMP, can be produced by platelets following exposure to stimulatory agonists (Nishizuka, 1984; Haslam et al, 1980) it is possible that DAG and cGMP act as endogenous modulators that function to limit or terminate platelet reactivity.

This study was supported by the MRC and by the Medical Research Funds of the University of Glasgow.

Bushfield, M. et al (1985) This meeting
Feinstein, M.B. et al (1983) Biochem.Biophys.Res.Comm. 113, 598-604
Haslam, R.J. et al (1980) in Platelets: Cellular Response Mechanisms and their Biological Significance (A. Rotman et al, eds.) pp. 213-231, Wiley, New York
Jones, R.L. et al (1985) Br.J.Pharmacol. 84, 148P
Lapetina, E.G. (1984) Biochem.Biophys.Res.Comm. 120, 37-44
MacIntyre, D.E. et al (1985a) FEBS Lett. 180, 160-164
MacIntyre, D.E. et al (1985b) Nouv.Rev.Fr.Hematol. (in press)
Nishizuka, Y. (1984) Nature 308, 693-698
Pollock, W.K. et al (1984) Biochem.J. 219, 833-842
Takai, Y. et al (1981) Biochem.Biophys.Res.Comm. 101, 61-67

BICUCULLINE: A $GABA_A$ ANTAGONIST WITH OTHER PHARMACOLOGICAL ACTIONS

A.Bartolini*, A.Galli, A.Giotti, S.Giuliani[1], P.Malmberg-Aiello, R.Patacchini & G.Renzi[2], Department of Preclinical and Clinical Pharmacology, [2]Department of Pharmaceutical Sciences, University of Florence and [1]Pharmaceutical Laboratories A.Menarini, Florence, Italy.

The pharmacological action of (+)bicuculline•HCl was studied in the following biological preparations: isolated rat atria, longitudinal muscle of guinea-pig ileum and mouse vas deferens. In isolated rat atria, bicuculline provokes a positive inotropic and a negative chronotropic dose-dependent effect from the concentration of 10 μM. The positive inotropic effect is more marked than the negative chronotropic one (+136±21% and -14±4% respectively at 100 μM). Neither effect is modified by pretreatment of the atria with (±)propranolol•HCl (1 μM) or by treatment of the rats with 6-hydroxydopamine•HBr 50 mg/kg i.v. for 2 days. Bicuculline dose-dependently increases the release of [^{3}H]-noradrenaline, induced by intramural electrical stimulation of the sympathetic nerve fibres, from in vitro isolated rat atria. The increase in [^{3}H]-noradrenaline release is 97±9% at 100 μM of bicuculline.

From 25 μM bicuculline dose-dependently inhibits the electrically-evoked contractions of the myenteric plexus longitudinal muscle preparation of guinea-pig ileum. Inhibition is almost total at 1 mM. 1 μM phentolamine mesylate does not antagonize the inhibitory action of bicuculline. At this dose phentolamine has no effect on ileum contraction, but blocks the inhibitory effect of noradrenaline (0.1 μM).

Bicuculline dose-dependently potentiates the electrically-evoked contractions of mouse vas deferens preparation from 25 μM upwards. 125 μM bicuculline increases evoked deferens contractions by 75±7%. Bicuculline (125 μM) exerts competitive antagonism against clonidine action on deferens. It neither contracts nor relaxes vas deferens preparation which have not been stimulated electrically.

The effects described for bicuculline are not reproducible with picrotoxin, the other antagonist molecule according to Hill and Bowery (1981) of the $GABA_A$ receptor. This would seem to rule out involvement of the GABAergic system in the actions described. On the other hand, the bicuculline induced potentiation of noradrenaline release as well as the structural similarity of its molecule and the adrenaline one could hint at involvement of the sympathetic system, at least in some of the effects observed.

Supported by CNR: Progetto finalizzato (applied project) Sp8 "Medicina Preventiva e Riabilitativa".

Hill, D.R. & Bowery, N.G. (1981) Nature 290, 149-152.

CHANGES OF SENSITIVITY OF PRESYNAPTIC α_2-ADRENOCEPTORS IN RAT BRAIN AFTER LONG-TERM CLONIDINE ADMINISTRATION

G. Bonanno, A. Gemignani, G. Maura and M. Raiteri*, Istituto di Farmacologia e Farmacognosia, Università di Genova, Viale Cembrano 4, 16148 Genova, Italy.

Clonidine is a centrally-active antihypertensive agent which possesses, in addition to the cardiovascular effects, a wide spectrum of pharmacological activities. Most of the clonidine effects appear to be mediated by alpha-2 adrenoceptors which are widespread throughout the body, including the brain. In the present study we have analyzed the effect of chronic clonidine on the sensitivity of the central alpha-2 presynaptic auto- and heteroreceptors mediating, respectively, inhibition of ^{3}H-noradrenaline (^{3}H-NA) and ^{3}H-5-hydroxytryptamine (^{3}H-5-HT) release'(Frankhuyzen & Mulder, 1982; Schlicker et al., 1983; Raiteri et al., 1983)'.

Adult male Sprague-Dawley rats were treated twice a day, for 12 days, with clonidine (100 µg/kg i.p.). Control animals received saline. The rats were killed 36 h after the last injection. The effects of chronic treatment were compared with those of an acute clonidine treatment (2 x 100 µg/kg i.p. in the 24 h). Crude synaptosomes were prepared from the whole cerebral cortex, labelled 10 min at 37 ° C with 0.04 µM ^{3}H-NA or with 0.04 µM ^{3}H-5-HT and superfused as previously described '(Raiteri et al., 1974)'. KCl (15 mM) was used as a depolarizing agent. The sensitivity of the presynaptic receptors was evaluated by comparing, in the various animal groups, the inhibitory action of NA on the K^+-evoked release of ^{3}H-NA or ^{3}H-5-HT, respectively.

In in vitro conditions, both NA and clonidine inhibited in a concentration-dependent way the K^+-induced release of ^{3}H-NA and of ^{3}H-5-HT. The pIC_{30} values (negative logarithm of the concentration causing 30% inhibition) amounted to 7.47 (^{3}H-NA release) and 6.47 (^{3}H-5-HT release). In synaptosomes from clonidine-treated rats NA was less effective in inhibiting ^{3}H-NA release than in synaptosomes from saline-treated animals. Similar results were obtained when NA was tested as an inhibitor of the K^+-evoked ^{3}H-5-HT release.

The finding that chronic clonidine treatment induced down-regulation of the alpha - 2 presynaptic receptors sited, respectively, on noradrenergic and serotonergic terminals in the rat CNS suggests that these receptors represent two of the in vivo targets of clonidine.

This work has been supported by Grant from the Italian Ministry of Education.

Frankhuyzen, A.L. & Mulder, A.H. (1982) Eur. J. Pharmacol. 81, 97-106.
Raiteri, M., Angelini, F. & Levi, G. (1974) Eur. J. Pharmacol. 25, 411-414.
Raiteri, M., Maura, G. & Versace, P. (1983) J. Pharmacol. Exp. Ther. 224, 679-684.
Schlicker, E., Göthert, M., Kostermann, F. & Clausing, R. (1983) Naunyn-Schmiedeb. Arch. Pharmacol. 323, 106-113.

