1P HIGH AFFINITY BINDING SITES FOR [^{35}S]ATPγS IN RAT VAS DEFERENS MAY BE P_{2X} PURINOCEPTORS

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[^{3}H]αβmeATP is the only well characterised radioligand that has been used to label the P_{2X} purinoceptor for ATP (Bo et al., 1994). However, another stable ATP analogue, ATPγS, has been identified as possessing high affinity for the [^{3}H]αβmeATP binding sites of rat vas deferens (Bo et al., 1994). Since [^{35}S]ATPγS is commercially available the aim of the present study was to determine if this radioligand could be used to label P_{2x} purinoceptors in rat vas deferens.

Rat vas deferens membranes were prepared as described previously (Michel & Humphrey, 1994). Assays were conducted at 4°C using a 50mM Tris 1 mM EDTA buffer (pH 7.4). [^{35}S]ATPγS (0.2 nM, Spec. Act 1500Ci/mmol) and membranes were incubated, usually for 3hr, in a volume of 250μl and assays were terminated by vacuum filtration (Michel & Humphrey, 1994). NSB was defined using 10μM ATPγS. Data are presented as the mean±s.e.m (n=3-5).

Specific [^{35}S]ATPγS binding reached a steady state in 2 hr ($t_{1/2}$ 38±6 min), was stable for 4hr and was reversible ($t_{1/2}$ 45±8 min) following addition of 10μM ATPγS. The kinetic data were best described by assuming association with, and dissociation from, a single population of non-interacting binding sites. Saturation studies, performed by isotopic dilution with unlabelled ATPγS, were also consistent with the radioligand labelling a single population of binding sites (K_D = 1.62 nM : B_{max} 14670±2100 fmol.mg.protein^{-1}).

Binding of [^{35}S]ATPγS was inhibited by a number of nucleotide analogues, cibacron blue and suramin (see table), but not by GTPγS (1μM), ouabain (10μM), thapsigargin (1μM), cyclopiazonic acid (10μM), NECA (10μM), cyclohexyladenosine (10μM), sodium thiosulphate (1mM) or pyrophosphate (100μM). Radioligand binding was increased 3.2±0.4 fold by 4mM $CaCl_2$, but about 75% of this binding was inhibited by 1μM GTPγS suggesting that additional non-P_{2X} sites may be labelled by [^{35}S]ATPγS under these conditions.

The rank order of potency (ATP = 2me-S-ATP ≥ ATPγS >αβ meATP>βγmeATP) at the high affinity [^{35}S]ATPγS binding sites in rat vas deferens was similar to that obtained at the P_{2X} purinoceptor of rat vagus nerve in the absence of divalent cations (Trezise et al., 1994). These rank orders of agonist potency differ from that obtained at the [^{3}H]αβmeATP binding sites in rat vas deferens (Michel & Humphrey, 1994), where αβmeATP was the most potent compound. This may be due to the presence of $CaCl_2$ in the later study with [^{3}H]αβmeATP, since $CaCl_2$ greatly increases the affinity of αβmeATP for its binding sites (Michel & Humphrey, 1994) and markedly influences metabolism of hydrolysable nucleotides (Khakh et al., 1995). Due to its high specific activity, [^{35}S]ATPγS may be a useful radioligand for the study of P_{2X} purinoceptors.

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Compound	ATP	2meSATP	ATPγS	αβmeATP	ADP	βγmeATP	Cib Blue	Suramin	UTP
pIC_{50}	9.01±0.08	8.79±0.12	8.73±0.10	7.57±0.14	7.24±0.11	7.18±0.13	5.76±0.01	5.20±0.10	4.7±10.13
Hill Slope	0.85±0.10	0.68±0.03	0.89±0.04	0.92±0.06	0.84±0.04	0.96±0.13	1.39±0.08	0.69±0.06	0.81±0.11

2P INHIBITION OF ECTOATPase AND Ca-ATPase IN RAT VAS DEFERENS BY P_2 PURINOCEPTOR ANTAGONISTS

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Previous studies have suggested that the low potency of ATP and some other nucleotides as P_{2X} purinoceptor agonists in rat vas deferens is due, in part, to their metabolism by ectoATPase (Khakh et al., 1995). In the present study we have examined the ability of some P_2 purinoceptor antagonists to inhibit ectoATPase and Ca-ATPase activity in the rat vas deferens.

Bisected vasa deferentia were obtained from 200-250g, male, Sprague-Dawley rats. Breakdown of ATP was assessed in 4ml of oxygenated Hepes buffer (Khakh et al., 1995) at 37°C using the luciferin-luciferase technique. The breakdown of [γ^{33}P]ATP (1nM) by vas deferens membranes (Khakh et al., 1994) was determined at 37°C in 50mM Tris, 4mM $CaCl_2$ buffer, pH 7.4, by measuring the release of the terminal phosphate group, as described by Beukers et al., (1993), over a 10 min period, in the presence or absence of varying concentrations of competing compounds. The data are presented as the mean±s.e.m of 3-5 experiments. Statistical significance was assessed by Student's t test.

The breakdown of ATP by bisected vasa deferentia was rapid ($t_{1/2}$ of 9.4±2.3, 9.1±1.2 and 22.3±4.8 mins at 1, 10 and 100 μM ATP respectively). Using a 1μM concentration of ATP, ATP levels significantly decreased (p<0.05) to 51.2±7.1% of their initial level after a 20 min incubation with tissue. In contrast, in the presence of 30μM pyridoxalphosphate-6-azophenyl-2',-4'-disulphonic acid (PPADS), 300μM suramin or 300μM pyridoxal 5'-phosphate (P5P) there was no significant (p>0.05) breakdown of ATP (i.e. in the presence of PPADS, suramin and P5P, respectively, ATP concentrations were 80.7±10.2, 104.7±15.9 and 76.5±10.0% of their initial level after the 20 min incubation with tissue).

To quantify the effect of the P_2 purinoceptor antagonists as ATPase inhibitors their action on the Ca-ATPase activity of rat vas deferens membranes was determined. In these membranes ATP was rapidly metabolised with an estimated K_m of 3μM. Metabolism was eliminated by omission of extracellular Ca^{2+} (+1mM EDTA), inhibited (pIC_{50} 5.1±0.1) by the ectoATPase inhibitor FPL67156 (Crack et al., 1995), but was not significantly affected by inhibitors of the sarcoplasmic reticulum Ca-ATPase (10μM cyclopiazonic acid), mitochondrial ATPase (NaN_3 100μM) or Na-K-ATPase (ouabain 10μM). The P_2 purinoceptor antagonists, cibacron blue, suramin, PPADS and P5P inhibited the Ca-ATPase activity with pIC_{50} values of 5.9±0.1, 5.4±0.1, 4.4±0.1 and 3.3±0.1.

This study demonstrates that a number of P_2 purinoceptor antagonists inhibit ectoATPase in rat vas deferens and can inhibit the Ca-ATPase activity of rat vas deferens membranes. While the identity of the Ca-ATPase in the membranes is not certain the inhibition by FPL67156 suggests it may represent, in part, an ectoATPase activity. Since the pIC_{50} values for cibacron blue and suramin as Ca-ATPase inhibitors were similar to their pK_i values of 5.6 and 5.5, respectively, determined in binding studies on the P_{2X} purinoceptor (Khakh et al., 1994), this could limit their usefulness as P_{2X} purinoceptor antagonists, when using agonists which are either susceptible to ectoATPase and/or which release ATP. This problem may be less pronounced with PPADS which is more potent as a P_{2X} antagonist in binding studies (pK_i =5.9; Khakh et al., 1994) than it was a Ca-ATPase inhibitor in this study.

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3P SYNERGISTIC INTERACTIONS BETWEEN HUMAN TRANSFECTED ADENOSINE A_1 RECEPTORS AND ENDOGENOUS CHOLECYSTOKININ RECEPTORS IN CHO-KL CELLS

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In Chinese hamster ovary cells (CHO-K1) activation of the human transfected adenosine A_1-receptor synergistically enhanced the accumulation of ^{3}H-inositol phosphates (^{3}H-IP) produced by the endogenous P_2-purinoceptor (Megson et al., 1995). In this study, we have investigated whether the transfected adenosine A_1-receptor potentiates the ^{3}H-IP response elicited by the endogenous cholecystokinin-receptor in CHO-K1 cells (Freund et al., 1994).

CHO-K1 cells transfected with the human adenosine A_1-receptor (CHO-A1) were grown as described previously (Townsend-Nicholson & Shine, 1992). Cell monolayers were loaded for 24 h with ^{3}H-myo-inositol (37 KBq/well) in 24 well cluster dishes in inositol-free DMEM containing 0.1% foetal calf serum and 2 mM L-glutamine. Cells were then washed once and incubated in Hanks/HEPES buffer (290 µl/well), pH 7.4, for 30 min in the presence of 20 mM LiCl. Agonists were added in 10 µl of medium and the incubation continued for 45 min at 37°C. Incubations were terminated with ice-cold methanol/0.12 M HCl (1:1 v/v) and total ^{3}H-inositol phosphates isolated as described previously (White et al., 1993). Data are shown as means ± s.e.mean. Statistical analysis was performed with Student's unpaired t-test. A P value <0.05 was considered as statistically significant.

Cholecystokinin (CCK; 1µM) elicited a 5.5 ± 0.7 fold increase in ^{3}H-IP in CHO-A1 cells (EC_{50} 18 ± 5 nM; n=5). The response to CCK (1 µM; EC_{50} 14 ± 2 nM; 6.0 ± 0.9 fold; n=4) was insensitive to pertussis toxin (PTX) pretreatment (100 ng/ml; 24 h). The A_1-receptor agonist N^6-cyclopentyladenosine (CPA; 1µM) produced a 1.6 ± 0.1 fold increase in ^{3}H-IP (n = 5) which was completely inhibited by PTX (100 ng/ml; 24 h). A combination of CPA (1 µM) and CCK (1 µM) produced a 9.5 ± 0.9 (n=5) fold increase in the accumulation of ^{3}H-IP which is significantly greater than the predicted 6.0 ± 0.6 (n=5) fold increase (P<0.05). In contrast, the ^{3}H-IP response to conjoint addition of 100 µM ATPγS and 100 nM CCK (7.25 ± 0.6; n=4) was similar to the predicted additive value (6.9 ± 0.6; n=4). In these experiments CCK (100 nM) and ATPγS (100 µM) alone produced responses of 4.7 ± 0.6 (n=4) and 3.1 ± 0.04 (n=4) fold increases in ^{3}H-IP respectively.

This study has shown that the human transfected adenosine A_1-receptor (which is coupled to PTX-sensitive G protein(s)) can interact synergistically with the endogenously expressed cholecystokinin receptor (which is coupled to PTX-insensitive G protein(s)) in CHO-K1 cells.

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4P SUBSTRATE SPECIFICITY OF ADENOSINE TRANSPORT IN PROCYCLIC FORMS OF *TRYPANOSOMA BRUCEI BRUCEI*

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The protozoan parasite *Trypanosoma brucei brucei* lacks the enzymes required for *de-novo* synthesis of purines (Hammond & Gutteridge, 1984). Thus, this organism relies entirely on salvage of exogenous purines from the mammalian host to satisfy its nucleotide requirement. Purine salvage therefore represents a possible chemotherapeutic target in these parasites. Membrane transport of purines is the first step in purine salvage and in contrast to mammalian cells, little is known about this process in *Trypanosoma brucei*. It has been reported recently that bloodstream forms of the parasite possess two high affinity adenosine transporters (Carter & Fairlamb, 1993). In this study we report evidence for a single adenosine transporter in cultured procyclic forms of *T. brucei brucei*.

Adenosine transport by mid-logarithmic *T. b. brucei* procylics cultured in SDM-79 media (Brun & Schoenberger, 1979) was determined by a rapid oil-stop method (Jarvis *et al.*, 1982). In brief, [^{3}H]adenosine (100 µl, 1 µCi) was overlayed onto 200 µl of a dibutylphthalate/mineral oil (7:1 v/v) mixture (density 1.018 g/ml at 25°C). To initiate transport, cells were added and the assay terminated by adding 1 ml of ice-cold buffer containing 1 mM adenosine. Cells were then spun through the oil and the pellet was counted for accumulated radioactivity.

Uptake of 1 µM adenosine by *T. b. brucei* was found to be linear at 25°C over a period of 20 s. Adenosine influx showed saturable kinetics with a high affinity (K_m 0.25 ± 0.02 µM, V_{max} 1.08 ± 0.4 pmol/10^7 cells s^{-1}; mean ± S.E. (n=3)). Adenosine, inosine and guanosine were potent inhibitors of 1 µM adenosine uptake with monophasic inhibition profiles (IC_{50} 1.0 ± 0.13 (3), 3.6 ± 0.32 (3) and 4.7 ± 0.18 µM (3), respectively). Adenine and hypoxanthine had no effect on adenosine uptake, neither did the pyrimidine nucleosides thymidine and uridine. Of the mammalian equilibrative nucleoside transport inhibitors tested, dipyridamole inhibited 1 µM adenosine transport in procyclics of *T. b. brucei* (IC_{50} 3.2 ± 0.13 µM (3)) but nitrobenzylthioinosine had no effect. Interestingly, tubercidin, an adenosine analogue substituted at the 7-position of the purine ring failed to block transport.

In conclusion, these results demonstrate that adenosine transport occurs via a single high-affinity transporter which has a substrate specificity similar to the P1 adenosine transporter in bloodstream forms of *T. b. brucei*. This permeant specificity differs from that of mammalian nucleoside transporters and this may facilitate the exploitation of purine salvage as a potential target of anti-parasitic drugs.

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5P RENAL ADENOSINE RECEPTORS IN RATS WITH ACUTE RENAL FAILURE

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We have previously demonstrated a selective increase in sensitivity to the renal vasoconstrictor actions of adenosine in glycerol but not in $HgCl_2$-induced acute renal failure (ARF) in the rat (Gould *et al.* 1995). One explanation for this phenomenon could be a change in the adenosine receptor population in the kidney. The aim of this work was to assess renal adenosine receptor binding characteristics during the development of ARF.

ARF was induced by injection of either glycerol (10 ml kg^{-1} of 50% v/v glycerol in saline i.m.) or $HgCl_2$ (2 mg kg^{-1} s.c.). Control animals were given equivalent volumes of saline. At various times, kidneys were removed and freeze clamped in liquid nitrogen. Kidney cell membranes were diluted to 1 mg ml^{-1} protein and preincubated with adenosine deaminase (5U ml^{-1}) for 30 minutes at 37°C; followed by 5 hours incubation at 4°C with concentrations of the selective A_1 antagonist ^{3}H-8-cyclopentyl-1,3-dipropylxanthine (^{3}H-CPX, 0.08-12 nM) or the A_{2a} agonist ^{3}H-CGS 21680 (1-500 nM) in Tris buffer (50 mM) at pH 7.4.

In rats with glycerol-induced ARF, there was a progressive increase in the number of ^{3}H-CPX binding sites (Bmax), which by 48 h had increased 2.4 fold compared to the control group (Table 1). In addition, the apparent dissociation constant (Kd) also increased during the development of ARF. By contrast to glycerol-induced ARF, there were no statistically significant changes in either Kd or Bmax following $HgCl_2$ injection although the severity of ARF, as indicated by plasma urea concentrations, was similar in the two forms of ARF. Specific binding of ^{3}H-CGS 21680 was not detected in either controls or rats with ARF.

Present findings suggest: (1) there are changes in the binding characteristics of renal adenosine A_1-receptors during development of glycerol-induced ARF; (2) these do not occur in $HgCl_2$-induced ARF and (3) there are few adenosine A_{2a}-receptors in rat kidney.

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Table 1 Binding characteristics of renal adenosine A_1 receptors and plasma urea concentrations in rats with acute renal failure

Group	Kd (pM)	Bmax (fmol mg^{-1})	Urea (mg 100ml^{-1})	Group	Kd (pM)	Bmax (fmol mg^{-1})	Urea (mg 100 ml^{-1})
Saline (i.m.) 0.5h	576 ± 82	11.9 ± 1.3	47 ± 8	Glycerol (i.m.) 0.5h	301 ± 62	9.8 ± 0.7	40 ± 6
Saline (i.m.) 16h	299 ± 24	7.5 ± 1.1	38 ± 2	Glycerol (i.m.) 16h	730 ± 52*	20.6 ± 2.0*	231 ± 17
Saline (i.m.) 48h	329 ± 23	13.1 ± 2.6	39 ± 6	Glycerol (i.m.) 48h	1196 ± 46*	31.2 ± 2.6*	258 ± 67
Saline (s.c.) 48h	507 ± 60	18.2 ± 2.1	32 ± 2	$HgCl_2$ (s.c.) 48h	817 ± 157	22.7 ± 5.9	220 ± 40

Estimate ± s.e. estimate (8 d.f.) from nonlinear least squares regression analysis of binding isotherm; * $p < 0.01$ (*t*-test) relative to saline controls

6P URIDINE 5'TRIPHOSPHATE STIMULATES MITOGEN-ACTIVATED PROTEIN KINASE IN EAhy 926 ENDOTHELIAL CELLS

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Endothelial cells are known to express the P_{2Y}-purinoceptor and effects of ATP have been attributed to activation of this receptor. However, recently another receptor responsive to both ATP and UTP, termed the nucleotide receptor (P_{2U}) has been identified on endothelial cells (O'Connor *et al.*, 1991) and stimulation of this receptor may account for some of the responses to ATP. ATP stimulates tyrosine phosphorylation of mitogen activated protein (MAP) kinase in renal mesangial cells (Huwiler & Pfeilschifter, 1994) and this may be important in the mitogenic effect of this nucleotide. We have investigated stimulation of MAP kinase by UTP in the endothelial cell line EAhy 926.

Experiments were performed on EAhy 926 cells cultured on six well plates and made quiescent in serum free conditions for 40 h. Protein tyrosine phosphorylation was examined by Western blotting using an anti-phosphotyrosine polyclonal antibody. MAP kinase and isoforms of protein kinase C (PKC) were identified using the anti-MAP kinase monoclonal antibody MK12 and specific monoclonal antibodies respectively. All antibodies were purchased from Affiniti Research Products Ltd, Nottingham, England. MAP kinase activity was assessed by *in vitro* kinase assay using the EGF receptor peptide as substrate (BIOTRAK assay kit, Amersham).

In EAhy 926 cells, the nucleotides ATP and UTP stimulated tyrosine phosphorylation of the pp42kDa isoform of MAP kinase in a concentration dependent manner (30μM maximum). For UTP an EC_{50} value of 3 ± 0.2μM was obtained. In contrast no activation of MAP kinase was observed on stimulation of the cells with the specific P_{2Y}-purinoceptor agonist 2-methylthio ATP. The time course of stimulation in response to both ATP and UTP was transient, with maximal activation occurring at 2 min and levels returning to basal by 60 min. The time course of tyrosine phosphorylation was closely matched by the shift in mobility of MAP kinase when phosphotyrosine blots were reprobed with a specific MAP kinase antibody. MAP kinase activity, as assessed by phosphorylation of the specific EGF receptor peptide assay, showed a similar time course (control 8482±55 dpm; UTP 2min 21705±5743; UTP 60min 9742±2009 dpm, n=3). 18 h pretreatment with 50 ng ml^{-1} pertussis toxin had no effect on MAP kinase activation induced by UTP. Pretreatment of the cells for 24 hr with the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA, 100 nM), downregulated PKCα and ε in these cells and resulted in over 90% reduction in the activation of MAP kinase in response to maximal concentrations of UTP.

These results show that both ATP and UTP stimulate the tyrosine phosphorylation and increased kinase activity of MAP kinase in EAhy 926 cells and suggest that this activation occurs through interaction with a P_{2U}-purinoceptor, rather than interaction with a P_{2Y}-purinoceptor. MAP kinase activation does not appear to involve activation of a pertussis toxin-sensitive G-protein but possibly involves PKC.

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7P THE *IN VIVO* PHARMACOLOGICAL PROFILE OF CP-122,288, A SELECTIVE INHIBITOR OF NEUROGENIC INFLAMMATION

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The inhibition of neurogenic inflammation within cranial tissues has been proposed as the mechanism of action of the anti-migraine agent, sumatriptan (Moskowitz, 1992). In contrast, the cardiovascular side-effects observed to sumatriptan in the clinic may be due to vasoconstriction. Recently, CP-122,288 has been shown to be a potent inhibitor of neurogenic inflammation in guinea-pig dura mater (Lee & Moskowitz, 1993). In this paper, we compare doses of CP-122,288 and sumatriptan that inhibit neurogenic inflammation with those which produce vasoconstriction.

In rat, plasma protein extravasation (PPE) was measured in the dura mater following electrical stimulation of the trigeminal ganglion; compound was administered either before or after ganglion stimulation. Haemodynamic parameters were also determined in rat. In the anaesthetised dog, a range of haemodynamic parameters were determined, including carotid blood flow (CBF), and coronary arterial diameter (CAD, by sonimicrometry). CP-122,288 (0.3-300ng/kg i.v.), administered prior to stimulation of the trigeminal ganglion, produced a dose-dependent inhibition of PPE in rat dura with a minimum effective dose (MED) of 3ng/kg (n=8); maximal inhibition occurred at 30ng/kg i.v. (PPE ratio, stimulated/unstimulated dura: control, 2.0±0.3, n=13; CP-122,288 30ng/kg i.v., 1.0±0.1, n=7, $P<0.05$). When compared with the MED for sumatriptan (100µg/kg i.v.), CP-122,288 is 30,000-fold more potent in this model. Low doses of CP-122,288 (30 & 300ng/kg i.v.) when administered during an ongoing and established nerve-driven PPE response in rat dura also produced a complete inhibition of neurogenic inflammation. Over the dose-range used (0.3-300ng/kg), CP-122,288 did not alter mean arterial pressure or heart rate in the anaesthetised rat. Similarly, low doses of CP-122,288 (1-100ng/kg i.v.) produced no haemodynamic changes in the anaesthetised dog. However, at higher doses (1-300µg/kg i.v.), both CP-122,288 and sumatriptan produced a similar dose-dependent reduction of CBF (ED_{50} value, CP-122,288, 8± 2 µg/kg; sumatriptan, 9±1 µg/kg, both n=10) and CAD (ED_{50} value, CP-122,288, 25±7 µg/kg, n=8; sumatriptan, 23±4 µg/kg, n=10). At the higher doses tested (100-300µg/kg i.v.), both compounds had moderate effects on heart rate and mean arterial blood pressure.

These data demonstrate that at doses which produce a maximal inhibition of neurogenic inflammation, CP-122,288 differs from sumatriptan in that it is completely devoid of vasoconstrictor activity in canine carotid and coronary vascular beds. If neurogenic inflammation is involved in the pathogenesis of migraine, CP-122,288 has the potential to be a safe and effective acute treatment.

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8P EFFECT OF CP-122,288 ON INCREASED SKIN BLOOD FLOW INDUCED BY ELECTRICAL STIMULATION OF THE RAT SAPHENOUS NERVE

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Electrical stimulation of the saphenous nerve leads to increased skin blood flow which is attenuated by the calcitonin gene-related peptide receptor antagonist ($CGRP_{8-37}$) in the rat hind paw (Escott & Brain, 1993). Recently, a C-3 conformationally restricted tryptamine analogue, CP-122,288 (Lee & Moskowitz, 1993) has been shown to be a potent inhibitor of neurogenic inflammation in the dura mater of rat (Gupta *et al.*, this meeting) and guinea-pig (Lee & Moskowitz, 1993). We have examined the effect of CP-122,288 on the increased blood flow induced by electrical stimulation (10V, 1ms, 2Hz for 30s) of the rat saphenous nerve. Male Wistar rats (200-250g), anaesthetised with sodium pentobarbitone (50mg kg^{-1}, i.p.), were prepared for electrical stimulation of the saphenous nerve of the test leg while the contralateral leg served as a sham control. Skin blood flow was measured continuously in both paws by a dual channel Moor laser Doppler flow meter. The saphenous nerve in the test leg was stimulated at least twice (once before and once after i.v. test agent, see Escott & Brain, 1993). The peak increase in blood flow after electrical stimulation of the saphenous nerve, in control (saline, 1ml kg^{-1} i.v.) rats was 118.5 ± 21.4 flux in the stimulated paw, -1.5 ± 1.1 flux in the sham paw for the first stimulation and 109.2 ± 20.4 flux in the stimulated paw, 1.8 ± 1.6 flux in the sham paw for the second stimulation. CP-122,288 (60ng kg^{-1}) significantly inhibited skin blood flow in the stimulated paw: peak skin blood flow before CP-122,288, 142.2 ± 28.4 flux, and after 32.2 ± 12.9 flux, n=6, $P<0.01$ (modified t-test). In further experiments, i.v. CP-122,288 (60ng kg^{-1}) did not effect systemic blood pressure (mean arterial pressure: before 82 ± 3 mmHg and 10 min after i.v. agent 84 ± 3 mmHg, n=5, $P>0.05$). Furthermore, in sequential measurements of paw skin blood flow, i.v. CP-122,288 (60ng kg^{-1}) did not significantly effect basal (i.d. Tyrode-injected site; before 40 ± 5 flux and 10 min after i.v. CP-122,288, 39 ± 5 flux, n=4, $P>0.05$) or CGRP (10pmol $site^{-1}$, i.d.)-induced (before 116 ± 7 flux and 10 min after i.v. CP-122,288, 107 ± 5 flux, n=4, $P>0.05$) cutaneous blood flow. These results demonstrate that neurogenic vasodilatation, induced by the release of CGRP, in rat paw skin is attenuated by CP-122,288. The absence of an effect of CP-122,288 on basal and exogenous CGRP-induced blood flow suggests that CP-122,288 inhibits neuropeptide release by acting *via* prejunctional receptors, as has been previously suggested in the dura mater (see Lee & Moskowitz 1993). These results indicate that CP-122,288 could be of use for the treatment of disease states in peripheral tissues where neurogenic inflammation is involved.

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9P CHARACTERISATION OF THE MECHANISM OF ENHANCED NITRITE FORMATION BY LIPOTEICHOIC ACID (FROM *S. AUREUS*) IN CULTURED J774.2 MACROPHAGE CELLS

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Lipoteichoic acid (LTA), a cell wall component of the gram-positive bacterium *S. Aureus* causes an enhanced formation of nitric oxide (NO) due to expression of the inducible isoform of nitric oxide synthase (iNOS) in murine peritoneal macrophages (Cunha *et al.*, 1993). Here, we characterise the mechanisms by which LTA causes an increased formation of NO in a murine macrophage cell line (J774.2).

J774.2 cells were cultured in 96-well plates with 200 µl of culture medium (DMEM) containing foetal calf serum (10%) and glutamine (4 mM) until cells reached confluence. LTA (10 µg/ml) was added to the cells to induce iNOS activity. Nitrite accumulation, an indicator of NO formation, was measured 24 h later in the supernatant of J774.2 cells by the Griess method. Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependant reduction of MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Szabo *et al.*, 1993).

Activation of J774.2 macrophages with LTA (10 µg/ml) caused an increase in nitrite concentration in the medium from 2±0.1 µM (baseline) to 35±1 µM (n=12, $p<0.01$). This increase in nitrite caused by LTA was not affected by treatment of cells with polymyxin B (0.05-0.5µg/ml), an agent which binds and inactivates endotoxin.

The rise in nitrite caused by LTA was dose-dependently reduced by the non-selective nitric oxide synthase (NOS) inhibitor N^G-methyl-L-arginine (L-NMMA, IC_{50}; 22±2 µM, n=10), or by the iNOS-selective NOS inhibitor aminoethyl-isothiourea (AE-ITU, IC_{50}; 6±1µM, n=10, Southan *et al.*, 1995). The inhibition by L-NMMA or AE-ITU of the increase in nitrite formation caused by LTA in J774.2 cells was (i) similar when these NOS inhibitors were given 10 h after LTA; and (ii) reversed by excess of L-arginine (3-30 mM), but not D-arginine (3-30 mM).

Dexamethasone, an inhibitor of iNOS induction, or cyclohexamide (protein synthesis inhibitor) prevented the rise in nitrite caused by LTA when given together with LTA (Table 1), but the inhibition was significantly less when given 10 h after LTA (91±2 and 96±2 % of LTA control, respectively, n=10, $p<0.01$). Addition to the medium (i) the tyrosine kinase inhibitor genistein, (ii) tricyclodecan-9-yl-xanthogenate (coded D609), an agent which inhibits phosphatidylcholine-dependent phospholipase C (PC-PLC, Baeuerle and Henkel, 1994) or (iii) pyrrolidine dithiocarbamate (PDTC; 10-50µM; Baeuerle and Henkel, 1994), an inhibitor of the activation of the transcription factor NFκB, attenuated the increase in nitrite caused by LTA when these drugs were given together with LTA (time 0), but the inhibition was significantly less when given 10 h after LTA.

Table 1. Effect of inhibitors of NOS activity or iNOS induction on the LTA induced nitrite formation in J774.2 cells.