BLOCK OF NERVE ACTIVITY BY ETOMIDATE

E. Benoit, M.R. Carratù[1], J.M. Dubois, D. Mitolo-Chieppa[*1] & P. Preziosi[2], Laboratoire de Physiologie Comparée, Université de Paris XI, 91405 - Orsay, France, [1]Istituto di Farmacologia, Università di Bari, 70124 Bari and [2]Istituto di Farmacologia, Università Cattolica del Sacro Cuore, 00168 Roma, Italia

Etomidate is a carboxylated imidazole administered intravenously for complete anesthesia of short duration (Janssen et al., 1975). No adverse effects are reported on blood pressure, heart-rate or respiration; involuntary myoclonic muscle movements are sometimes observed during etomidate-induced anesthesia but they are not associated with EEGs epileptiform discharges. In order to investigate the mode of action of etomidate, experiments were carried out on frog voltage clamped myelinated nerve fibers as a model of excitable membranes. The sodium current was reversibly blocked by external etomidate with an apparent dissociation constant of 0.6 mM. In the presence of 0.5 mM of drug the steady state sodium inactivation-voltage curve was shifted by about 12 mV towards negative potentials. The block of the sodium current was partially removed by high frequency (1 and 10 Hz) depolarizations (Figure 1). The unblock was increased when a given test pulse was preceeded by a 50 ms hyperpolarization to -120 mV. These observations suggest that the drug molecules block the Na channels in resting and inactivated states. In some respects, the effects of etomidate resemble those of local anesthetics. The present results on the frog node of Ranvier could explain the anesthetic action of etomidate.

Janssen, P.A., Niemegeers, C.J.E. & Morsboom, R.P.H. (1975) Arch. Int. Pharmacol. Ther. 214, 92.

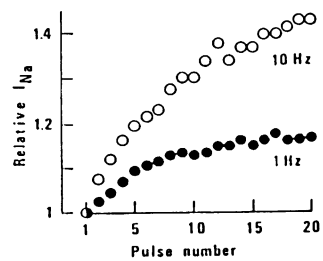

Figure 1 Removal of etomidate (0.5 mM)-induced block of Na current by high frequency (1 and 10 Hz) stimulations. The sodium current was recorded during depolarizations to 0 mV preceeded by 50 ms hyperpolarizations to -120 mV.

MICROIONTOPHORETIC APPLICATION OF THE HALLUCINOGEN DOM FACILITATES FACIAL MOTOR NEURONE EXCITATION

N.J. Penington* & R.J. Reiffenstein, Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7.

2,5-Dimethoxy-4-methylamphetamine (DOM, "STP") is a potent hallucinogen proposed to be a serotonin (5HT) agonist. Direct evidence that a drug is a 5HT agonist in the CNS has been difficult to obtain due to the often observed failure of known 5HT antagonists to prevent 5HT induced inhibition of cell firing (Haigler and Aghajanian 1974) in areas that contain $5HT_1$ binding sites. McCall and Aghajanian (1980) have characterised the facial motor neurone (FMN) response to 5HT and NA (a depolarisation facilitating excitatory input) and its blockade by certain antagonists. This site appears to have some properties in common with the $5HT_2$ binding site (Fozard 1984). We have examined the effect of iontophoretically applied DOM to FMNs to find out if its binding to $5HT_2$ sites (Glennon et al. 1984) is expressed as an agonist effect, using methysergide, chlorpromazine and ketanserin.

Adult Sprauge Dawley rats (39) were anaesthetised with urethane (1.25 g/kg i.p.) and placed in a stereotaxic apparatus. The animals temperature was maintained at 37°C. Single FMNs were found at ≈2.5 mm P, 2 mm L to λ and 8 mm deep from the brain surface. Five barrelled microelectrodes, often with automatic current compensation, were used. Controls for current, pH and local anaesthetic effects were routinely done. Results were measured as average of 3 responses each in the control, antagonist and recovery periods. Data was pooled and averaged for each antagonist applied. Each recording site was stained and examined histologically.

Table 1 FMN firing during antagonism & recovery as % of control

Methysergide	Cells	Plus Antagonist	Recovery	Mean Current
5HT	7*	29 ± 9	91 ± 8	10 nA
NA	7	100 ± 3	100 ± 5	8 nA
DOM	5†	18 ± 7	89 ± 13	9 nA
Chlorpromazine				
DA	4Δ	38 ± 12	98 ± 2	7 nA
5HT	2	99	101	8 nA

Mean ± s.e. mean, * 4/4 cells selectively blocked vs. NA, † 3/3 cells selectively blocked vs. NA, Δ 2/2 cells selectively blocked vs. 5HT

5HT excited a total of 27/27 cells, NA excited 12/12 cells DA excited 12/13 cells and DOM 19/20 cells. Methysergide (MS) antagonized 5HT responses selectively relative to NA responses (in the same cell) (Table 1). MS also reversibly blocked DOM responses selectively relative to NA responses. Ketanserin blocked 5HT but was not selective vs. NA responses. Chlorpromazine reversibly blocked DA but did not affect 5HT responses. DOM did not sensitize the cells to NA and 5HT [as does LSD and mescaline (McCall and Aghajanian 1980)] but behaved as a pure agonist. Evidence gathered to date is consistant with a $5HT_2$ agonist effect of DOM but some agonist effect at DA receptors has not yet been ruled out. Chlorpromazine would appear to be an adequate tool to differentiate a DA from a 5HT receptor agonist effect of DOM in this preparation.

N.J.P. holds an Alberta Heritage Foundation for Medical Research Studentship.

Fozard, J.R. (1984) Neuropharmacology 23, (12B), 1473-1486.
Glennon, R.A. et al. (1984) Life Sci. 35, 2505-2511.
Haigler, H.J. and Aghajanian, G.K. (1974) J. Neural Transm. 35, 257-273.
McCall, R.B. and Aghajanian, G.K. (1980) Life Sci. 26, 1149-1156.

DISTRIBUTION OF HIGH AFFINITY [^{3}H]-SOMATOSTATIN BINDING SITES IN THE RAT BRAIN

JM Candy[1], CR Snell[2] and CA Whitford[1*], [1]MRC Neuroendocrinology Unit, Newcastle General Hospital, Westgate Road, Newcastle upon Tyne NE4 6BE, UK and [2]Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6NE, UK.

In a previous communication (Brundish et al, 1984) we reported the presence of two high affinity binding sites for [4-^{3}H(Phe^{6})]-somatostatin-14 in whole rat brain. We now report their gross regional distribution and the autoradiographic localisation of ^{3}H-somatostatin binding sites in the rat brain.

Rat brains were dissected into 7 regions (Glowinski and Iversen, 1968) and P_2 membrane fractions prepared. The binding assay was carried out as previously described (Weightman et al, 1985) using increasing concentrations of somatostatin-14 to displace specifically bound ^{3}H-somatostatin. The results were subjected to computer-assisted Scatchard analysis, and showed that two classes of high affinity binding sites for ^{3}H-somatostatin were present in the cerebral cortex, hippocampus and midbrain (Table 1).

Table 1.

Regional distribution of ^{3}H-somatostatin binding sites

Region	Kd_1 (nM)	$Bmax_1$ (fmol/mg protein)	Kd_2 (nM)	$Bmax_2$ (fmol/mg protein)
Cerebral Cortex	0.25 ± 0.05	96.4 ± 3.3	33.18 ± 9.01	342.6 ± 110.0
Hippocampus	0.23 ± 0.02	106.9 ± 8.0	36.33 ± 6.46	334.8 ± 55.1
Midbrain	0.28 ± 0.04	25.0 ± 2.2	49.68 ± 9.36	224.1 ± 89.4
(n=5)				

Variable non-specific binding in the striatum did not allow reproducible analysis, and the low density of binding sites present in the hypothalamus, brainstem and cerebellum precluded Scatchard analysis.

The distribution of ^{3}H-somatostatin binding sites was studied using tritium film autoradiography. Air dried, 10μm, coronal, cryostat sections were labelled with 1nM ^{3}H-somatostatin using a protocol to be described (Whitford et al, submitted). Conditions were used which eliminated breakdown of ^{3}H-somatostatin and which predominantly labelled the higher affinity binding site. Areas with the highest density of binding sites included the deep layers of cerebral cortex, lateral septal nucleus, amygdala, claustrum and habenula. Low densities of binding sites were observed in the thalamus, hypothalamus, brainstem and cerebellum.

We conclude that 2 high affinity binding sites for ^{3}H-somatostatin are present in the cerebral cortex, hippocampus and midbrain. Autoradiography reveals a discrete distribution of ^{3}H-somatostatin binding sites in the rat brain similar to that recently reported using an iodinated ligand (Leroux and Pelletier, 1984).

Brundish, DE et al. (1984) Brit. J. Pharmacol. 83, 369P.
Glowinski, J and Iversen, LL (1968) J. Neurochem. 13, 655.
Leroux, P and Pelletier, G (1984) Peptides 5, 503.
Weightman, DR et al. (1985) Neurosci. Lett., 55, 161.

552P

D_2 DOPAMINE RECEPTORS AND THE REGULATION OF ALDOSTERONE PRODUCTION

P. Liberini, C. Missale*, M. Memo, M. Pizzi, P.F. Spano, Inst. of Pharmacology and Experimental Therapeutics, University of Brescia, Via Valsabbina 19, 25100 Brescia Italy.

There is growing evidence for an influence of dopamine on adrenal glomerulosa production of aldosterone: metoclopramide induces a prompt rise in plasma aldosterone and this effect is markedly attenuated by dopamine infusion; on the same line, dopamine inhibits aldosterone biosynthesis induced by angiotensin II, but not basal aldosterone production by both bovine and rat dispersed adrenal glomerulosa cells.

In vivo studies with various dopaminergic drugs show that in rats administration of D_2 antagonists increase plasma aldosterone levels with (-)sulpiride more active than metoclopramide and (+)sulpiride; haloperidol induces a slight increase in plasma aldosterone, while the D_1 antagonist SCH 23390 is completely inactive. Since these data suggest an involvement of D_2 receptors in the regulation of aldosterone production, we characterized dopamine receptors in the adrenal cortex by using ^{3}H-(-)sulpiride, a highly selective ligand for D_2 receptors.

^{3}H-(-)sulpiride binding fulfills the criteria of saturability, reversibility, stereospecificity and regional distribution; the Scatchard analysis of saturation curve reveals one binding component with K_d of 6.2 nM and B_{max} of 8 fmol/mg protein. The binding is highly dependent on the presence of cations in the incubation medium and decreases as an inverse function of temperature. Sulpiride and butaclamol displace ^{3}H-(-)sulpiride binding stereospecifically, with (+)sulpiride one 300th as active as (-)sulpiride and l-butaclamol completely inactive; the ergot derivatives are the most potent displacers of ^{3}H-(-)sulpiride binding, while SCH 23390 is almost inactive.

This binding site appears to be coupled to the inhibition of adenylate cyclase. In fact, when D_1 receptors in the adrenal cortex are blocked by SCH 23390, dopamine elicits a concentration-dependent inhibition of adenylate cyclase (IC_{50} 10 uM); bromocriptine, dihydroergotoxine and lisuride inhibit the cyclase activity as well and these effects are completely antagonized by 1 uM (-)sulpiride, but not by 1 uM (+)sulpiride.

These data indicate that D_2 receptors coupled to the cyclic AMP generating system in an inhibitory way are present in the adrenal glomerulosa and are functionally involved in the regulation of aldosterone production.

SOME PROPERTIES OF AMINE OXIDASE ACTIVITIES IN THE RAT INTESTINE

B.A. Callingham*, P. Mazel and J.C. Porter, Department of Pharmacology, University of Cambridge, Hills Road, Cambridge, CB2 2QD.

Both forms of monoamine oxidase (MAO-A and MAO-B) are known to be present in the gastrointestinal tract of many animals where they appear to limit the absorption of potentially pressor amines from the gut contents. However the distribution of these two enzymes and of the semicarbazide-sensitive amine oxidase (SSAO) in the rat gastrointestinal tract is not uniform and most activity is located in the ileum (Hasan & Tipton, 1984). In addition, although the common dietary amine, tyramine is a substrate for all three amine oxidases in many tissues including rat gut, it is only the inhibition of MAO-A that leads to severe hypertensive episodes following ingestion of this amine (Sandler et al, 1980).

The relative importance of these three enzymes in two segments of rat gut I, from 0.25 to 0.375 and II, from 0.375 to 0.625 of the stomach to caecum distance of male Wistar rats weighing between 125 and 300g has been examined. Specific enzyme activities were assayed radiochemically in crude tissue homogenates with 500μM 5-hydroxytryptamine 5-HT), 500μM benzylamine (BZ) and 1μM benzylamine as substrates for MAO-A, MAO-B and SSAO respectively. In addition 500μM tyramine was also examined. Relative proportions of each enzyme activity were also determined by the use of the MAO-A selective irreversible inhibitor, clorgyline. Cells from villi and crypts were separated from the remainder of the gut segments by a method modified from that of Hoffman & Kuksis (1979). Degradation rate constants for the enzymes were found by measuring the rates of recovery of enzyme activity in the gut segments of groups of 4 rats killed at 3h, 1, 2, 5, 7, 14, and 21d following the intraperitoneal injection of 30mg/kg pargyline HCl. Control animals received similar volumes of 0.9% NaCl solution.

Table 1 Kinetic parameters of enzymes in two segments of rat gut

Segment	Parameter	5-HT (MAO-A)	BZ (MAO-B)	BZ (SSAO)
I	Km (μM)	180 ± 10	35·8 ± 4·5	3·8 ± 1·3
II	Km (μM)	204 ± 27	86·6 ± 4·5	3·7 ± 1·2
I	Vmax (nmol.mg prot .h)	1124 ± 71	1746 ± 55	1399 ± 186
II	Vmax (nmol.mg prot .h)	1190 ± 71	1697 ± 170	1297 ± 161

Measurements of specific activities of the enzymes and the use of clorgyline with the above substrates and concentrations, confirmed earlier observations that there was at least twice as much MAO-A as MAO-B and that both BZ and tyramine were substrates for SSAO. The recoveries of MAO-A and -B activities following irreversible inhibition by pargyline in the two segments were found to be different yielding degradation rate constants of: MAO-A; 0·31 ± 0·18 and 0·33 ± 0·15, MAO-B; 0·09 ± 0·03 and 0·09 ± 0·2d^{-1} (mean ± confidence limits at P=0·05.) for segments I and II respectively ($t_{\frac{1}{2}}$ of about 2·2d for MAO-A and 7·7d for MAO-B). These compare with a $t_{\frac{1}{2}}$ of about 3·3d for both enzymes in the livers. Cells from villi and crypts showed a greater proportion of MAO-A to MAO-B, while in the gut residue, there was little or no difference.

From these results it would appear that dietary amines are preferentially exposed to MAO-A during passage through the rat gut and that contact with MAO-B may be limited.

Hasan, F & Tipton K.F. (1984) J.Pharm.Pharmacol. 36, 20W
Hoffman, A.G.D. & Kuksis, A. (1979) Can.J.Physiol.Pharmacol. 57, 832-842
Sandler, M et al (1980) J.neural.Trans. 48, 241-247

RELATIONSHIP BETWEEN LIPOPHILIC CHARACTER AND URINARY EXCRETION IN RATS OF A SERIES OF NITROHETEROCYCLIC COMPOUNDS

A.M. Barbaro, G.L. Biagi*, P.A. Borea[1], G. Cantelli Forti, M.C. Guerra and P. Hrelia, Istituto di Farmacologia, Università di Bologna, Bologna, and [1]Istituto di Farmacologia, Università di Ferrara, Ferrara, Italia.