Inhibition of	Inhibitor	Concentration	Nitrite (% control)	n
NOS activity	L-NMMA	10µM	68±2	10
	AE-ITU	10µM	29±1	10
iNOS induction	cyclohexamide	0.3µg/ml	10±1	10
	dexamethasone	0.5µM	51±1	10
Tyrosine kinase	genistein	100µM	26±6	10
PC-PLC	D609	30µg/ml	14±2	5
activation of NFκB	PDTC	25µM	10±2	5

Thus, the enhanced formation of NO by LTA in J774.2 is due to the induction of iNOS. In addition our results suggest that the signal transduction mechanism leading to the induction of iNOS caused by LTA involves the activation of (i) PC-PLC (ii) tyrosine kinase, and (iii) NFκB.

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10P PROTEIN KINASE C REGULATION OF LIPOPOLYSACCHARIDE-INDUCED NITRIC OXIDE SYNTHASE ACTIVITY IN RAW 264.7 MURINE MACROPHAGES

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In macrophages an inducible isoform of nitric oxide synthase (iNOS) regulates NO synthesis following cell stimulation with cytokines or the active component of endotoxin, lipopolysaccharide (LPS) (Mitchell *et al.*, 1992). The intracellular signalling pathways by which LPS stimulates NOS induction are largely unresolved though activation of protein kinase C (PKC) has been implicated (Severn *et al.*, 1992). In this study we have examined the role of PKC in LPS- and IFNγ-stimulated induction of NOS activity in RAW 264.7 macrophages.

RAW 264.7 murine macrophages were stimulated with LPS or IFNγ. NOS activity was estimated by conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline as described by Mitchell *et al.*, (1992). Induction of NOS protein and expression of PKC isoforms were assessed by Western blotting using specific anti-iNOS and anti-PKC antibodies.

LPS (1µgml^{-1}) stimulated an increase in iNOS activity in RAW 264.7 macrophages which was maximal 8-12h following LPS addition. IFNγ (10-100 IUml^{-1}) alone or in combination with LPS also stimulated an increase in NOS activity. (Control: 3019±42dpm, LPS 1µgml^{-1}:30109±3223dpm, IFNγ 30IUml^{-1}:9823±453dpm, LPS 1ngml^{-1} & IFNγ 10IUml^{-1}:37371±3029dpm, n=4). The increase in iNOS activity was also reflected in an increase in the levels of a protein which corresponded immunologically to the 130kDa inducible isoform of NOS.

The phorbol ester, 12-o-tetradecanoyl phorbol 13-acetate (TPA) alone did not stimulate an increase in iNOS activity, however chronic TPA pretreatment (30nM; 8h pre-LPS stimulation) resulted in down regulation of PKC α, β and ε but not ζ in these cells and corresponded to a 39±11.3% reduction, a 20±6.3% increase and 66±4.3% increase in LPS-, IFNγ- and LPS&IFNγ-induced NOS activity respectively (Control: 2086±62dpm, Control TPA: 2250±140dpm, LPS 12 h: 19639±523dpm, LPS/TPA: 12974±1211dpm, IFNγ 12h: 8841±249dpm, IFNγ/TPA: 10668±68dpm, LPS & IFNγ: 7086±322, LPS & IFNγ/TPA : 10522±448, n=3). The observed modulation of iNOS activity under each of these conditions was also reflected at the level of protein expression. However, co-incubation with the PKC inhibitor Ro-318220 (Davis *et al.*, 1989), at a concentration of 10µM, abolished both iNOS activity and iNOS protein expression in response to LPS, IFNγ and LPS and IFNγ in combination (n=4).

These results show that LPS and IFNγ stimulate induction of NOS activity in RAW 264.7 macrophages in a PKC-dependent manner and would appear to involve both DAG-sensitive and insensitive isoforms of PKC.

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11P IMMUNODETECTION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN RATS AFTER LIPOPOLYSACCHARIDE ADMINISTRATION: CHARACTERISATION OF A NOVEL ANTIBODY

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The administration of *E. Coli* lipopolysaccaride (LPS) to rats causes a fall in blood pressure and induction of cytokine inducible nitric oxide synthase (iNOS; Kilbourn et al., 1990). The effects of LPS *in vivo* may be prevented by pre-treatment of the animals with dexamethasone (DEX, Szabo et al., 1993). Here we have raised and characterised a selective antibody to iNOS, investigated the effects of LPS on iNOS expression in a range of tissues *in vivo*, and the potential inhibitory effect of a pre-treatment with dexamethasone (DEX).

A unique 20 amino acid peptide sequence from macrophage iNOS (TGTAQNVPESLDKLHVTSTRC; Lyons et al., 1992) was conjugated to Keyhole Limpet haemocyanin and used to immunise male New Zealand White rabbits. After a second immunisation the resulting antisera was characterised using Western blot analysis for selectivity to iNOS. Male Wistar rats (220-250g) were treated with LPS (1-10mg/kg, i.p.) for 3 h. A dose response curve of DEX (-1h; 0.1-1mg/kg, s.c.) on LPS (1mg/kg) induced iNOS expression was also prepared. The animals were killed and the lungs, liver, kidney, spleen and aorta frozen in liquid nitrogen. The organs were homogenised and the resulting sample subjected to Western blot analysis for iNOS (dilution of 1:2000) as previously described (Bryant et al., 1993). Staining density of the protein band was subjected to densiometric analysis. Peritoneal cells (>80% macrophages) were taken from animals treated with LPS (1mg/kg) alone or pre-treated with DEX (0.3mg/kg), cultured overnight and the release of nitrite into the medium measured using the Griess reaction (Gross et al., 1991).

The iNOS antibody recognised a single protein band at a molecular weight of approximately 130kDa in LPS activated J774 macrophage cell lysate, but recognised no protein bands in bovine aortic endothelial cell lysate (a source of endothelial NOS) or rat cerebellar homogenate (a source of neuronal NOS). Thus the antibody shows no cross reactivity with other NOS isoforms. LPS induced iNOS in rat tissues, with the maximum induction occurring at 1mg/kg (64% greater than that observed at 10 mg/kg in the lung, n=2-3). Expression of iNOS was prominent in the lung, less so in the aorta, liver and spleen, and low in the kidney. DEX caused a dose dependent reduction in iNOS expression in both lung and aorta (58% reduction in the lung at 0.3 mg/kg dose compared with LPS alone, n=2-3). *Ex vivo* nitrite release from cultured peritoneal cells was 78±1μM from rats treated with LPS (1mg/kg) which was reduced to 34±2μM by animal pre-treatment with DEX (0.3mg/kg, n=3).

Thus using this selective iNOS antibody the induction of iNOS expression *in vivo* by LPS can be demonstrated in a number of organs. The iNOS expression is dose dependently inhibited by dexamethasone and the suppression of iNOS expression is accompanied by decreased activity in peritoneal macrophages

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12P GROWTH OF MESANGIAL CELLS FROM THE *H-2K*^{b}-tsA58 TRANSGENIC MOUSE

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Introduced by A.W.Cuthbert

The *H-2K*^{b}-tsA58 transgenic mouse (Jat *et al.* 1991) harbours a temperature-sensitive mutant of the immortalizing SV40 T antigen under control of the γ-interferon inducible *H-2K*^{b} promoter sequence. This has enabled direct derivation of conditionally immortalized cell lines to be established by cell isolation procedures. We have investigated the effects of different growth conditions on renal glomerular mesangial cells obtained from this mouse.

Cells were cultured at 33°C in the presence of 50U/ml murine γ-interferon in Dulbecco's Minimum Essential Medium supplemented with 10% Foetal Calf Serum (FCS) and 100mU/ml penicillin/streptomycin. Cells (1 × 10^5 cells/ml) were plated out into 96 well plates in serum-containing or serum-free media, with or without 50U/ml interferon (IFN) and were cultured at either 33°C or 37°C. A modified colorimetric assay using the tetrazolium compound MTT which is converted to a dark blue formazan product in mitochondria of living cells was used to determine cell growth (after Mosmann 1983). 20µl of 5mg/ml stock MTT was added to 100µl of cells for 3 hours after 24, 48, 96 and 168 hours for cells grown in FCS and after 24, 48, 72, and 96 hours for cells grown in serum-free conditions. The product was then solubilized for 24 hours using 20% w/v lauryl sulphate dissolved in 50% v/v dimethyl formamide and water at pH 4.7. Plates were read on a Titertek Multiskan MCC/340. Neither IFN or FCS had any effect on optical density. The results are shown in Table 1 as mean optical densities from 3 different experiments of 24 wells each. After 168 hours in serum+ media optical densities were higher for cells grown at 33°C than at 37°C which suggests the tsA58 may be active. At 33°C optical density was greater for cells grown in IFN, but this was not the case at 37°C. One explanation of this could be inhibition of protein synthesis by IFN causing cell death (Farrell *et al.* 1978). However, at 33°C this is overridden by the activated oncogene. In the absence of FCS cells grew best at 33°C with IFN, though some growth was observed at 33°C without IFN and at 37°C with IFN. Minimal growth was seen in non-permissive conditions (37°C without IFN). A dose-response curve for IFN was established at 72 hours for cells grown at 33°C in FCS-free conditions and had an EC_{50} of 17.6U/ml.

This study shows that in serum-free conditions mesangial cells from the *H-2K*^{b}-tsA58 transgenic mouse grow as expected, that is at 33°C and in the presence of interferon, but not at 37°C in the absence of interferon. The EC_{50} for interferon is 17.6U/ml. In the presence of serum different growth rates are observed and this is essential to maintain viable cells at 37°C.

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Cells kindly supplied by the Ludwig Institute for Cancer Research, London.

Table 1.

	Serum+				Serum-free			
	33°C/IFN+	33°C/IFN-	37°C/IFN+	37°C/IFN-	33°C/IFN+	33°C/IFN-	37°C/IFN+	37°C/IFN-
24 hours	0.654±0.008	0.685±0.007	0.653±0.009	0.696±0.010	0.296±0.013	0.148±0.013	0.263±0.015	0.032±0.002
48 hours	0.815±0.013	0.885±0.008	0.873±0.008	0.891±0.004	0.383±0.012	0.097±0.009	0.141±0.014	-0.003±0.02
72 hours	---	---	---	---	0.406±0.016	0.106±0.012	0.109±0.010	0.002±0.001
96 hours	1.013±0.010	0.970±0.018	0.683±0.004	0.762±0.006	0.365±0.012	0.088±0.009	0.127±0.029	0.001±0.002
168 hours	1.401±0.031	1.245±0.016	0.465±0.009	0.604±0.011	---	---	---	---

13P DO GELATINASES CONTRIBUTE TO LOSS OF EPITHELIAL COHESION IN EOSINOPHIL-MEDIATED INJURY OF THE BRONCHIAL MUCOSA?

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We have previously shown that guinea pig and human eosinophils mediate injury of the bronchial epithelium, and that this can be inhibited by the broad spectrum antiproteinase α_2-macroglobulin (Herbert *et al.*, 1991; 1994). We have therefore investigated the presence of extracellular proteinases during the interaction between eosinophils and the bronchial mucosa. We were particularly interested in enzymes with substrate specificities directed towards basement membrane proteins because these might disrupt the cohesion of epithelial cells at sites of inflammation. Peritoneal eosinophils elicited by polymyxin B were harvested from guinea pigs as previously described and placed on the basolateral aspect of sheets of bovine bronchial mucosa mounted in heated bathing chambers (Herbert *et al.*, 1991). After exposure for 60 min to 3.3 x 10^6 eosinophils per ml, the incubation medium was then withdrawn, the eosinophils removed by centrifugation and the medium stored for assay of gelatinolytic activity. Injury of the bronchial mucosa caused by eosinophils was measured using serum albumin flux as an index of transepithelial permeability (Herbert *et al.*, 1991).

Addition of unstimulated eosinophils to the bronchial mucosa did not change the net flux of albumin (1.94 ± 0.44 v. 2.44 ± 0.40 fmol cm^{-2} min^{-1} in the absence and presence of cells respectively, n=5 for each), but net flux was significantly increased when the eosinophils had been stimulated by 5μM A23187 (5.07 ± 0.57 fmol cm^{-2} min^{-1}, n=5, P<0.05). Gelatin substrate zymography indicated a complex pattern of proteolytic activity that was blocked by chelation of divalent cations and by specific metalloproteinase inhibitors. These enzymes included the type IV collagenases known as gelatinase A (matrix metalloproteinase (MMP)-2) and gelatinase B (MMP-9), together with their presumptive activation products. Quantitative assay of total gelatinase activity (latent proenzyme plus activated forms of gelatinases A and B) was performed radiochemically on samples treated with 4-aminophenylmercuric acetate (APMA). Gelatinase was released from the airway mucosa in the absence of eosinophils (37.2 ± 11.4 mU ml^{-1}), but levels of activity were increased following the addition of unstimulated eosinophils (103.3 ± 20.4 mU ml^{-1}, n=5, P<0.05). Stimulation of eosinophils did not produce any further increase in gelatinase activity (93.0 ± 7.1 mU ml^{-1}, n=5, P<0.05 with respect to the eosinophil-free control). Trivial quantities of gelatinase (<5mU ml^{-1}) were detected from the eosinophils themselves, whether assayed as enzyme activity in conditioned media from stimulated or unstimulated eosinophils, or in eosinophil sonicates. This implied that the major source of the enzymatic activity was probably the airway mucosa. In the absence of eosinophils, isolated sheets of bronchial mucosa released gelatinase activity from both the apical and basolateral aspects of the tissue, without any apparent sidedness (after APMA activation, apical: 17 ± 6.7 mU ml^{-1}; basolateral: 15.4 ± 8.5 mU ml^{-1}, n=8 for each) and this release was not stimulated by the addition of 5μM A23187 (after APMA activation, apical: 20.1 ± 12.3 mU ml^{-1}; basolateral: 13.0 ± 7.8 mU ml^{-1}, n=8 for each). In view of our previous observations that exogenous recombinant gelatinase A causes epithelial injury and increase the responsiveness of isovolumic bronchial segments to ACh (Herbert *et al.*, 1994), there may be a role for gelatinases in compromising epithelial cohesion. This would be expected to render the airway mucosa more susceptible to other agents released by stimulated eosinophils, such as oxidants and toxic proteins, that are also incriminated in the process of tissue injury.

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14P CHARACTERISATION OF MUSCARINIC RECEPTOR SUBTYPE MEDIATING CONTRACTIONS OF THE UTERUS OF OESTROGEN-TREATED AND PREGNANT RATS

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Radioligand binding studies using uterine membranes from mature virgin rats indicated the presence of M_2 muscarinic receptor binding sites (Pennefather *et al.*, 1994). The aim of the present study was to characterize the muscarinic receptor subtype mediating contractions of uteri from oestrogen-primed and late pregnant rats.

Uterine horns were taken from (i) virgin female Sprague Dawley rats (210-275g) pretreated with oestradiol cypionate 20μg/kg s.c. 24 h previously, and (ii) 17-20 day pregnant Sprague Dawley rats (300-350g). Tissues were set up for recording contractions of the longitudinal muscle layer. Log concentration-response curves to carbachol were constructed in the absence and presence of three concentrations of seven muscarinic receptor antagonists.