In recent years there has been a considerable interest in the study of the biological activity of nitroheterocyclic drugs. In previous reports we have investigated the mutagenic activity of a series of nitroimidazoles and nitrothiazoles, and its relationship with physico-chemical parameters (Guerra et al., 1981, Cantelli-Forti et al., 1983, Biagi et al., 1983). In the present work we have studied the relationship between the lipophilic character and the urinary excretion of the unchanged forms of a series of 24 nitroimidazoles and 2 nitrothiazoles. The cumulative excretion data at 18, 36, 54 and 72h after i.p. administration are parabolically related with the log P values of the compounds. In particular at 18h the relationship is described by equation 1:

$$\log BR = 2.570\ (\pm 0.042) + 0.050\ (\pm 0.054)\ \log P - 0.964\ (\pm 0.089)(\log P)^2$$

A similar parabola is obtained with the excretion data from the urine of the 36h after administration as shown by eq. 2:

$$\log BR = 2.661\ (\pm 0.045) - 0.119\ (\pm 0.058)\ \log P - 0.499\ (\pm 0.096)(\log P)^2$$

The optimal lipophilic character for urinary excretion is expressed by the log P_o of the fitted curves. It is interesting to note that the log P_o value from eq 1 is very close to that from eq 2. This means that the optimal lipophilic character for urinary excretion does not change in a significant way, as one could have expected. The main difference between the two equations is represented by the lower coefficient of the $(\log P)^2$ term in eq 2 which is due to a relative higher excretion of the most lipophilic and most hydrophilic compounds, taking place between the 18 and 36h. However all this has to be explained. The ideal lopophilic character could fulfill one or more of the following conditions: higher absorption from the i.p. site of inoculation, lower biotransformation in the liver, lower degree of protein binding and/or tubular reabsorption. The relative higher increment in the excretion of the most hydrophilic compounds between 18 and 36h could be due to their slower absorption from the site of administration; for the most lipophilic compounds it could be due to their higher protein binding and/or tubular reabsorption. The present results seem to be one of the first reports relating on a quantitative base urinary excretion and physical chemical parameters. They could help in designing compounds aimed to the treatment of urinary tract diseases.

Guerra, M.C., Barbaro, A.M., Cantelli Forti, G., Foffani, M.T., Biagi, G.L., Borea, P.A., Fini, A. (1981) J. Chromatogr. 216, 102.

Cantelli Forti, G., Aicardi G., Guerra, M.C., Barbaro, A.M., Biagi, G.L., (1983) Teratog. Carcinog. Mutagen. 3, 51-63.

Biagi, G.L., Barbaro, A.M., Guerra, M.C., Cantelli Forti, G., Aicardi, G., Borea, P.A. (1983) Teratog. Carcinog. Mutagen. 3. 429.

INFLUENCE OF VESICLE LIPID COMPOSITION ON DRUG METABOLISM BY A RECONSTITUTED MIXED FUNCTION OXIDASE ENZYME SYSTEM

P.Skett* and S.Cuthill, Department of Pharmacology, The University, GLASGOW G12 8QQ Scotland.

There is evidence to suggest that membrane lipids, as well as the amount and type of enzyme present in the membrane, are responsible for the differences in drug metabolism seen in various conditions.The elegant work of Feuer et.al.(1979) has shown a direct relationship between development of membrane lipids and drug metabolism in the rat liver and early work showed that phospholipids are needed for the activity of a number of drug-metabolising enzymes (Strobel et.al.,1970).

In this study, a microsomal fraction was prepared from male and female rat livers by differential centrifugation (Berg & Gustafsson,1973) and the fraction solubilised by adding sodium cholate solution dropwise to a concentration of 1.8% while stirring the mixture, keeping it on ice and gassing with oxygen-free nitrogen. The solubilised fraction was delipidated using a Bio-gel P-30 column which retains the lipids while allowing the larger protein molecules to be eluted. The delipidated enzymes were reconstituted into lipid vesicles by the method of Ingelman-Sundberg & Glaumann (1980) using mixtures of known synthetic lipids. Pure dilaurylphosphatidylcholine (DLPC),DLPC and dilaurylphosphatidylethanolamine (DLPE) mixture (3:1) and DLPC and cholesterol (94:6) mixture were used as lipid matrix.Cytochrome P-450 NADPH-cytochromeP-450 reductase and cytochrome b5 were measured using previously described methods (Omura & Sato,1962; Philips & Langdon,1962; Estabrook & Werringloer,1978). Drug metabolising enzyme activity was assayed using lignocaine as substrate as previously described (Skett et.al.,1980).

Delipidation resulted in a 75% loss of phospholipid (as assayed by phospholipid phosphorus) from the enzyme complex and the final reconstituted vesicles contained the various enzymes is approximately the same proportions as in the original microsomes. The incorporation of DLPE into the DLPC vesicles resulted in a marked decrease in N-deethylase activity (0.81 ± 0.21 v 2.20 ± 0.47 pmoles/min/nmole P-450) and a smaller, non-significant ($p > 0.05$) fall in 3-hydroxylase (1.54 ± 0.54 v 2.10 ± 0.26 pmoles/min/nmole P-450).The incorporation of 6% cholesterol into the DLPC vesicles, however, gave a somewhat different result in increasing N-deethylase activity (3.48 ± 0.41 v 2.25 ± 0.35 pmoles/min/nmole P-450) and decreasing 3-hydroxylase (4.23 ± 0.46 v 7.46 ± 0.26 pmoles/min/nmole P-450).Both of these effects were significant ($P < 0.05$).

The results indicate that the composition of the membrane can influence the activity of the drug-metabolising enzymes and that the effects can be specific for certain enzyme activities. It is not certain from these experiments whether the changes in lipid composition affect different enzymes in different ways or change the activity of a single enzyme to give different products. Further work using purified species of cytochrome P-450 would be necessary to answer this question.

Berg,A. & Gustafsson,J-Å.(1973) J.Biol.Chem. 248, 6559.
Estabrook,R. & Werringloer,J.(1978) Methods in Enzymol. 52, 212.
Feuer,G.(1979) Drug Metab.Rev. 9, 147.
Ingelman-Sundberg,M.& Glaumann,H.(1980) Biochim.Biophs.Acta 599,417.
Omura,T.& Sato,R.(1962) J.Biol.CHem. 237, 1375.
Philips,A.H. & Langdon,R.G.(1962) J.Biol.Chem. 237, 2652.
Skett,P.et.al.(1980) Biochem.Pharmacol. 29, 2759.
Strobel,H.W.et.al. (1970) J.Biol.Chem. 245, 4851.

THE KINETICS OF PLATELET AND FIBRIN(OGEN) DEPOSITION ON TO DAMAGED RABBIT CAROTID ARTERIES IN VIVO

K.D. Butler, J.A. Davies[1], V.C. Menys[1], R.A. Shand* and R.B. Wallis, Research Centre, CIBA-GEIGY Pharmaceuticals, Horsham, West Sussex, RH12 4AB and [1]University Department of Medicine, The General Infirmary, Leeds, LS1 3EX

A single clamping injury to rabbit carotid arteries in vivo results in deposition of platelets and fibrin (Menys and Davies, 1984). We have now determined the time course of platelet and fibrin(ogen) deposition using iodine crystal scintillation detectors linked to an automated isotope monitoring system (AIMS 8000). Rabbits were injected with either ^{51}Cr-platelets (PL), red cells (RBC), ^{125}I-labelled human fibrinogen (F) or albumin (A). A detector was then placed over the carotid artery and control counts recorded at 1 min intervals for 5 min prior to the vessel being clamped for 5 min. After restoration of flow, recordings were made for a further 45 x 1 min periods. The counts following injury were then expressed as a percentage of the mean count obtained during the control period. The area under the curve (AUC) was calculated and peak heights recorded.

During the 45 min period following injury PL associated radioactivity increased by 12% (median value, range 7.6-15.5% $p = \leq .001$. Mann-Whitney U-test) and F associated radioactivity by 21.4% (range 14.3-49.6 $p = \leq .001$) from the control values. There were no significant increases in either RBC or A associated radioactivity. PL and F deposition on the injured vessel were verified using electron microscopy.

	AUC (Per cent Mins)		
	SHAM	INJURED	APAS
^{51}Cr Platelet	114 ± 2009	15956 ± 2512*	N.D.
^{51}Cr RBC	-5832 ± 3993	-4209 ± 1748	N.D.
^{125}I-Fibrin(ogen)	5650 ± 3315	31422 ± 7956*	17662 ± 5649
^{125}I-Albumin	748 ± 721	-1028 ± 3719	9890 ± 7947

All results are the mean of 5 experiments ± s.e. *Significant increase from sham operated animals. $p \leq 0.01$ (Student's t-test). N.D. not determined.

In rabbits pre-treated with guinea-pig anti-rabbit platelet antiserum (APAS) to deplete the circulating platelet count (by 95%) there was a significant delay in fibrin deposition on the damaged vessel although the circulating fibrinogen concentration was not impaired. This data implicates an involvement of the platelets in the initial fibrin deposition at the site of injury.

Menys, V.C. and Davies, J.A. (1984). Clin.Sci. 66, 22P

PURIFICATION OF INTESTINAL PEROXIDASE AND ITS ACTIVITIES TOWARDS XENOBIOTICS

L. Della Corte, G.P. Sgaragli[1], K.F. Tipton[2] & M. Valoti[1*], Dipartimento di Farmacologia Preclinica e Clinica "M. Aiazzi-Mancini", 50134 Firenze, Italy; [1]Centro Ricerca Interdisciplinare sul Metabolismo dei Farmaci Psicotropi, Universita di Siena, 53100 Siena, Italy and [2]Department of Biochemistry, Trinity College, Dublin 2, Ireland.

Considerable peroxidase activity is associated with the intestine although it is uncertain whether this enzyme is produced by the intestinal cells themselves or arises from eosinophil infiltration (Rytömaa & Teir, 1961). In order to investigate the possible involvement of this enzyme in the metabolism of ingested xenobiotics we have purified it from rat small intestine by a procedure involving hydrophobic chromatography on phenyl-Sepharose and affinity chromatography on concanavalin A-agarose. The purified enzyme appeared to be homogeneous by the criterion of polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate and the ratio of haem absorbance to aromatic amino-acid absorbance (A_{415}/A_{280}) was 0.65. Crude extracts of the enzyme are associated with considerable amounts of material absorbing at 260nm but its removal was effected by the affinity chromatography step.

2-t-butyl-4-methoxyphenol, a commonly used antioxidant food additive, has been shown to be toxic to the rat intestine by a mechanism that may involve its peroxidase-catalysed oxidation (Sgaragli et al, 1980). This compound was shown to be a substrate for the purified intestinal peroxidase in a reaction leading to oxidative ring coupling to produce the considerably less hydrophilic dimer. The product was, however, found to be further oxidised by peroxidase presumably to form a higher aggregate. A similar oxidative ring coupling of morphine was shown to be catalysed by peroxidase.

Rytömaa, T. & Teir, H. (1961) Nature (London) 192, 271-272
Sgaragli, G. et al (1980) Biochem. Pharmacol. 29, 763-769

DISPLACEMENT OF (^{3}H)-ALFENTANIL FROM PLASMA PROTEIN BINDING SITES BY DI-ISOPROPYLFLUOROPHOSPHATE

P.G. Green and I. Kitchen, Department of Biochemistry, University of Surrey, Division of Pharmacology & Toxicology, Guildford, Surrey, GU2 5XH.

The irreversible anticholinesterase agent, di-isopropylfluorophosphate (DFP) potentiates the antinociceptive activity of alfentanil in mice (Kitchen & Green, 1983). Further studies have indicated that DFP enhances entry of alfentanil into the brain (Green & Kitchen, 1985) and this may account for the observed increase in antinociception. Alfentanil has been shown to be highly plasma protein bound in rat, dog and man (Meuldermans et al, 1982) and as only free unbound drug is able to enter the brain, we have investigated the effect of DFP on alfentanil plasma protein binding in vitro and in vivo.

Male albino mice (CD-1 strain, 25-30 g) received either DFP (1 mg/kg) or 0.9% saline subcutaneously. Alfentanil (400µg/kg containing 7µCi of tritium-labelled opioid) was injected subcutaneously 55 minutes later. Animals were decapitated after a further 5 minutes and trunk blood collected. Plasma was prepared by centrifugation and a 20µl aliquot ultrafiltrated in an Amicon micropartition system (MPS-1) for separation of free and protein bound drug. Free and bound alfentanil were determined by liquid scintillation counting. In addition, a second experimental protocol was employed to investigate protein binding in vitro. The procedure was identical to that described above with the exception that alfentanil (150ng containing 17nCi of tritium-labelled opioid) was incubated in vitro with 250µl plasma (37^{0}C for 30 min) obtained from animals pretreated with DFP.

Table 1. Effect of DFP on Plasma Protein binding of [^{3}H]Alfentanil in vivo

	Alfentanil(ng/ml)	
	Control	DFP-treated
Total	485 ± 25.2	286 ± 40.2
Free	118 ± 6.1	110 ± 13.0
% Bound	75.7%	61.5%

Values are the mean ± s.e.m. of six observations.

DFP pretreatment caused a decrease in plasma protein binding of alfentanil in vivo (Table 1). This was accompanied by a decrease in total alfentanil in the plasma indicating redistribution of free drug throughout the animal. Addition of [^{3}H] alfentanil to plasma in vitro confirmed that DFP treatment displaces this opioid from plasma proteins. Under these experimental conditions a 39% increase in free drug concentrations was observed.

DFP is highly protein bound (Martin, 1985) and the displacement of alfentanil from plasma protein binding sites and resultant increase in free drug may account for the enhanced entry into the central nervous system.

Green, P.G. and Kitchen, I. (1985) Br. J. Pharmac. 84, 657-661.
Kitchen, I. and Green, P.G. (1983) Life Sci. 33, 669-672.
Martin, B.R. (1985) Toxicol. appl. Pharmac., 77, 275-284.
Meuldermans, W.E.G., Hurkmans, R.M.A. and Heykants, J.J.P. (1982) Arch. int. Pharmacodyn. 257, 4-19.

CYTOTOXICITY OF MENADIONE AND BENZO(A)PYRENE-3,6-QUINONE IN CULTURED HUMAN FIBROBLASTS

D. Di Monte[4], M.H. Grant[1*], B. Jernstrom[3] and M. Nordenskjold[2] (introduced by G.M. Hawksworth) Department of Therapeutics and Clinical Pharmacology[1], University Medical Buildings, Foresterhill, Aberdeen AB9 2ZD, Departments of Clinical Genetics[2] and Toxicology[3], Karolinska Institute, Stockholm S-104 01, Sweden and Clinica Medica I, Universita degli Studi, Bari, Italy[4].

Quinones can undergo either one-electron reduction to yield semiquinone radicals, or two-electron reduction to more stable hydroquinones. The cytotoxic and anti-tumour properties of quinoid drugs are thought to be mediated through one-electron reduction. In contrast, two-electron reduction, catalysed by DT-diaphorase, is considered to be a detoxifying reaction.

The cytotoxicity of menadione (2-methyl-1,4-naphthoquinone) (MQ) and benzo(a)-pyrene-3,6-quinone (BQ) was compared in cultured human fibroblasts from healthy donors. Toxicity was measured in terms of (i) DNA damage, detected as the increase in % single stranded DNA using the alkaline unwinding method, (ii) cell membrane damage, assessed by Trypan blue penetration and (iii) depletion of reduced glutathione (GSH).