Mean neg. log molar EC_{50} values and s.e.means for carbachol were 5.46 ± 0.47 (n=30) and 5.57 ± 0.70 (n=23) in tissues from oestrogen-treated and pregnant animals, respectively. Mean pA_2 values for antagonists and s.e.means of 4 - 5 experiments are shown in Table 1. Slopes of Schild plots did not differ from unity.

These data indicate that the muscarinic receptor subtype mediating carbachol-induced contractions of rat uterus is the same in oestrogen-treated and pregnant animals. The pA_2 values for atropine, pirenzepine and methoctramine are similar to those reported by Eglen *et al.*, (1989) for antagonism of carbachol on guinea-pig uterus and are consistent with the presence of an M_2 receptor subtype. The pA_2 values for AF-DX 116 and himbacine are similar to those reported for guinea-pig atria by Doods *et al.*, (1993). The relatively high pA_2 value for hexahydrosiladifenidol is not so obviously compatible with the presence of receptors of the M_2 subtype. Nevertheless it would seem premature to propose, on the basis of this single discrepancy, that receptors of the M_4 subtype may mediate carbachol-induced contractions of the rat uterus.

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Table 1. pA_2 values ± s.e.means versus carbachol (n = 4-5)

	Atropine	Himbacine	Hexahydro-siladifenidol	Methoctramine	AF-DX 384	AF-DX 116	Pirenzepine
Oestrogen-treated	9.42 ± 0.17	8.73 ± 0.22	8.61 ± 0.26	8.49 ± 0.26	7.91 ± 0.21	7.36 ± 0.27	7.26 ± 0.29
Pregnant	9.48 ± 0.22	8.37 ± 0.21	8.22 ± 0.31	8.01 ± .0.25	7.44 ± 0.23	7.73 ± 0.22	6.92± 0.28

15P CELL CYTOTOXICITY AND CHANGES IN INTRACELLULAR CALCIUM IONS MEDIATED BY CLONED NMDA RECEPTOR SUBTYPES

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We have previously reported that the expression of NMDAR1-1a/R2A heteromeric receptors in human embryonic kidney (HEK) 293 cells results in cell death (Cik *et al.*, 1993). Cytotoxicity was reduced by the inclusion of either NMDA receptor antagonists, DL-2-aminophosphonopentanoic acid (AP5), 5,7-dichlorokynurenic acid (DKA), MK801, Mg^{2+} or the calcium chelator, EGTA, in the cell culture medium post-transfection. Further, the expression of the mutant NMDAR1-1a(N598Q)/R2A, a point mutation known to reduce the Ca^{2+} permeability of the ion channel, resulted in an increase in cell viability compared to the expression of wild-type NMDAR1-1a/R2A receptors (Cik *et al.*, 1994; Burnashev *et al.*, 1992). These observations were explained by the presence of l-glutamate in the cell culture medium resulting in sustained receptor activation and cell death via an increase in intracellular Ca^{2+}. The prevention of cell death following the expression of NMDAR1-1a/R2B receptors required significantly higher concentrations of AP5 compared to NMDAR1-1a/R2A receptors whereas the NMDAR1-1a/R2C combination did not result in cell death post-transfection (Chazot *et al.*, 1994). (Note that for all cloned receptor subunits, similar levels of expression were found by both radioligand binding assay and immunoblotting using subunit-specific antibodies). Molecular size determinations showed that the R1 and R2C subunits coassemble to form a receptor with a similar size to native forebrain and NMDAR1-1a/R2A receptors, i.e. 700 000 - 850 000 daltons. Further, these NMDAR1/R2C receptors are functional but show a marked reduction in the increase in intracellular Ca^{2+} following receptor activation compared to NMDAR1/R2A receptors. For these studies cells were loaded with 0.5 μM FURA-2 AM, 100 μM glutamate with 10 μM glycine were applied by perfusion and changes in intracellular Ca^{2+} assessed. For the NMDAR1-1a/R2A combination, 13 out of 18 fields chosen (72%) responded with a mean increase in intracellular calcium of 11.8 ± 6.9% (n=25). Responses were blocked by 100 μM AP5 and 10 mM Mg^{2+}. For the NMDAR1-1a/R2C combination, 2 out of 14 fields (14%) responded with an increase in intracellular Ca^{2+} of 7.7 ± 0.6% (n=3). Thus, we have established a novel method to study the properties and functional expression of cloned NMDA receptor subtypes by a cell cytotoxicity assay.

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16P EFFECT OF NITRIC OXIDE ON BASAL AND NMDA-EVOKED RELEASE OF GLUTAMATE AND ASPARTATE RELEASE *IN VIVO*

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Nitric oxide (NO) is generally accepted as a messenger molecule in the periphery and central nervous system (CNS). Within the CNS an association has been made between activation of glutamatergic N-methyl-D-aspartate (NMDA) receptors and production of NO (Garthwaite, 1991). NO has been shown to increase release of the excitatory amino acids glutamate (GLU) and aspartate (ASP) in rat striatum in vivo (Guevara-Guzman et al., 1994). However, these experiments were performed under anaesthesia which itself will alter transmitter release.

In the present study we have investigated the effects of infusion, via dialysis probes, of NMDA, the nitric oxide synthase (NOS) inhibitor L-nitro-arginine methyl ester (L-NAME) on GLU and ASP release in rat hippocampus. The effect of the NO donor drug S-nitroso-N-penicillamine (SNAP) was also studied.

Male Wistar rats (280-320g) were implanted under chloral hydrate anaesthesia (400mg/kg) with concentric dialysis probes into the ventral hippocampus. The following day rats were dialysed (0.5μl/min) with artificial cerebrospinal fluid. Four samples were collected prior to drug infusion. Amino acids were determined using HPLC followed by fluorimetric detection. Basal levels of ASP and GLU were 0.36 ± 0.04 μM and 3.7 ± 0.2 μM respectively (n = 46).

NMDA (10-1000μM) caused concentration-dependent increases in both GLU, up to 638 ± 76 % (mean ± s.e.mean, n = 6) and ASP, up to 389 ± 80 % (mean ± s.e. mean) above basal values. When L-NAME (100μM) was infused for 60 min, dialysate GLU and ASP were increased by up to 231± 26 % and 200 ± 17 % respectively (mean ± s.e.mean). When 200μM L-NAME was infused the magnitude of GLU and ASP release was less than that seen with 100μM L-NAME. 1mM L-NAME did not significantly alter extracellular GLU but decreased ASP to 50 ± 9 % below basal level. L-NAME infusion was observed to have profound effects on NMDA-evoked amino acid release. When 100μM L-NAME was co-infused with 100μM NMDA the increase in extracellular GLU was 490 ± 58 % compared with 240 ± 20 % for NMDA alone. In the case of ASP the increases were 647 ± 88 % following L-NAME + NMDA (100μM each) and 178 ± 26 after NMDA alone. However, in the presence of 1mM L-NAME NMDA failed to significantly increase either dialysate GLU or ASP. SNAP infused at 500μM decreased extracellular GLU and ASP to 55 ± 3 % and 41± 12 % below basal respectively. In contrast higher concentrations of SNAP (1.0 and 2.0mM) increased dialysate GLU and ASP up to 534 ± 86 % and 486 ± 96 respectively, at the highest SNAP dose.

These data suggest that the level of NOS activity and concentration of NO alter basal and NMDA evoked GLU and ASP release in a biphasic manner.

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17P CHARACTERISATION OF LY293558 AS A STEREOSELECTIVE AND COMPETITIVE AMPA RECEPTOR ANTAGONIST ON RAT NEOCORTICAL SLICES

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LY215490, (3RS, 4aRS, 6RS, 8aRS)-6-[2-(1(2)H-tetrazole-5-yl) ethyl] decahydroquinoline-3-carboxylic acid) is a selective AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor antagonist (Ornstein et al., 1993). The compound has also been shown to be neuroprotective following middle cerebral artery occlusion in the rat (Gill and Lodge, 1994) and cat (Bullock et al., 1994).

We have used the rat cortical wedge preparation (Harrison and Simmonds, 1985) to characterise its mode of action. LY215490 proved to be a dose-dependent, reversible and selective AMPA antagonist. Concentration-response curves for AMPA, quisqualate, kainate and N-methyl-D-aspartate were shifted rightward in a near parallel manner by increasing doses of LY215490. This resulted in pA2 values of 6.3±0.1, 6.0±0.1, 5.1±0.1 and 4.7±0.1 for the above agonists respectively. Schild slopes were close to unity. Combination studies with NBQX (6-nitro-7-sulfamoylbenzo(F)quinoxalinedione) and GYKI52466 (1-(aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine) showed additive and multiplicative dose-ratios on AMPA concentration-response curves. Thus dose-ratios for 3.16uM LY215490, 1uM NBQX and 10uM GYKI52466 were 4.4, 9.6 and 2.8 respectively and for the combination of LY215490 with NBQX and with GYKI52466 were 11.6 and 19.6 respectively. When the isomers of LY215490 were separated, activity was seen to reside in the (-) isomer. Thus (-)LY293558 and (+)LY293559 had IC50s against AMPA 40uM of 1.8±0.2 and >100uM respectively. This stereoselectivity agrees with that seen following parenteral administration (Bond and Lodge, 1995).

These studies confirm the stereoselective and competitive nature of AMPA antagonism of LY293558.

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18P MICRODIALYSIS APPLICATION OF L-*TRANS*-PYRROLIDINE-2,4-DICARBOXYLATE (L-*TRANS*-PDC): EFFECT ON EXTRACELLULAR GLUTAMATE AND LOCAL FIELD POTENTIAL

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Impairment of glutamate uptake in the CNS is often proposed to play a major role in excitotoxic neuronal death (Szatkowski & Attwell, 1994). Here we examine the effects of application by microdialysis of L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-PDC), a selective inhibitor of high-affinity glutamate uptake (Griffiths *et al.*, 1994), on extracellular glutamate and field potential, both recorded at the same tissue site.

Rats were anaesthetized throughout with halothane in N_2O:O_2 (1:1). Microdialysis probes incorporating an electrode were implanted in the parietal cortex and perfused at 1 μl/min with artificial CSF (ACSF) (Obrenovitch *et al.*, 1994). After 2 h of stabilization, increasing concentrations of L-*trans*-PDC were applied through the probe for 20 min. Each drug application was followed by 15 min of perfusion with ACSF alone. Dialysate glutamate was monitored by enzyme-fluorescence. The extracellular field potential was recorded between the microdialysis electrode and a reference electrode.

Basal levels of glutamate in the cortical perfusate were 1.3 ± 0.2 μM (mean ± s.e.mean, n = 8). L-*trans*-PDC markedly and dose-dependently increased the dialysate concentration of glutamate, but these changes were associated with only minor negative shifts of field potential (Figure 1). The increase of dialysate glutamate by 10 mM L-*trans*-PDC (1700 % of basal level) produced a depolarization of 1.7 ± 0.09 mV (n = 8), i.e. around 1/10 that of spreading depression.

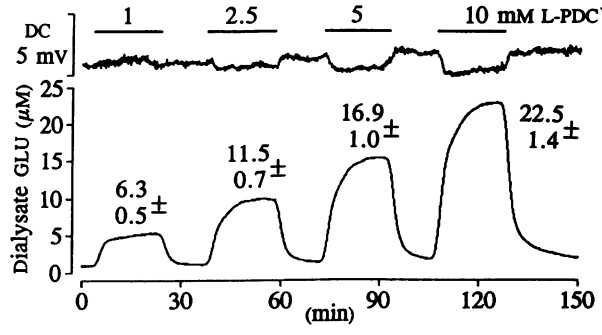

Figure 1. Effects of glutamate uptake inhibition by L-*trans*-PDC on extracellular glutamate (bottom) and field potential (top). Traces are from a single representative experiment. Values next to the glutamate trace are maximum glutamate levels (mean ± s.e.mean, n = 8; $P<0.001$, Student's paired *t*-test) reached with perfusion of the corresponding L-*trans*-PDC concentration.

These data confirm that very high extracellular concentrations of glutamate are necessary to produce local depolarization in the intact brain (Obrenovitch *et al.*, 1994). They do not support the hypothesis that high extracellular glutamate may underlie spreading depression and anoxic depolarization.

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19P CHARACTERISATION OF THE BINDING OF [^{125}I)α-DENDROTOXIN TO RAT BRAIN

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The peptide α-dendrotoxin (α-DTX) from the green mamba snake (*Dendroaspis angusticeps*) blocks certain classes of voltage-gated potassium channels (Stansfeld *et al.*, 1987). [^{125}I]α-DTX labels specific binding sites in rat synaptosomes (Stansfeld *et al.*, 1987) and hippocampal homogenates (Schechter *et al.*, 1994) but these sites have only been partially characterised. We report here on the identification of a similar binding site for [^{125}I]α-DTX in a crude rat brain homogenate and have used 15 potassium channel blockers to characterise this site.

Whole rat brains minus cerebellum were homogenised in 10mM TrisHCl/ 0.32M sucrose (pH 7.2 at 4°C), washed and resuspended in incubation buffer (5mM TrisHCl, 154mM NaCl, 5.6mM KCl, 1mM $CaCl_2$, pH 7.2 at 25°C) then stored at -80°C until use. Incubations (60 min at room temperature) were terminated by rapid filtration. Non-specific binding was determined using 0.2μM α-DTX.

The binding of [^{125}I]α-DTX (0.02 to 5nM) was saturable and consistent with a single population of binding sites: K_d=0.13±0.02nM and B_{max}=5.5±0.3pmol.mg^{-1}protein (n=3). Competition experiments were performed using 0.2nM [^{125}I]α-DTX. Specific binding was normally 85 - 95% of total. Binding of [^{125}I]α-DTX was displaced by 10 peptides known to block voltage-gated potassium channels (Table 1), whereas iberiotoxin, apamin and scyllatoxin had no effect up to 1μM. The non-peptide potassium channel blockers 4-aminopyridine and tetraethylammonium also had little effect on [^{125}I]α-DTX binding up to 10mM which is consistent with observations that these compounds act at a different site to α-DTX.

This profile supports the proposal that [^{125}I]α-DTX is labelling a voltage-gated potassium channel in rat brain.