Both quinones damaged fibroblast DNA after 30 min exposure. The cells were more susceptible to BQ induced strand breaks; the threshold concentration of BQ being 1 µM compared with 15 µM for MQ. Dicoumarol, a specific inhibitor of DT-diaphorase, potentiated the response to MQ, but not that to BQ (see Table 1).

Table 1. DNA damage induced by MQ and BQ in human fibroblasts

Treatment	Increase in % single stranded DNA	
Control	0 ± 0.4	(16)
BQ 10 µM	19.4 ± 2.9	(7)*
BQ 10 µM + Dicoumarol 30 µM	14.1 ± 2.2	(6)
MQ 20 µM	20.9 ± 3.0	(6)*
MQ 20 µM + Dicoumarol 30 µM	35.0 ± 1.7	(6)**

Values are Mean ± S.E. mean, with the number of experiments in parentheses.
* P <0.001, compared with control, ** P <0.005, compared with incubations without dicoumarol. (Wilcoxon-Mann-Whitney test).

Treatment with 25 µM MQ for 2h increased the number of cells allowing Trypan blue penetration by 26%. Dicoumarol potentiated this MQ induced damage; 100% of cells being damaged within 2h. BQ did not affect membrane integrity, even in the presence of dicoumarol. 50 µM MQ decreased cell GSH to 5.5 nmol/mg protein after 30 min (control cells contain 15.5 nmol GSH/mg protein). In contrast, BQ did not deplete GSH.

MQ is more toxic to fibroblasts than BQ, and the sequence of toxic events is DNA damage, followed by GSH depletion and ultimately cell membrane damage. MQ induced GSH depletion is due to oxidative stress caused by the redox recycling of semi-quinone radicals (Morrison et al., 1984). BQ does not appear to cause oxidative stress in this system. DT-diaphorase protects fibroblasts agains MQ induced toxicity probably by competing with one-electron reduction.

Morrison, M.H. et al (1984) Biochem. Pharmac. 33, 1763-1769

560P

EFFECTS OF MEDROXYPROGESTERONE ACETATE ON cAMP PHOSPHODIESTERASE ACTIVITY IN RAT MAMMARY TUMOURS

F. Di Carlo, S. Racca, A. Crispino, G. Conti, M. Giubertoni & P. Portaleone, Institute of Pharmacology, Faculty of Medicine, University of Turin, C.so Raffaello 30, Turin (Italy).

An antagonistic action between cAMP and estrogen in rat mammary tumor growth control was demonstrated some years ago by Cho-Chung et al. (1978). Recently, it has been observed that an antiestrogen (tamoxifen) inhibits the activation of cAMP phosphodiesterase (PDE) by calmodulin (H.Y.P. Lam, 1984). Therefore it has been speculated that the antitumor activity of tamoxifen could be in part dependent on the antagonism of calmodulin.

In the present study we investigated the effects of medroxyprogesterone acetate (MPA, a progestin widely used in the treatment of breast cancer) on calmodulin-dependent cAMP PDE activity in rat DMBA-induced mammary tumors.

Mammary tumors were induced in 50 day old Sprague-Dawley female rats by a single oral dose of 20 mg DMBA in olive oil. Ninety days later, about 70% of rats resulted tumor-bearing. These animals were treated for 15 days with MPA at doses of 15 or 75 mg/kg i.m..

The highest dose induced a significant inhibition of tumor growth or, in some cases, the complete disappearance of tumors. Minor inhibitory effect was induced by 15 mg/kg MPA. On the other hand, some tumors continued to grow in spite of the administration of MPA.

Basal cAMP PDE activity was significantly reduced in all tumors taken from MPA-treated rats. On the other hand, the addition of calmodulin (1 ug/ml) and Ca++ (2 mM) to the incubate completely reversed the inhibition of PDE induced by MPA. In fact cAMP PDE activity increased of 98% and 61% in non-responsive and responsive tumors, respectively.

Our results suggest that MPA is able to reduce tumor cAMP PDE activity in the rat and that this effect is obtained, at least in part, via the inhibition of calmodulin.

At present investigations are in progress to establish whether the different ability of calmodulin to reactivate cAMP PDE in responsive and non-responsive tumors is important for the antitumoral activity of MPA.

5-METHYLTETRAHYDROFOLATE (MeTHF) ENHANCES FLUOROPYRIMIDINE CYTOTOXICITY AGAINST HUMAN LEUKEMIA CELLS IN VITRO

Teresita Mazzei, E. Mini* & P. Periti, Department of Preclinical and Clinical Pharmacology, University of Florence, Florence 50134, Italy.

Fluoropyrimidine, 5-fluorouracil (FUra) and 5-fluorodeoxyuridine (FdUrd), are among the most effective agents for the treatment of various solid tumors. FUra is also active in adult acute leukemia (Costanzi et al., 1979). It has been shown that modulation of metabolic processes in tumor cells might alter the intracellular pharmacokinetics and pharmacodynamics of these drugs, and hence their chemotherapeutic efficacy. Since fluoropyrimidines exert cytotoxicity in most cells due to inhibition of thymidylate (dTMP) biosynthesis as a consequence of covalent binding of 5-fluorodeoxyuridylate (FdUMP) to dTMP synthase in the presence of the folate coenzyme 5,10-methylenetetrahydrofolate (5,10-CH_2FH_4) (Santi et al., 1974), the use of an exogenous reduced folate, which might increase intracellular folate pools, including 5,10-CH_2FH_4 (Houghton et al., 1982), and potentially increase the inhibitory action of fluoropyrimidines on dTMP synthase (Ullman et al., 1979), appears of potential therapeutic importance. This prompted us to examine the interactions of MeTHF, the predominant form of serum folate, and FUra or FdUrd in human leukemic lymphoblasts, CCRF-CEM.

When cells were exposed to MeTHF (1-100 µM) for 4 h and to FUra (250 µM) or FdUrd (0.5 µM) during the last 2 h, synergistic inhibitory effects on cell growth were observed. No clear dependence on sequence was observed with FUra and MeTHF (4 h exposure, MeTHF→FUra, FUra→MeTHF, or MeTHF+FUra), while with MeTHF-FdUrd combinations synergy was noted to be dependent on sequence of administration; that is, LV→FdUrd and LV+FdUrd were synergistic, but FdUrd→MeTHF was not. These differences in sequence-dependence of synergy might be explained by a different rate of conversion of the two fluoropyrimidines to the common active metabolite, FdUMP. Hypoxanthine (100 µM) administered concomitantly to MeTHF did not protect cells from cytotoxicity of MeTHF-FUra combination. Thymidine (0.1 µM) partially rescued cells from the cytotoxicity of this combination. L-methionine (1500 mg/L) greatly protected cells from cytotoxicity of combined MeTHF-FdUrd.

These data suggest that the mechanism by which MeTHF potentiates fluoropyrimidine cytotoxicity is the enhancement of ternary complex formation between FdUMP and dTMP synthase, as a consequence of an increase of intracellular levels of 5,10-CH_2FH_4, generated from MeTHF. These results encourage further studies both on the biochemical mechanism of synergy and on the definition of the therapeutic index in vitro utilizing leukemic and normal bone marrow cells from patients.

This work was supported by a National Research Council grant. We thank M. Coronnello, L. Criscuoli & M. Gualtieri for expert technical assistance.

Costanzi, J.J., Gagliano, R., Coltman, C.A. & Bickers, J.N. (1979) Cancer Treat. Rep. 63, 2126-2128.

Houghton, J.A., Schmidt, C. & Houghton, P.J. (1982) Eur. J. Clin. Oncol. 18, 347-354.

Santi, D.V., McHenry, C.S. & Sommer, H. (1974) Biochemistry 13, 471-481.

Ullman, B., Lee, M., Martin, D.W. & Santi, D.V. (1978) Proc. Natl. Acad. Sci. USA 72, 980-983.

562P

EFFECT OF ANTHRACYCLINES ON SUPEROXIDE PRODUCTION AND METABOLIC ACTIVITY OF HUMAN NEUTROPHILS

A. Bertelli & M.L. Schinetti, Institute of Pharmacology, Medical School, University of Pisa, Pisa, Italy.

Cancer chemotherapy is toxic to several types of cells, including polymorphonuclear leukocytes, which play a key role in host defence. Anthracycline antibiotics inhibit human neutrophil phagocytic and antimicrobial activity as revealed by the chemi= luminescent response and superoxide (O_2^-) production during cell activation.

Doxorubicin (DXR) epidoxorubicin (epiDXR) and thepirubicin (THP) pretreatment of the cells, before stimulation with particulate or soluble stimuli leads to a signifi= cantly reduced chemiluminescent response (inhibition ranging between 71% - 78%) revealing a comparable inhibiting power on the bactericidal activity of the cells.

On the other hand the anthracyclines tested exhibited a different behaviour as far as O_2^- generation from both resting and stimulated neutrophils is concerned.

O_2^- production from human neutrophils was determined by using the SOD-inhibitable cytochrome C reduction test.

DXR (10^{-4} M) elicited superoxide production from unstimulated cells is comparable to that induced by phorbol-myristate acetate (PMA) (23.7 n moles/10^6 cells/15'), whereas epi-DXR (10^{-4} M) is far less effective (9.6 n moles/10 cells/15'). THP (10^{-4} M) is completely devoid of superoxide inducing power. No cytochrome C re= duction could be detected during the continuous assay in a 30' period, when neutro= phils were exposed to the drug.

Since there is a growing body of evidence correlating the cardiotoxicity of the anthracyclines to the generation of free radicals, it seems reasonable to correlate the different cardiotoxic effect of these drugs, to their effect on superoxide production.

The more cardiotoxic DXR is the most potent inducer of superoxide generation whereas epi-DXR, less cardiotoxic, has a relatively little effect on superoxide production.

THP which have been shown not to induce delayed cardiomiopathy is ineffective on superoxide generation from the cells.

THE EFFECT OF DEAZAADENOSINE DERIVATIVES ON CELL GROWTH, NUCLEIC ACIDS AND PROTEIN SYNTHESIS IN CULTURED CELLS

W. Balduini, O. Cantoni, F. Cattabeni, G. Cristalli°, M. Grifantini°, G. Piedimonte°°, and P. Sestili. Istituto di Farmacologia e Farmacognosia, Univ. di Urbino; °Istituto di Chimica Farmaceutica, Univ. di Camerino; °°Istituto di Patologia Generale, Univ. di Parma.

Several adenosine analogues resistant to adenosine deaminase have demonstrated cytotoxic activity in numerous types of cells in culture (Lim and Klein, 1981). Among these agents, the newly synthesized pyrrolopyrimidine, 9-deaza-A was shown to be the most cytotoxic, displaying potent growth inhibitory effects against murine and human leukemia cell lines (Lim and Klein, 1981), nine human solid tumours, a human pancreatic carcinoma xenograft (Chu et al., 1982) and a human colon carcinoma (Glazer et al., 1983). In the present report we have studied the activity of A analogues lacking the nitrogen atom in position 1-3-7 and 1,3 of the adenine moiety. The approach taken was to study the effect on growth rate, nucleic acids and protein synthesis in cultured mammalian cells. Vero cells, in exponential growth were exposed for two hours to 1, 3, or 10 uM concentrations of the different drugs, in serum free medium; the rate of macromolecules synthesis was estimated after 1 hr pulse treatment with the radiolabelled precursors. At the concentrations used 1-deaza-A and 3-deaza-A produced no or very little changes in DNA, RNA or protein synthesis. Conversely, 7-deaza-A was a potent inhibitor of the synthesis of macromolecules. 1,3-dideaza-A did not significantly affect RNA and protein metabolism but produced a dramatic increase in DNA synthesis. We have therefore investigated for a possible mitogenic activity of this compound. Vero cells and normal human limphoblasts were treated according to the previously described experimental protocol and the cell number was determined 72 hr after exposure to the drug. In both cell lines the chemical produced a concentration dependent increase in growth rate. The effects of 1,3-dideaza-A were also investigated in three transformed cell lines, such as SV40 3T3 and two Burkitt lynphomas (Namalwa and Daudi). Again, an increase in cell proliferation was found. In other experiments we have attempted to further characterize the effect of 1,3-dideaza-A on Vero cells DNA synthetic functions. The increase in DNA synthesis was not dependent on an increased uptake of ^{3}H-Thymidine. Time course experiments have shown that the effect promptly occurs following addition of the chemical to the growth medium and is accompained by a decrease in the intracellular pool of radiolabelled thymidine. Transport of low molecular weigth metabolites was also investigated. Transport of thymidine was reduced by ca. 30%. Transport of glucose resulted increased by 20-30%. No effect was found on the transport of leucine.

Chu,M.Y, Landry,S.R., Lim,M.I., Badgen,A.E. and Grabtree,G.W. (1982) Proc. Am. Ass. Cancer Res. 23, 25.
Glazer,R.I., Hartman,K.D. and Knode,M.C. (1983) Mol. Pharmacol. 24, 309.
Lim,M.I. and Klein,R.S. (1981) Tetrahedron Lett. 22, 25.

ION TRANSPORT ACTIVATED BY α-LATROTOXIN AND CONGENER TOXINS AT THE PRESYNAPTIC MEMBRANE

L. Madeddu*, J. Meldolesi*, H. Scheer*, and E. Wanke°, Department of Pharmacology* and of Biochemistry and Physiology°, University of Milano, Milano, Italy.

We have studied a group of neurotoxins which, applied at 10^{-10}-10^{-9}M concentrations, massively stimulate release of a wide variety of neurotransmitters. These toxins (purified from different sources: αlatrotoxin, αLTx, from the venom of the black widow spider; leptinotoxin-h, LPTx, from the hemolymph of the potato beetle, Leptinotarsa haldemani; glycerotoxin, GCTx, from the venom of the sea worm Glycera convoluta) are high (55-300 KD) M_r proteins devoid of enzyme activity. At vertebrate synapses of the central or peripheral system, these toxins stimulate exocytosis. This effect occurs with and, although attenuated, without Ca^{++} in the incubation fluid. αLTx and LPTx are active also in a line of neurosecretory cells (PC12). The three toxins induce: large depolarization of the plasma membrane insensitive to tetrodotoxin (measured by $TPMP^+$ distribution as well as bis-oxonol fluorescence); increased ^{45}Ca influx (insensitive to verapamil); large rises of cytosolic free Ca^{++} (when applied in Ca^{++}-containing medium). Single αLTx-induced channels have been studied in PC12 cells by patch clamp. Their conductance is of 40 pS, and they do not discriminate between Na^+ and K^+. Specific, high affinity binding sites (receptors) of αLTx exist in brain synaptosomes membranes, PC12 cells and neuromuscular junctions where they are localized exclusively in the presynaptic membrane. αLTx receptors, from bovine synaptosomal membranes, were purified by affinity chromatography. The pure receptor (a 200 KD integral membrane protein composed of several subunits) is able to bind αLTx with unchanged affinity. After reincorporation into lipid bilayers the receptor mediates membrane conductance changes upon application of αLTx. Receptors of LPTx and GCTx have not yet been studied. They seem however different from αLTx receptors because: a) the various toxins do not compete for ^{125}I-αLTx binding and b) some differences exist in target and activity specificity. We conclude that the three toxins activate ion transport through cation selective channels (different from the classical Na^+, K^+ and Ca^{++} channels) that in neurons seem to exist (or to form) exclusively in presynaptic membanes.