Table 1. Binding potencies (pK_i) against [^{125}I]α-DTX binding to rat brain homogenate (mean ± s.e.mean):

Toxin	pK_i	n
α-DTX	9.82 ± 0.09	4
β-DTX	8.48 ± 0.09	4
γ-DTX	8.24 ± 0.09	4
δ-DTX	8.69 ± 0.10	4
charybdotoxin	8.33 ± 0.03	4
mast cell degranulating peptide	8.34 ± 0.03	4
toxin K	8.55 ± 0.15	4
toxin I	10.60 ± 0.05	4
stichodactyla toxin	10.70 ± 0.25	3
kaliotoxin	9.77 ± 0.06	4

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20P SELECTIVITY OF ACTION OF THIOPENTONE AND PROPOFOL ON COMPONENTS OF DELAYED RECTIFIER POTASSIUM CURRENTS IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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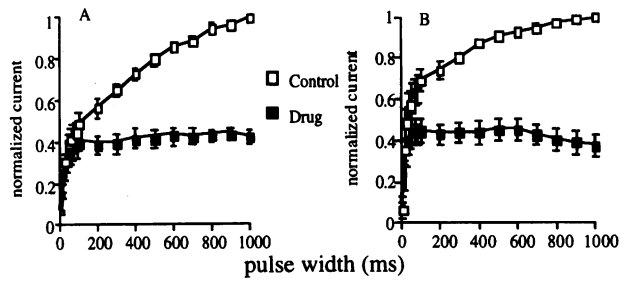
Delayed rectifier potassium currents in cardiac ventricular muscle are thought to comprise fast (I_{Kr}) and slow (I_{Ks}) components (Connors *et al.*, 1990; Sanguinetti & Jurkiewicz, 1990). The aim of these experiments was to test for possible differential effects of the intravenous anaesthetics, thiopentone and propofol, on the two components of delayed rectifier currents.

Myocytes were isolated from guinea-pig ventricular muscle and superfused with a balanced salt solution containing 2.5 mM Ca and 5.4 mM K (36°C). Patch electrodes (containing, in mM: KCl, 13.2; K glutamate, 109; NaCl, 5; $MgCl_2$, 1; Na_2ATP, 2, EGTA, 1.1; free Ca 10^{-5}; pH 7.2) were used for whole-cell voltage-clamp; delayed rectifier currents were activated by step depolarizations (10-1000 ms duration) to +40 mV and measured as amplitudes of de-activating tail currents at -40 mV.

Figure 1A shows that 100 μM thiopentone suppressed the currents at times longer than 100 ms (reduction by 58±4% at 1000 ms, P<0.05, n=9 cells) with little effect at shorter times. Effects of 300 μM thiopentone were similar (65±9% reduction at 1000 ms, n=9). Figure 1B shows that 100 μM propofol also selectively suppressed the current at long times (63±5% at 1000 ms, n=6); effects of 25 μM propofol were less (35±6% at 1000 ms, n=8), while the effects of the solvent were negligible (decrease of 1±2% at 1000 ms, n=6).



The observations are consistent with selective effects of thiopentone and propofol on the slow component (I_{Ks}) of delayed rectifier current leaving a drug resistant component which reaches a peak in less than 100 ms and is maintained for periods up to 1000 ms. The current resistant to thiopentone and propofol thus resembles the current sensitive to E4031 (a drug which is thought to be selective for I_{Kr}, Sanguinetti & Jurkiewicz, 1990).

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21P SELECTIVITY OF ACTION OF E4031 AND CLOFILIUM ON COMPONENTS OF DELAYED RECTIFIER POTASSIUM CURRENTS IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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Components of the delayed rectifier potassium current (I_K) were separated using two voltage protocols to activate I_K with time. The blocking effects of 1 μM E-4031 and 3 μM clofilium on the rapid (I_{Kr}) and slow (I_{Ks}) components were investigated.

Single cells were isolated from guinea-pig ventricle. I_K was studied (balanced salt solution, 2.5 mM Ca at 36°C; micro-electrodes contained 40-60 mM BAPTA, 0.5 M K_2SO_4 and 10 mM KCl). Protocol 1: Cells voltage clamped at -40 mV were step depolarized to +40 mV (for 10-600 ms) and tail currents were measured upon repolarization to -40 mV. Protocol 2: Cells voltage clamped at -50 mV were depolarized to +30 mV (for 10-600 ms); I_{Ks} was measured as a deactivating tail current at -10 mV and I_{Kr} as the tail current subsequently deactivating at -50 mV.

E-4031 and clofilium almost completely abolished the current activated by pulses shorter than 100 ms in protocol 1 (figure 1), while the effect at longer pulses was smaller ($p<0.01$; n=5).

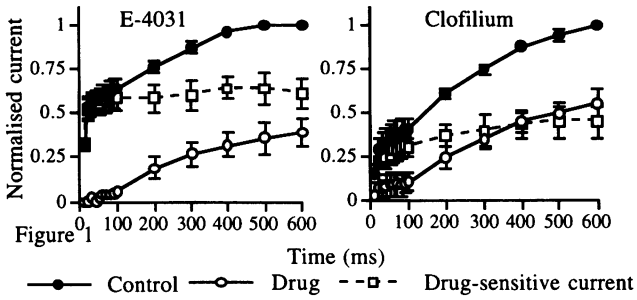

Figure 1

The drug-sensitive current (I_{Kr}) activated rapidly (after 10 ms pulses) and reached a peak amplitude at the 100 ms pulse which was maintained at longer pulses. The residual current (thought to be I_{Ks}) developed over a much longer time course. This interpretation was further tested using protocol 2 (modified from Carmeliet, 1992). Figure 2 shows that the current which deactivated at -10 mV (I_{Ks}) increased with longer pulses and was little affected by either E-4031 or clofilium. The current which subsequently deactivated at -50 mV (I_{Kr}) was largely time-independent and was substantially reduced by both E-4031 ($p<0.01$; n=5) and clofilium ($p<0.01$; n=5). Conclusions from the two protocols are thus consistent.

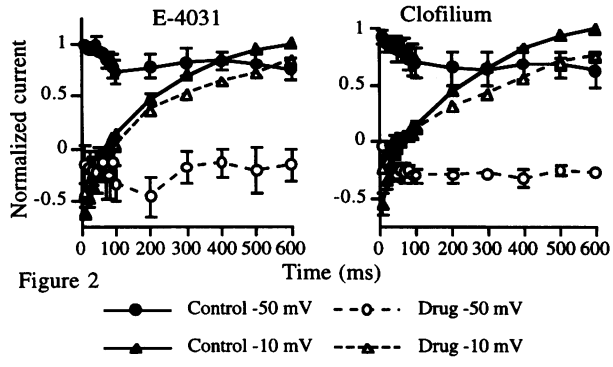

Figure 2

The observations provide further support for the selective action of E-4031 on I_{Kr} (Sanguinetti & Jurkiewicz, 1990) but show that the action of clofilium (reported as a non-selective blocker of I_K) can exert a selective action on I_{Kr} at relatively low concentrations.

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22P ACTIONS OF THAPSIGARGIN ON CALCIUM TRANSIENTS, CALCIUM CURRENTS, ACTION POTENTIALS AND CONTRACTIONS IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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Thapsigargin is thought to be a selective inhibitor of the calcium ATP-ase of the sarcoplasmic reticulum (Thastrup *et al.*, 1990; Kirby *et al.*, 1992). The purpose of the present study was to investigate its effects on cytosolic calcium transients, L-type calcium currents, action potentials and contractions in guinea-pig ventricular myocytes.

Myocytes were isolated from guinea-pig ventricle, superfused with a balanced salt solution (containing 2.5 mM calcium) at 34-36°C, and stimulated to fire action potentials at a frequency of 1 Hz. Calcium transients were constructed from the magnitude of inward currents, thought to be carried predominantly by Na:Ca exchange, recorded when action potentials were interrupted by application of a voltage clamp to -70 mV (see Terrar & White 1989); currents were expressed as a percentage of that at 50 ms in the absence of drugs. Contractions accompanying action potentials were measured from the video image of cells, using an edge detection system. L-type calcium currents were recorded in cells voltage-clamped at -40 mV and step depolarized to 0 mV for 200 ms at a frequency of 0.3 Hz.

Application of 1 μM thapsigargin for 3 min caused a significant prolongation of action potential duration (measured as time to 90% repolarization) from 169±5 ms to 260±13 ms ($P<0.05$, n=12 cells). The early part of the calcium transient was suppressed whereas the later part of the calcium transient was elevated by exposure to thapsigargin (reduced from 83±7% to 50±8% at 10 ms, and increased from 22±6% to 74±8% at 150 ms; $P<0.01$ in both cases, n=12 cells). In 5 cells where shortening was measured, exposure to thapsigargin (3 min) increased time to peak shortening (from 192±22 ms to 296±47 ms), and reduced the amplitude of contraction (from 11.3±0.9% of initial length to 7.8±0.6%). In a further 10 cells thapsigargin did not reduce L-type calcium currents (4.6±0.4 nA before and 4.8±0.3 nA after 3 min exposure to thapsigargin).

The observations are consistent with an inhibitory action of thapsigargin on calcium uptake into the sarcoplasmic reticulum, leading to suppression of the calcium transient at early times in the action potential (reflecting a reduced loading of the sarcoplasmic reticulum with calcium), and to an elevation of cytosolic calcium at later times in the action potential (reflecting suppression of calcium removal from the cytoplasm by stores uptake) with a corresponding increase in time to peak contraction. Such a prolongation of the calcium transient could account for the observed action potential prolongation if this were to induce additional inward Na:Ca exchange current to maintain the plateau.

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23P DIRECT INHIBITION OF 5-HT_3 RECEPTOR FUNCTION BY L-TYPE Ca^{2+} CHANNEL ANTAGONISTS

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We have previously shown, using single cell video imaging of undifferentiated, fura-2-loaded N1E-115 neuroblastoma cells, that activation of 5-HT_3 receptors produces a significant increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Similar responses were seen in human embryonic kidney (HEK) cells transfected with either the long (A) or short (A_s) forms of the 5-HT_3 receptor. Several lines of evidence demonstrated that the increase in $[Ca^{2+}]_i$ stimulated by the 5-HT_3 receptor agonist, m-chlorophenyl biguanide (mCPBG), was not due to activation of voltage-operated Ca^{2+} channels (Hargreaves *et al.*, 1994).

In N1E-115 cells, application of 1-10 μM (+)verapamil, (-)verapamil or nimodipine caused concentration-dependent inhibition of the mCPBG-evoked increase in $[Ca^{2+}]_i$. Similar results were obtained in HEK cells transfected with the A or A_s forms of the $5HT_3$ receptor, suggesting that these Ca^{2+} channel antagonists directly inhibit 5-HT_3 receptors. Equilibrium radioligand binding studies using membranes prepared from Sf9 cells expressing the mouse 5-HT_3A subunit, demonstrated that (+)verapamil, (-)verapamil and diltiazem all displaced binding of 0.1 nM [^{3}H]mCPBG with IC_{50} values (mean ± s.e. mean) of 1.2 ± 0.1 (n=3), 11.8 ± 1.4 (n=3) and 37.5 ± 4.5 μM (n=5), respectively. However, nimodipine (≤ 300 μM) (n=3), had no effect on [^{3}H]mCPBG binding.

We conclude that a variety of L-type Ca^{2+} channel antagonists directly inhibit 5-HT_3 receptor function by at least two distinct mechanisms. Verapamil and diltiazem interact directly with the agonist-binding site of the 5-HT_3 receptor. Nimodipine inhibits the influx of Ca^{2+} through 5-HT_3 receptors by a different mechanism.

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Hargreaves, A.C., Lummis, S.C.R. & Taylor, C.W. (1994) *Mol. Pharmacol.* 46, 1120-1128

24P RADIOLIGAND BINDING CHARACTERISTICS OF 5-HT_3 RECEPTOR SPLICE VARIANTS: EVIDENCE THAT PHOSPHORYLATION DOES NOT AFFECT RECEPTOR BINDING AFFINITY

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5-HT_3 receptor proteins, which are members of the family of ligand-gated ion channels, may be homomeric receptors in vivo. So far only a single receptor subunit has been cloned, initially from the NCB20 neuroblastoma x Chinese hamster cell line and later from other neuronal cells (Maricq *et al.*, 1989, Hope *et al.*, 1993, Werner *et al.*, 1993). The latter studies have shown the subunit exists in two forms which differ by 6 amino acids containing a potential phosphorylation site in the intracellular loop. Expression of homomeric receptors constructed from either the long (5-HT_3R-A) or short (5-HT_3R-As) subunits reveals differences in agonist potency and efficacy (Sepulveda & Lummis, 1994; Downie *et al.*,1994). Here we examine if this is reflected in radiolabeled antagonist binding, and as phosphorylation is an important method of modifying receptor activity for other ligand-gated ion channels, we have also examined the effect of removing the potential phosphorylation site in the extra 6 amino acids contained in the 5-HT_3R-A subunit.

Full length 5-HT_3 R-As DNA was obtained from N1E-115 neuroblastoma cell mRNA using PCR. Coding sequence for the additional 6 amino acids in the 5-HT_3R-A subunit was inserted using site-directed mutagenesis, as was the same coding sequence with serine at position 386 replaced by alanine. All three sequences were inserted into the eukaryotic expression vector pRc/CMV and transfected into human embryonic kidney 293 cells using calcium phosphate precipitation. Radioligand binding studies with [^{3}H]granisetron were then performed on membranes prepared from the transfected cells. Relative potencies of a selection of 5-HT_3 receptor selective ligands were similar in the 3 preparations: GR65630 (3-(5-methyl-imidazolyl)-1-(methyl-indolyl)-1-propanone) > m-chlorophenylbiguanide > MDL 72222 (tropanyl 3,5-dichlorobenzoate) > 5-HT, but the affinity of [^{3}H]granisetron binding differed between the long and short forms of the receptor; 5-HT_3R-As: K_d = 0.24 ± 0.02, 5-HT_3R-A: K_d = 0.38* ± 0.03, S386A-5-HT_3R-A: K_d = 0.43 ± 0.02. (Values = mean ± s.e.mean, n = 4, *Significantly different to 5-HT_3R-As, $p<0.05$).

Thus the short form of the receptor binds [^{3}H]granisetron with higher affinity than either the long form or the mutant, where serine 386 was replaced by alanine. The results show that the change in receptor affinity can be identified using radioligand binding, and that it is not due to a change in phosphorylation in the 6 amino acid portion which is missing in the short form of the receptor.

SCRL is a Royal Society University Research Fellow.

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25P THE EFFECTS OF AMPHETAMINE AND COCAINE IN THE RAT ELEVATED ZERO-MAZE TEST OF ANXIETY: A COMPARISON WITH DIAZEPAM

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Amphetamine has previously been reported to produce both anxiolytic (Crawford et al., 1994) and anxiogenic (Pellow et al., 1985) effects in the rat elevated plus-maze model. The purpose of the present studies was to investigate the effects of amphetamine sulphate (AMPH; 0.15-5 mg/kg) and a pharmacologically similar psychostimulant, cocaine hydrochloride (COC; 3-30 mg/kg), in the rat elevated zero-maze test of anxiety. This model is a modification of the plus-maze test and incorporates both traditional and ethological measures to provide a more detailed behavioural profile and facilitate the interpretation of drug effects. Previous studies have demonstrated that the zero-maze test is sensitive to both anxiolytic and anxiogenic drug action (Shepherd et al., 1994).

Group-housed (4 per cage) male Sprague-Dawley rats (290-350g) were injected subcutaneously with either drug or vehicle (10% polyethyleneglycol/90 % saline) and thirty minutes later were placed onto a closed quadrant of the zero-maze. A 5 min test session was then recorded on video-tape for subsequent analysis. The following behavioural parameters were scored: (a) percentage of test time spent in the open quadrants (%TO), (b) the frequency of head dips over the edge of the platform (HDIPS), (c) the frequency of stretched attend postures (SAP) exhibited when animals, in the closed quadrants, investigated the open areas with at least their snouts crossing the closed-open divide, and (d) the frequency of rears. A treatment group receiving a single dose of diazepam (DZP; 0.5 mg/kg) was included in each study as a positive control.

The results are presented in Table 1. DZP exhibited a reliable and consistent anxiolytic-like profile, significantly increasing %TO and HDIPS, and decreasing SAP. COC (3-30 mg/kg) displayed an identical profile to DZP, whilst AMPH (0.5-5.0 mg/kg) also significantly increased %TO and decreased SAP, and produced marked, but non-significant, increases in HDIPS. None of the drugs tested had any significant effects on rearing activity. Thus, the present data indicate that the psychomotor stimulants, amphetamine and cocaine, exhibit effects in the rat elevated zero-maze consistent with an anxiolytic-like profile. Further studies are required to elucidate whether these behavioural profiles are typical of stimulant drug action *per se*.

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Table 1. Effects of amphetamine, cocaine and diazepam in the rat elevated zero-maze test.

Dose (AMPH)	n	%TO	HDIPS	SAP	Dose (COC)	n	%TO	HDIPS	SAP
0 mg/kg	12	20.8 ± 3.0	5.0 ± 1.0	2.4 ± 0.7	0 mg/kg	12	13.6 ± 2.5	2.1 ± 0.7	4.6 ± 1.3
0.15 mg/kg	11	27.5 ± 2.9	4.8 ± 1.0	2.8 ± 0.8	3 mg/kg	12	31.3 ± 4.1*	5.4 ± 1.2*	1.0 ± 0.5*
0.5 mg/kg	12	45.8 ± 3.9*	8.8 ± 1.9	0.5 ± 0.3*	10 mg/kg	12	30.2 ± 4.1*	6.7 ± 1.4*	1.4 ± 0.7*
1.5 mg/kg	9	44.3 ± 3.1*	10.2 ± 2.6	0.2 ± 0.1*	30 mg/kg	10	32.4 ± 4.7*	8.0 ± 1.5*	0.9 ± 0.4*
5.0 mg/kg	11	45.8 ± 8.0*	2.5 ± 0.9	0*	DZP (0.5)	12	39.8 ± 4.0*	18.5 ± 1.7*	0*
DZP (0.5)	11	35.8 ± 3.4*	17.0 ± 2.3*	0.3 ± 0.1*					

The data are presented as mean scores ± s.e.mean. *P<0.05 versus vehicle-control group (one-way ANOVA/Dunnetts test).

26P DOPAMINE OUTPUT DURING L-DOPA INFUSION INTO THE SUBSTANTIA NIGRA OF RESERPINISED RATS IS AUGMENTED BY DIZOCILPINE

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It is widely accepted that degeneration of the nigrostriatal dopaminergic pathway underlies many of the clinical manifestations of Parkinsons Disease (PD). The mainstay of treatment for PD remains L-DOPA, although continued use of this drug does little to halt the progress of the disease and is often associated with side effects. Recently, it was demonstrated that very low doses of the glutamate antagonist dizocilpine potentiated the motor effects of L-DOPA, but not DA receptor agonists, in reserpine treated rats (Kaur et al, 1994). This finding suggests that dizocilpine potentiates L-DOPA presynaptically. Using in vivo microdialysis of reserpinised rats, we examined this possibility by measuring the conversion of L-DOPA to DA in the substantia nigra pars reticulata (SNr).

Male Wistar rats (250-300g) were anaesthetised with chloral hydrate (400 mg/kg) and concentric microdialysis probes implanted into both SNr. Groups 1-3 (n=4 each) received reserpine (5mg/kg), whilst group 4 animals received saline.The following day all rats were dialysed with artificial cerebrospinal fluid (ACSF;0.5 µl/min) and ten 30 min. dialysates collected. After 90 min, all rats received α-methyl-*p*-tyrosine (αMPT; 200mg/kg). Following a further 60 min, drugs were infused via probes for 90 min such that group 1 rats received dizocilpine (150 nM) and L-DOPA (10µM), group 2 rats L-DOPA (10µM) and group 3 rats dizocilpine (150 nM). Group 4 rats received normal ACSF. Dialysates were analysed immediately for monoamine content using HPLC with electrochemical detection. Probe placements were confirmed histologically.

Basal DA output from SNr of control and reserpinised rats was 38±13 and 2.20±0.64 fmol/10µl, respectively. In all groups, αMPT treatment resulted in a reduction of basal DA output, which fell to undetectable levels in groups 1-3. In group 2, infusion of L-DOPA alone resulted in a modest elevation of DA output (peak 305±39% of basal; $p<0.05$) which was maintained for the duration of infusion. DA remained undetectable during infusion of dizocilpine alone (group 3). However, in animals where dizocilpine and L-DOPA were co-infused (group 1), DA output was massively potentiated, such that peak DA levels were comparable to basal values noted in control animals (peak 3638±980% of basal; $p<0.001$). Significantly, these animals were the only subjects to exhibit obvious signs of motor activation during drug infusion.

The present data suggest that dizocilpine substantially increases the output of DA during L-DOPA infusion into the SNr of DA-depleted rats. This potentiation occured at a dose of dizocilpine which we calculated was equivalent to that potentiating the anti-akinetic action of L-DOPA in behavioural studies (Kaur et al, 1994). The mechanism of the pro-dopaminergic action of dizocilpine in the SNr requires further evaluation.

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27P MODIFICATION OF L-DOPA-INDUCED DYSKINESIAS BY THE D-1 AGONIST A-77636 IN THE MPTP-TREATED COMMON MARMOSET (*CALLITHRIX JACCHUS*)

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The common marmoset (*Callithrix jacchus*) shows parkinsonian motor disability following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure and develops dyskinesias during chronic L-DOPA dosing (Pearce *et al.*, 1993). We have previously shown that the D-1 agonist A-77636 [(1R, 3S) 3-(1'-adamantyl)-1-aminomethyl-3,4-dihydro-5,6-dihydroxy-1H-2-benzopyran HCl] exhibits potent antiparkinsonian activity in the MPTP-treated marmoset (Kebabian *et al.*, 1992). We now report the effects of combined L-DOPA and A-77636 administration upon L-DOPA-induced dyskinesias in this model.

MPTP-treated parkinsonian marmosets were treated with L-DOPA (12.5 mg/kg bid PO) plus carbidopa (12.5 mg/kg bid PO) until they showed dyskinesias. Several months later they received L-DOPA with carbidopa in the same doses for seven days followed by the D-1 agonist A-77636 (3 µmol/kg OD SC) for 10 days and then both drugs together for three days. Dyskinesias were rated on a semiquantitative dyskinesia scoring system (0= absent to 4= severe).

Both L-DOPA and A-77636 were highly effective in reversing MPTP-induced akinesia. L-DOPA administration produced dyskinesias, with prominent limb chorea and dystonia, abundant climbing, stereotypy and a pressured hyperkinesis. Initially, A-77636 reproduced the dyskinesias seen after L-DOPA administration but with an intensification of dystonia, and then gradually abolished all dyskinesias while preserving antiparkinsonian activity, although some behavioral tolerance was detected. When L-DOPA was reintroduced and given concurrently with A-77636, dystonia, but no significant chorea was seen and there was a marked reduction in L-DOPA-induced climbing, stereotypy and hyperkinesis (Figure 1).

The suppression of dyskinesia and differential effects upon choreic and dystonic L-DOPA-induced dyskinesias exercised by A-77636 may relate to adaptive changes in the D-1 receptor-bearing direct striatopallidonigral pathway, with secondary alterations in activity of the pedunculopontine nucleus and subthalamus.

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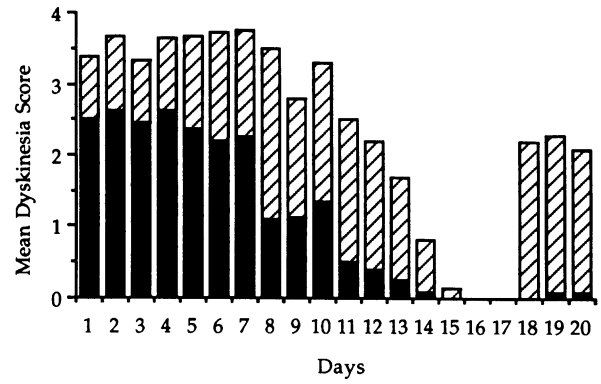
Figure 1. Mean dyskinesia scores (chorea= solid bar; dystonia= hatched bar) in MPTP-treated common marmosets (n= 4) receiving L-DOPA with carbidopa on days 1-7 and 18-20 (both at 12.5 mg/kg bid) and A-77636 on days 8-20 (3 µmol/kg OD).



28P INHIBITION OF MITOCHONDRIAL RESPIRATION BY ISOQUINOLINE DERIVATIVES

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We have shown that isoquinoline derivatives exert MPP^+-like activity as inhibitors of complex I and α-ketoglutarate dehydrogenase in rat brain mitochondrial fragments (McNaught *et al.*, 1994a,b). Now we examine the effects of 19 isoquinoline derivatives from our previous study on respiration in intact mitochondria. This is important since the toxicity of MPP^+ depends on its active mitochondrial accumulation.

Liver mitochondria were isolated from male Wistar rats (200-250g) as described elsewhere (Schnaitman & Greenwalt, 1968). Oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Rank Bros. Ltd., UK) in a thermostated incubation chamber at 25 °C containing 3.0 ml reaction medium and 0.8 mg mitochondrial protein (Clark, 1992). Oxygen consumption was supported by 10 mM glutamate + 2.5 mM malate, 10 mM succinate + 100 µM rotenone or 50 µM tetramethylparaphenylenediamine (TMPD) + 2 mM ascorbate. State 3 respiration was initiated by the addition 0.25 mM ADP.

In experiments to determine the time-dependent inhibition of respiration, the isoquinoline derivatives or MPP^+ (0.5 mM) were added to the reaction medium with substrates and allowed to incubate for up-to 20 min before state 3 respiration was initiated. To determine the concentration-dependent inhibition of respiration supported by glutamate + malate, the compounds (0.2-2 mM) were added with substrates and allowed to incubate for 5 min before state 3 respiration was initiated. Respiratory rates are expressed as ng atoms O/min/mg. All measurements were performed in triplicate and data presented as mean ± SEM. Results were analysed statistically using the Mann-Whitney *U*-test.

None of the compounds examined inhibited respiration supported by either succinate + rotenone or TMPD + ascorbate. In contrast, with glutamate + malate as substrates, fifteen of the isoquinoline derivatives and MPP^+ inhibited state 3, and to a lesser extent state 4 respiration, in a time- and concentration-dependent manner. However, none of the isoquinoline derivatives were more potent than MPP^+ which inhibited state 3 respiration by 83.6% and 100% ($p < 0.05$) after 5 min and 10 min, respectively.

In the isoquinoline series, 6,7-dimethoxyisoquinoline was inactive, while the others inhibited state 3 respiration with varying potencies. 6-Methoxyisoquinoline and N-methyl-6,7-dimethoxyisoquinolinium, the most and least potent isoquinoline derivatives, respectively, at a concentration of 0.5 mM and incubation time of 5 min, inhibited state 3 respiration by 82.4% and 13.2% ($p < 0.05$). Norsalsolinol was inactive; the other tetrahydroisoquinolines at 0.5 mM produced only a small inhibition (8-18%) of state 3 respiration and only after 20 min, except for 1,2,3,4-tetrahydroisoquinoline which inhibited state 3 respiration by 36% after 5 min ($p < 0.05$). Amongst the two dihydroisoquinolines studied, N-methyl-6-methoxy-1,2-dihydroisoquinoline was inactive, while 6,7-dimethoxy-1-styryl-3,4-dihydroisoquinoline was found to be a mitochondrial uncoupler rather than an inhibitor. In the presence of glutamate + malate, 6,7-dimethoxy-1-styryl-3,4-dihydroisoquinoline at 0.5 mM and 1.0 mM (10 min incubation), stimulated state 4 respiration by 90% and 122% ($p < 0.05$), respectively.

In conclusion, isoquinoline derivatives are less potent in inhibiting respiration in intact mitochondria than complex I in mitochondrial fragments. This suggests that these compounds are not accumulated by mitochondria as avidly as MPP^+. The inhibition of respiration by both charged and neutral isoquinoline derivatives suggests that active and passive membrane transport may play a role in the mitochondrial accumulation of these compounds.

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29P DIFFERENTIAL EFFECT OF DOPAMINE DEPLETION ON THE LEVEL OF D2 AND D3 RECEPTOR EXPRESSION IN RAT BASAL GANGLIA

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Interruption of dopaminergic neurotransmission by neurotoxic lesions differentially alters the expression of dopamine D1 and D2 receptors in rat basal ganglia (Graham et al., 1990). Less is known about the effect of altering dopaminergic neurotransmission on the dopamine D3 receptor. Within the basal ganglia the D3 receptor is expressed highly in the nucleus accumbens. The nucleus accumbens can be divided into two regions, the shell and core, on the basis of anatomical and neurochemical criteria (Zahm and Brog, 1992). It is therefore pertinent to investigate whether the D3 receptor is also subject to regulation by dopamine within the basal ganglia. This question was addressed using *in situ* hybridization and receptor autoradiography in rats with unilateral lesions of the dopaminergic neurons in the medial forebrain bundle.

Adult male rats (Sprague-Dawley, 300g) were anaesthetised with halothane and an intracranial injection of 6-OHDA (8µg 6-OHDA base in 4 µl 0.9% NaCl containing 0.1% ascorbic acid) was made into the left medial forebrain bundle (P 4.16mm, V 8.4mm, L 1.6mm). Three weeks after surgery brains were removed, immediately frozen (isopentane -45°C), and coronal sections (12µm) cut with a cryostat. Dopamine D2 and D3 receptor mRNA was visualized using [^{35}S]-labelled oligonucleotide probes. Dopamine D2 and D3 receptors were labelled with 0.5 nM [^{3}H]-spiperone and 1.0 nM [^{3}H]-7-OH-DPAT respectively. Non-specific binding was defined using 10 µM sulpiride and 10 µM dopamine for D2 and D3 receptors respectively.