DEPRESSION OF CONTRACTILE RESPONSES BY SPONTANEOUSLY RELEASED EDRF IN RAT AORTA

R.F. Furchgott, D. Jothianandan, W. Martin[*1] and G.M. Villani, Department of Pharmacology, S.U.N.Y. Downstate Medical Centre, New York 11203 U.S.A. and [1]Department of Cardiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN. (Introduced by J.A. Smith)

Eglème et al (1984) showed that the endothelium depresses contractions of rat aorta induced by α-adrenoceptor agonists, particularly the preferential α_2-agonist clonidine and proposed that stimulation of endothelial α_2-adrenoceptors evokes release of the endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980) which counteracts the vasoconstriction. Using the EDRF blocking agent haemoglobin, we showed that spontaneously released EDRF depresses contractile responses in rabbit aorta (Martin et al, 1985). We wished therefore to determine the role of spontaneous and evoked release of EDRF on the endothelium-dependent depression of contraction in rat aorta.

Endothelium-containing rings of rat aorta were 4-fold less sensitive (EC_{30} 4.7×10^{-8}M, n=11) to the contractile effects of phenylephrine than endothelium-denuded rings (EC_{30} 1.2×10^{-8}M, n=6) but the maximum contractile responses of endothelium-containing and endothelium-denuded rings were similar (E_{max} 3.5 $\pm$ 0.2 g, n=11, and 4.0 $\pm$ 0.2 g, n=6, respectively). Endothelium-containing rings were less sensitive (EC_{30} 1.7×10^{-7}M, n=9) to the contractile effects of clonidine than endothelium-denuded rings (EC_{30} 4.0×10^{-8}M, n=10) and the maximum response was depressed 73% (E_{max} 0.5 $\pm$ 0.1 g, n=9) when compared with endothelium-denuded rings (E_{max} 1.9 $\pm$ 0.2 g, n=10). Exposure to haemoglobin (10^{-5}M, 10 min) selectively abolished the depressions of phenylephrine - and clonidine - induced contraction in endothelium-containing rings but did not affect the responses of endothelium-denuded rings, suggesting that these depressions were mediated by EDRF. We found no evidence, however, that phenylephrine or clonidine could elicit EDRF release: the resting level of cyclic GMP in endothelium-containing rings was 309 $\pm$ 60 pmol g^{-1} protein (n=6) and this was not affected by phenylephrine (10^{-7}M, 1 min) or clonidine (10^{-6}M, 1 min or 10 min), whereas acetylcholine (10^{-5}M, 1 min) which elicits EDRF release induced a 4-fold rise in cyclic GMP content. Haemoglobin (10^{-5}M, 10 min) reduced the cyclic GMP content of endothelium-containing rings to 150 $\pm$ 20 pmol g^{-1} protein (n=6); a level comparable to that of endothelium-denuded rings (121 $\pm$ 29 pmol g^{-1} protein, n=6), suggesting that EDRF was tonically active in unstimulated aortic rings.

As the E_{max} for clonidine was about 48% of that for phenylephrine in endothelium-denuded rings we considered the possibility that spontaneously released EDRF could depress contractions to a partial agonist more than a full agonist. Following partial irreversible α-adrenoceptor blockade using dibenamine (2×10^{-7}M, 20 min) we reduced the E_{max} of endothelium-denuded rings to phenylephrine to 56 $\pm$ 17% of control (ie, comparable to E_{max} for clonidine) but the E_{max} of endothelium-containing rings was reduced disproportionately more to 10 $\pm$ 1% of control, (n=4). Thus, by reducing the E_{max} of phenylephrine we could mimic the profound endothelium-dependent depression of E_{max} observed with clonidine.

In conclusion, spontaneously released EDRF depresses contraction induced by α-adrenoceptor agonists in rat aorta and this depression is greater for agonists with a low efficacy. There is no evidence that rat aortic endothelial cells possess α_2-adrenoceptors functionally linked to EDRF release.

Eglème et al. (1984) Br. J. Pharmac. 81, 16-18
Furchgott, R.F. & Zawadzki, J.V. (1980) Nature 288, 373-376
Martin et al. (1985) J. Pharmac. Exp. Ther. 232, 708-716

EFFECTS OF COCAINE ON CATECHOLAMINE-INDUCED INHIBITION OF NERVE-INDUCED RESPONSES IN RAT VAS DEFERENS

A. MacDonald* & J.C. McGrath[1], Department of Biological Sciences, Glasgow College of Technology, Glasgow G4 0BA and [1]Institute of Physiology, University of Glasgow, Glasgow G12 8QQ.

Previously we reported that cocaine had no effect on the pre-junctional α-adrenoceptor-mediated inhibition by noradrenaline of the non-adrenergic contractile response to single pulse field stimulation in the isolated rat vas deferens (MacDonald & McGrath, 1982). This is difficult to reconcile with current ideas of "co-transmission" in the rat vas deferens (French & Scott, 1983; Sneddon & Westfall, 1984). We have therefore re-examined the effects of cocaine.

Contractile responses of prostatic portions of isolated rat vas deferens were elicited by single pulse (0.5 ms) field stimulation. The pulses were delivered at 5 minute intervals in order to avoid the complication of endogenous feedback. Cocaine (3 μM) itself had little effect on the prostatic peak responses. Noradrenaline (0.03-3 μM) produced a concentration-related inhibition of the responses. The presence of EDTA (57 μM) and ascorbic acid (27 μM) in the Krebs medium had little or no effect on the inhibition by noradrenaline. The inhibition was clearer in the presence of WB 4101 (0.1 μM) to block post-junctional excitatory α_1-adrenoceptor effects. Cocaine (3 μM) significantly potentiated the inhibition by noradrenaline (e.g. mean responses expressed as % control: noradrenaline 1 μM, 82 $\pm$3; Noradrenaline 1 μM plus cocaine 3 μM, 52 $\pm$8; $p<0.01$, $n=5$).

The noradrenaline-induced inhibition of nerve-induced responses could be partly reversed by the α-adrenoceptor antagonists yohimbine and rauwolscine (0.6-6 μM). An unexpected effect of cocaine was that it potentiated the antagonism of noradrenaline (e.g. mean responses expressed as % control after reversal with yohimbine 0.6 μM: no cocaine, 35 $\pm$4; cocaine 3 μM, 60 $\pm$5; $p<0.01$, $n=4$). Adrenaline (0.01-30 μM) and dopamine (0.01-100 μM) also produced concentration-related inhibition of the prostatic nerve-induced responses. Antagonism of these inhibitory effects by yohimbine was again more marked in the presence of cocaine.

In conclusion, the present results show that cocaine can potentiate the inhibitory effect of noradrenaline on nerve-induced responses in isolated rat vas deferens which differs from our earlier conclusions (MacDonald & McGrath, 1982). The discrepancy is probably due to the fact that the effect of cocaine is relatively small and is not always seen, in contrast to the clear potentiation of post-junctional α_1-adrenoceptor-mediated excitatory effects (MacDonald & McGrath, 1982). The reason for the potentiation of yohimbine by cocaine is not clear. Two possibilities arise. Firstly that cocaine unmasks post-junctional α_1-adrenoceptor excitatory effects of the catecholamines. Secondly that in the absence of cocaine exogenous noradrenaline acts partly on yohimbine-insensitive receptors (Baker et al, 1984) whereas in the presence of cocaine the main action of exogenous noradrenaline is on yohimbine-sensitive α_2-adrenoceptors.

Baker, D.J. et al (1984) Br. J. Pharmac. 81, 457-464.
French, A.M. & Scott, N.C. (1983) Experentia, 39, 264-266.
MacDonald, A. & McGrath, J.C. (1982) Br. J. Pharmac. 76, 211P.
Sneddon, P. & Westfall, D.P. (1984) J. Physiol. 347, 561-580.