In the nucleus accumbens shell (NAs) the level of both D3 receptor mRNA and [^{3}H]-7-OH-DPAT binding sites were reduced by the 6-OHDA lesion. In contrast, the level of D2 receptor mRNA and [^{3}H]-spiperone binding sites were unaltered in the NAs. In the nucleus accumbens core (NAc) the level of expression of both receptors was unaltered. In the dorsolateral caudate-putamen (CPdl) the level of D3 receptor mRNA and protein was not changed whilst the level of D2 receptor mRNA and protein was elevated. The results are summarized in Table 1.

Table 1 D2 and D3 receptor mRNA and protein in rat brain
*P < 0.05 intact vs. lesion, Student's t-test, n = 4-5

	D3 mRNA (amol/mg)		D2 mRNA (amol/mg)	
	Intact side	Lesion side	Intact side	Lesion Side
NAs	0.9 ± 0.05	0.7 ± 0.07*	0.6 ± 0.03	0.6 ± 0.01
NAc	0.4 ± 0.02	0.4 ± 0.03	0.6 ± 0.01	0.6 ± 0.03
CPdl	0.4 ± 0.02	0.4 ± 0.04	0.7 ± 0.02	0.8 ± 0.04*
	D3 receptor (fmol/mg)		D2 receptor (fmol/mg)	
	Intact side	Lesion side	Intact side	Lesion side
NAs	10.3 ± 0.6	7.2 ± 0.5*	23.1 ± 4.6	22.3 ± 4.5
NAc	3.3 ± 0.3	2.6 ± 0.3	20.4 ± 2.3	20.5 ± 3.1
CPdl	1.9 ± 0.2	1.4 ± 0.3	49.6 ± 4.6	61.6 ± 4.3*

This study demonstrates that the D3 receptor, but not the D2 receptor, is downregulated in the NAs after dopaminergic denervation. The decrease in receptor density is accompanied by a reduction in the level of mRNA, indicating that the D3 receptor is present on neurons intrinsic to the nucleus accumbens. Thus the alteration is an adaptive response to, rather than a direct result of, the 6-OHDA lesion. This provides evidence that the reduction in D3 receptor expression is due to a decrease in the rate of receptor synthesis and that the majority of D3 receptors in the NAs are located post-synaptically.

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Zahm D. & Brog J., (1992) *Neuroscience* 50, 751-767

30P EFFECTS OF GABAPENTIN ON ETHANOL WITHDRAWAL SIGNS IN MICE

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The novel anticonvulsant drug gabapentin was examined for its activity in an animal model of ethanol withdrawal convulsions with the aim of determining the selectivity of any effects.

Male TO mice (25-35 g) were made physically dependent on ethanol by a 10 day schedule of liquid diet (Watson and Little, 1995). The daily intake of ethanol was 22-30 g/kg. Immediately after withdrawal from ethanol, the mice were injected i.p. with gabapentin (5, 20, 50 or 100 mg/kg) or saline vehicle. Control mice received saline. The mice were then rated hourly on a scale of 0 - 5 for their handling-induced convulsive activity (Watson and Little, 1995). Control mice gave scores of 0 - 1. Mice undergoing ethanol withdrawal showed significant increases in handling scores, peaking 3-5 h from withdrawal ($P < 0.01$ Mann Whitney U-test). Ataxic effects of gabapentin were determined in naive animals using a rotarod rotating at 4.5 rpm. The time mice were able to stay on the rotating rod was measured, at 10 min intervals, for 1 h after gabapentin administration.

Administration of gabapentin at 100 mg/kg but not at the lower doses significantly reduced the handling-scores over the whole 12 h period of the experiment ($P < 0.001$ Non-parametric ANOVA; Meddis, 1984). When the area under the handling-time curve for each mouse was analysed for the first 4 h of the experiment, gabapentin at 20, 50 and 100 mg/kg significantly reduced the severity of the withdrawal ($P < 0.05$, 0.05, 0.01 respectively Mann-Whitney U test, see table). No ataxic effects of gabapentin were seen at doses of 50, 100 or 200 mg/kg.

The doses of gabapentin reported to be anticonvulsive in this study are lower than those reported for other types of convulsion e.g. electroshock 78 mg/kg, NMDA seizures >240 mg/kg (Chadwick, 1994). Gabapentin therefore showed some selectivity in protecting against ethanol withdrawal hyperexcitability, both compared with its effects in other types of seizures and in its lack of ataxic actions at the effective doses.

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Meddis, R. (1984) Statistics using ranks: A unified approach. Basil Blackwell, Oxford, New York.
Chadwick, D. (1994) Lancet, 343, 89-91.

Treatment	Area under handling curve (1 -4 h)
Control and Saline	3.0 ± 0.5
Ethanol and Saline	8.8 ± 0.2 ($P < 0.05$ c.f. control and saline)
Ethanol and GP 5 mg/kg	6.4 ± 0.8
Ethanol and GP 20 mg/kg	5.6 ± 1.2 ($P < 0.05$ c.f. ethanol and saline)
Ethanol and GP 50 mg/kg	5.9 ± 0.8 ($P < 0.05$ c.f. ethanol and saline)
Ethanol and GP 100 mg/kg	2.4 ± 0.6 ($P < 0.01$ c.f. ethanol and saline)

GP = gabapentin

31P THE EFFECT OF TERMINAL 5-HT AUTORECEPTOR ANTAGONISTS ON 5-HT RELEASE IN THE GUINEA-PIG BRAIN

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We report here the effects of three 5-HT autoreceptor antagonists, GR 127935 (Skingle et al., 1993), methiothepin and 1-(1-naphthyl)piperazine (NP) on *in vitro* and *in vivo* release of 5-HT from guinea-pig hypothalamus and substantia nigra. For *in vitro* release studies, hypothalamic slices from male Hartley guinea-pigs (300-350 g) were labelled with [^{3}H]5-HT, superfused with Krebs solution and stimulated (S_1 and S_2) electrically at 5 Hz for 2 min or under pseudo-one-pulse (POP) conditions (4 pulses at 100 Hz), where the stimulation is too brief for released 5-HT to activate the autoreceptor (Singer, 1988). *In vivo* release from the substantia nigra or hypothalamus of freely moving guinea-pigs was measured by microdialysis at a flow rate of 0.32 µl/min and samples collected every 30 min and analyzed with HPLC-ECD.

In vitro, [^{3}H]5-HT release induced by 5 Hz stimulation represented 2.49 ± 0.14 % (n = 75) of the total tissue radioactivity and 0.41 ± 0.03 % (n = 100) under POP stimulation. The S_2/S_1 ratio was close to unity under both conditions. The inhibition of release by the autoreceptor agonist, 5-carboxamidotryptamine (5-CT, 10 nM) was antagonised by GR 127935, methiothepin and NP. Their approximate IC_{50} values were 10, 50, and 20 nM, respectively. When added alone before S_2, GR 127935 and methiothepin significantly increased the evoked release of [^{3}H]5-HT at 0.1 µM (+ 71% and + 63 %, respectively) whereas NP had no effect even at 1 µM. Under POP stimulation, where the autoreceptor is not stimulated by released 5-HT, GR 127935 and methiothepin behaved as silent antagonists and did not change [^{3}H]5-HT release, whereas NP showed intrinsic agonist activity significantly decreasing release by 82 % at 1 µM. In slices of substantia nigra, methiothepin and NP behaved in the same manner as in hypothalamus at 5 Hz.

In vivo, basal extracellular levels of 5-HT, measured by microdialysis were (fmol/15 µl) 33.6 ± 2.1 (n = 79) and 13.7 ± 1.2 (n = 15), respectively in substantia nigra and hypothalamus. When perfused through the dialysis probe in substantia nigra at 100 µM, GR 127935 had no effect on 5-HT levels in perfusates whereas methiothepin and NP enhanced these levels to a maximum of 166 ± 50 % (n = 5) and 545 ± 167 % (n = 4) of basal levels. Extracellular 5-HT was not changed by infusion of GR 127935 at 10 µM into hypothalamus. None of the drugs had any action when administered systemically.

The full antagonist, methiothepin, and the partial antagonist, NP, increase 5-HT release *in vivo* when administered locally whereas the full antagonist, GR 127935, does not. These results suggest that *in vivo* activity of these compounds may depend more on their receptor selectivity (all have been shown or suggested to act at sites other than the 5-HT1D receptor) than their full or partial antagonist properties.

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32P [^{3}H]-RS-45041-190, A POTENT AND SELECTIVE RADIOLIGAND FOR I_2 IMIDAZOLINE RECEPTORS

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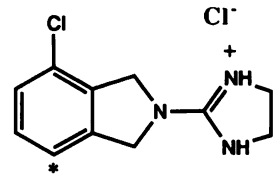
This report describes the binding characteristics of the radiolabelled selective I_2 imidazoline compound RS-45041-190 to rat kidney membranes (MacKinnon *et al.*, 1995) and its autoradiographical distribution in rat brain. [^{3}H]-RS-45041-190 (26 Ci mmol^{-1}, 0.2-25 nM) was incubated with EDTA-washed rat kidney membranes (prepared as described in MacKinnon *et al.*, 1993) to equilibrium (90 min at 25°C). Non-specific binding was defined with 10 µM cirazoline. The distribution of [^{3}H]-RS-45041-190 binding in rat brain sections was studied by incubating thawed-cryostat sections (20 µm) with 6 nM [^{3}H]-RS-45041-190 in Tris EDTA buffer for 60 min. Non specific binding was defined by co-incubation with 10 µM cirazoline.

[^{3}H]-RS-45041-190 bound to a single site in the rat kidney with high affinity (K_d = 2.71±0.59 nM, B_{max} = 223.1±18.4 fmol mg protein^{-1}). Idazoxan and cirazoline had high affinity for 60-70% of the binding (pIC_{50} = 7.85 and 8.16 respectively), and low affinity for the remaining 30-40% (pIC_{50} = 5.80 and 6.11 respectively). Guanabenz and naphazoline had moderate affinity (pK_i =7.23 and 6.61 respectively). Noradrenaline, delequamine (RS-15385-197), prazosin, dopamine, histamine, rilmenidine and *p*-aminoclonidine had low affinity (pK_i < 5.60) for [^{3}H]-RS-45041-190 binding suggesting that [^{3}H]-RS-45041-190 does not label α-adrenoceptors, dopamine, histamine or imidazoline receptors of the I_1 subclass. A comparison of affinities of nine compounds for [^{3}H]-RS-45041-190 binding and [^{3}H]-idazoxan binding to I_2 receptors in the same



RS-45041-190 * ^{3}H

preparation revealed an excellent correlation (r=0.97) confirming that [^{3}H]-RS-45041-190 labels I_2 receptors. In autoradiography studies, [^{3}H]-RS-45041-190 labelled discrete regions of rat brain corresponding to the distribution of I_2 receptors (Mallard *et al.*, 1992) *i.e.* the area postrema, interpeduncular and arcuate nuclei, and the lateral ventricles. Additional sites (>150 fmol mg^{-1} tissue) were also observed in the subfornical organ, locus coeruleus, medial habenular nucleus and dorsomedial hypothalamic nucleus, with moderate binding in the dorsal raphe, cortical amygdaloid zone and the nucleus of the solitary tract (>100 fmol mg^{-1} tissue). These results suggest that [^{3}H]-RS-45041-190 labels I_2 receptors on rat kidney and brain with high affinity, and an additional site which is as yet undefined. The discrete anatomical distribution of the receptors suggests a range of possible functions for I_2 receptors in the CNS.

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33P EFFECTS OF METABOTROPIC GLUTAMATE RECEPTOR AGONISTS ON SECOND MESSENGER RESPONSES IN THE CEREBELLUM

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Metabotropic glutamate receptors (mGluRs) may be divided into three groups based on similarities in primary sequence, signal transduction properties and agonist potencies (Nakanishi, 1992). Thus, 1-aminocyclopentane-1S,3R-dicarboxylate (1S,3R-ACPD) is a potent Group I agonist, coupling to a stimulation of phosphoinositide turnover. Group II and Group III receptors are both linked to inhibition of adenylyl cyclase activity, and may be selectively stimulated by (2S,1'R,2'R,3'R)-2,2',3'-dicarboxycyclopropylglycine (DCG-IV) and L-serine-O-phosphate (L-SOP). We have recently described responses to mGluR agonists which suggest the presence of the three different groups of mGluRs in the guinea-pig cerebral cortex (Cartmell *et al.*, 1994). In this report, we have investigated second messenger responses to mGluR agonists in the guinea-pig cerebellum.

Accumulation of [^{3}H]-cAMP and [^{3}H]-inositol phosphates ([^{3}H]-$InsP_x$) was monitored at 37°C in guinea-pig cerebellar slices labelled with [^{3}H]-adenine or [^{3}H]-inositol, respectively, as previously described (Hernández *et al.*, 1993). Data (means ± SEM) presented were derived from experiments conducted on at least three separate occasions.

Basal accumulation of [^{3}H]-$InsP_x$ in guinea-pig cerebellar slices was 1308 ± 579 dpm. [^{3}H]-$InsP_x$ accumulation was stimulated in the presence of 1S,3R-ACPD and L-glutamate with EC_{50} values of 32 ± 3 and 1040 ± 44 μM with maximal responses of 396 ± 130 and 397 ± 145 % basal, respectively. The accumulation of [^{3}H]-cAMP was examined under basal conditions (0.17 ± 0.04 % conversion) and in the presence of 30 μM forskolin (9.89 ± 0.81 %), 1 μM isoprenaline (1.48 ± 0.17 %) and 30 μM 2-chloroadenosine (2.95 ± 0.34 %). DCG-IV (10 μM, 100 ± 15 % control) and 1S,3R-ACPD (300 μM, 89 ± 4 % control) failed to significantly alter the response to forskolin. [^{3}H]-cAMP accumulations elicited by isoprenaline and 2-chloroadenosine were also not significantly altered in the presence of 1S,3R-ACPD (87 ± 23 % and 89 ± 6 % control). 10 mM L-glutamate significantly inhibited [^{3}H]-cAMP accumulations evoked by forskolin (59 ± 5 %), isoprenaline (32 ± 5 %) and 2-chloroadenosine (72 ± 4 %). 3 mM LSOP also significantly inhibited responses to forskolin (37 ± 5 %), isoprenaline (11 ± 3) and 2-chloroadenosine (58 ± 9 %).

These data indicate the presence of Group I and III mGluRs in the guinea-pig cerebellum. However, we are unable to observe significant effects of the selective Group II agonist DCG-IV, suggesting either the absence of these receptors or coupling to other signal transduction systems. The lack of significant potentiation of receptor-stimulated responses by L-glutamate and 1S,3R-ACPD contrasts with the 2-fold enhancement observed in the cerebral cortex (Alexander *et al.*, 1992), implying distinct cellular localizations of Group I mGluRs and these cAMP-linked receptors in the cerebellum.

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34P CENTRAL ANTINOCICEPTION BY SPINAL ACTIONS OF A SYSTEMICALLY-ADMINISTERED NSAID: MEDIATION BY ENDOGENOUS OPIOIDS

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The concept that non-steroidal anti-inflammatory drugs (NSAIDs) exert their antinociceptive actions purely in the periphery has recently been challenged by the effectiveness of intrathecal NSAIDs (McCormack, 1994), but the degree to which central actions may contribute to the effects of normal doses of systemically-administered NSAIDs remains uncertain. We have investigated this in electrophysiological experiments.

Single motoneurone withdrawal reflexes were recorded as reported previously (Herrero & Headley, 1991) in α-chloralose anaesthetized rats (50 $mg.kg^{-1}$ initially; approx. 20 $mg.kg^{-1}.hr^{-1}$ maintenance) in response to regular cycles of pinch (15s, 2.5N over 19 mm^2, activating nociceptors) and electrical stimuli (2 ms, 10-20T for the reflex, at 0.2 and 1 Hz; bypassing nociceptor endings) both applied to the foot. Inflammation of one paw was induced by intraplantar injection of carrageenan (100 μl of 10 $mg.ml^{-1}$ in water). The effects of i.v. drugs (n = 6-10 per test) were measured as % change from control (mean of 3 responses prior to drug; based on counts of evoked spikes). Cumulative doses of the μ-opioid fentanyl (4-16 $\mu g.kg^{-1}$) were followed by at least an hour for full recovery, after which the NSAID flunixin (Finadyne, Schering-Plough; 1-16 $mg.kg^{-1}$; an NSAID commonly used in veterinary practice at 2.2 $mg.kg^{-1}$) was given whilst recording the same units. Naloxone (1 $mg.kg^{-1}$) was administered either 12 min before or 12 min after flunixin, and fentanyl retested as the last drug.

In spinalized non-inflamed rats fentanyl reduced all responses (ED_{50} 7-10 $\mu g.kg^{-1}$) whereas flunixin reduced responses to only 75-85 % control at 8-16 $mg.kg^{-1}$. Naloxone reversed the fentanyl but not flunixin effects. In spinalized rats with an inflamed paw, fentanyl was similarly effective, whereas flunixin was much more effective (ED_{50} 4-11 $mg.kg^{-1}$). Moreover, under these conditions naloxone post-treatment reversed, and pre-treatment prevented, the effects of flunixin as well as fentanyl. In spinally intact rats with an inflamed paw, flunixin and fentanyl were similarly effective, but, surprisingly, naloxone no longer reversed the effects of flunixin. Neither fentanyl nor flunixin distinguished between those responses to electrical stimulation showing wind-up (1 Hz stimulation) and those that did not (0.2 Hz stimulation).

The results with these protocols indicate that the μ-opioid fentanyl was effective, and naloxone-sensitive, under all conditions, as expected. In the absence of peripheral inflammation the NSAID flunixin had little if any central antinociceptive action. In the presence of inflammation, however, the reduction of responses to electrical stimuli (which should have bypassed the peripheral nociceptor endings at which NSAIDs would be expected to act) indicates a central action; the effectiveness in spinalized rats indicates a spinal site. The block of flunixin antinociception by naloxone indicates that an endogenous opioid mediated the flunixin effects. Central wind-up was not influenced by the NSAID. The unexpected difference in naloxone effectiveness between spinally-intact and spinalized rats cannot at present be explained. The results suggest that systemic doses of NSAIDs close to those used clinically can have central antinociceptive actions and that these effects are mediated by the release of endogenous opioids.

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35P CHARACTERISATION OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR BINDING BY SCINTILLATION PROXIMITY ASSAY

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In Scintillation Proximity Assays (SPA†), scintillant is incorporated into small beads to which receptors and other biological molecules can be attached. Since low energy β particles emitted from a [^{3}H]-labelled ligand are detected only when the radioligand is in close proximity to the bead, the method allows receptor-ligand interactions to be measured without the need to separate bound from free ligand. We have established a SPA for inositol 1,4,5-trisphosphate (InsP_3) receptors using SPA beads coated with wheatgerm agglutinin (WGA) to allow purified InsP_3 receptors to attach *via* their covalently bound carbohydrate residues.

InsP_3 receptors were purified from rat cerebella by sequential heparin and concanavalin A affinity chromatography as previously reported[1]. InsP_3 bound with high affinity to a single class of sites in both cerebellar membranes (K_d=7.7nM [5.3-11nM], geometric mean [95% confidence limits], n=9; B_{max}=12.7 ± 3 pmol/mg protein, n=6) and the purified receptor preparation (K_d=5.7nM [4.4-7.5nM], B_{max}=1318 ± 357 pmol/mg, n=3). Silver-staining of gels after SDS PAGE analysis confirmed that the purified receptor migrated as a single band of apparent molecular weight 245kDa. Incubation of the purified receptors with WGA SPA beads for 2 hours at 4°C resulted in attachment of 97 ± 4 % (n=7) of the receptors; the coupling was stable for at least 12 hours at 4°C. D-[^{3}H]InsP_3 (5nM, 30-40Ci/mmol) binding to the InsP_3 receptor-beads was performed in TrisHCl (50mM), EDTA (1 mM), pH 8.6 at 4°C. Non-specific binding (typically 10% of total binding) was determined in the presence of 1μM D-InsP_3. Samples (200μl) were counted (30s) in a Wallac 1209 Rackbeta Liquid Scintillation counter at either 4°C (for equilibrium binding) or in dry ice/methanol (for frozen samples).

From equilibrium competition binding experiments, the K_d for D-InsP_3 was 0.9nM [0.2-3.9nM] (n=3), and for heparin, a competitive antagonist of the InsP_3 receptor, the K_d was 1.4nM [1.1-1.8nM] (n=3). L-InsP_3 (1μM) displaced only 5.2 ± 3.8% (n=4) of specific D-[^{3}H]InsP_3 binding, confirming the stereospecificity of binding to the InsP_3 receptor-beads. Association and dissociation of D-[^{3}H]InsP_3 from the InsP_3 receptor-beads were too fast to be reliably determined by scintillation counting as they occurred. Instead, at appropriate times, samples were rapidly frozen in liquid nitrogen and then counted while still frozen; freezing did not affect the affinity of the receptor for InsP_3 as determined by equilibrium competition binding. This kinetic analysis identified rapid components of D-[^{3}H]InsP_3 association and dissociation that were essentially complete within 4s and which could not be further characterised. For the slower phases, the bimolecular association rate constant (k_{+1}) was 1.6 ± 0.4 x $10^6 s^{-1}M^{-1}$ (n=4) and the dissociation rate constant (k_{-1}) was 0.020 ± 0.005s^{-1} (n=4); similar results were obtained when 10nM D-[^{3}H]InsP_3, rather 5nM, was used for the kinetics analysis.

We conclude that SPA, with the many advantages it provides, can be successfully employed to measure the interactions between purified InsP_3 receptors and their ligands.

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†SPA technology is covered by US Patent No. 4568649; European Patent No. 0154734; and Japanese Patent Application No. 84/52452.

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36P MEASUREMENT OF GUANINE NUCLEOTIDE BINDING PROTEIN ACTIVATION BY NEUROTRANSMITTERS IN BOVINE CAUDATE MEMBRANES

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Heterotrimeric guanine nucleotide binding (G) proteins serve critical roles in the regulation of neuronal function in the brain. To date, interactions between neurotransmitters and their G-proteins have been studied indirectly either by radioligand binding techniques or by stimulation/inhibition of second messenger systems. In this study we describe an assay for the direct measurement of G-protein activation by measuring the binding of the poorly hydrolysable GTP analogue, [^{35}S]GTPγS.

Membranes were prepared from fresh bovine caudate tissue (Farrell & O'Boyle, 1994). Procedures for measuring [^{35}S]GTPγS binding were modified from those described by Lorenzen et al., (1993). Briefly, 2.5 μg aliquots of membrane protein were pre-incubated on ice with 10 μM GDP to suppress basal binding, then incubated with neurotransmitter and 50,000 cpm (0.3-0.5 nM) [^{35}S]GTPγS in a Tris based buffer for 45 min at 30°C. When used, antagonists were included in the pre-incubation step. Non specific binding was defined using 10 μM GTPγS.

Dose-response curves were constructed for acetylcholine (ACh), 5-hydroxytryptamine (5-HT) and dopamine (DA). Table 1 summarises the responses to 100 μM neurotransmitter, and the concentration required to increase [^{35}S]GTPγS binding by 30% over basal (Δ_{30}).

neurotransmitter	% ↑ over basal (100 μM)	Δ_{30} (μM)	n
acetylcholine	98±13	1.97±0.4	4
5-HT	83±6	0.016±0.002	3
dopamine	63±4	5.92±1.8	4

mean ± s.e.mean of n independent experiments.

Responses to 100 μM ACh or DA or 1 μM 5-HT were examined following pretreatment of membranes with 1 μM of the following antagonists: SCH 23390 (D_1 dopamine), eticlopride (D_2 dopamine), piflutixol and (+)butaclamol (non-selective dopamine), ketanserin (5-HT_2), methiothepin (non-selective 5-HT), propranalol (non-selective ß adrenergic) and atropine (muscarinic cholinergic). Statistical significance of antagonist effects were tested using repeated measures ANOVA followed by Dunnett's test. Atropine was the only agent to attenuate the response to ACh. Eticlopride, (+)butaclamol and piflutixol were the only agents to attenuate the response to DA ($p<0.05$). The response to 1 μM 5-HT was significantly attenuated by methiothepin ($p<0.01$) but not by ketanserin, suggesting that the 5-HT response was not being mediated by 5-HT_2 receptors. Thus the agonist responses had the appropriate pharmacological profiles.

In summary, this study describes an assay for the direct measurement of receptor-mediated stimulation of G-proteins in bovine caudate homogenates which has the advantage of being independent of any effector system.

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37P INTRAVENOUS ADMINISTRATION OF SUMATRIPTAN PRODUCES MARKED SAPHENOUS VENOCONSTRICTION IN THE ANAESTHETISED DOG MEDIATED BY 5-HT_1-LIKE RECEPTORS

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Isolated tissue studies have demonstrated the presence of vasoconstrictor 5-HT_1-like receptors in the cerebral (Parsons, 1991) and coronary arteries of various species and in canine saphenous vein (Humphrey *et al.*, 1988). In vivo, the selective 5-HT_1-like agonist, sumatriptan, produces preferential vasoconstriction of the carotid vascular bed with relatively little effect on systemic or most other regional vascular resistances (Feniuk et *al.*, 1989). The possible effects of sumatriptan on venous function in vivo have received little attention. We have performed a haemodynamic study in anaesthetised dogs which demonstrates that low intravenous (i.v.) doses of sumatriptan cause marked saphenous venoconstriction.

Chloralose-anaesthetised artificially ventilated dogs were instrumented to monitor mean aortic pressure (MAP), left ventricular end diastolic pressure (LVEDP), total peripheral resistance (TPR) and heart rate (HR). Drugs were administered into the right femoral vein. The diameter of the left lateral saphenous vein was measured continuously using sonomicrometry and saphenous vein pressure (SVP) was monitored proximally from a branch. Animals were randomized into 3 experimental groups (n=6 per group). Each animal received sumatriptan (1 - 30 $\mu g\ kg^{-1}$ i.v. ascending bolus doses) followed by either methiothepin 0.3 $mg\ kg^{-1}$ i.v., ketanserin 0.3 $mg\ kg^{-1}$ i.v. or vehicle. Following the treatment, the dose-response curve to sumatriptan was repeated.

Sumatriptan (1 - 30 $\mu g\ kg^{-1}$ i.v.) produced marked dose-related reductions in saphenous vein diameter (SVD, -43% ± 4% at 30 $\mu g\ kg^{-1}$ i.v., basal value 2728 ± 166 µm) with no change in SVP (-1 ± 2% at 30 $\mu g\ kg^{-1}$ i.v., basal value 9.9 ± 0.4 mmHg). The reductions in SVD were generally accompanied by modest and transient increases in LVEDP, TPR and MAP. The venoconstrictor potency of sumatriptan was evaluated by calculating the dose necessary to decrease saphenous vein diameter by 20% (ED_{20} SVD). Saphenous venoconstriction produced by sumatriptan was unchanged by vehicle (ED_{20} SVD control 4.7 ± 1.3 $\mu g\ kg^{-1}$ i.v., vehicle 6.0 ± 1.6 $\mu g\ kg^{-1}$ i.v.) or ketanserin treatment (ED_{20} SVD control 6.0 ± 1.7 $\mu g\ kg^{-1}$ i.v., ketanserin 8.1 ± 2.1 $\mu g\ kg^{-1}$ i.v.) but was significantly antagonised by methiothepin (ED_{20} SVD control 5.0 ± 1.9 $\mu g\ kg^{-1}$ i.v., methiothepin 71 ± 23 $\mu g\ kg^{-1}$ i.v., $p<0.05$). Methiothepin displaced the sumatriptan dose-response curve (SVD) rightwards in a parallel fashion with no apparent change in maximum effect.

Hence, in accordance with in vitro data (Humphrey *et al.*, 1988), i.v. sumatriptan produces marked saphenous venoconstriction in the anaesthetised dog. This effect is sensitive to antagonism by methiothepin but not by ketanserin, suggesting that it is mediated by activation of 5-HT_1-like receptors. With a threshold of ≈ 1 $\mu g\ kg^{-1}$ i.v. this represents one of the most potent haemodynamic properties of sumatriptan so far recorded.

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38P THE EFFECT OF FORSKOLIN, U46619 AND EXPERIMENTAL PROTOCOL ON THE 5-HT_1-LIKE RECEPTOR-INDUCED CONTRACTIONS IN THE RABBIT ISOLATED SAPHENOUS VEIN

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Many vascular 5-HT_1-like receptor-mediated contractile responses are susceptible to potentiation by elevated vascular tone (MacLennan & Martin, 1992) and are associated with inhibition of cyclic AMP formation (Sumner *et al.*, 1992; Randall *et al.*, 1994). In this study we have investigated the interaction between the thromboxane A_2-mimetic U46619, forskolin (a direct activator of adenylyl cyclase) and experimental protocol on the 5-HT_1-like responses of the rabbit isolated saphenous vein.

4 mm ring segments of rabbit saphenous vein were set up in organ baths containing Krebs-Henseleit buffer solution maintained at 37°C and gassed continuously with 95%O_2: 5%CO_2, according to the method of Martin & MacLennan (1990). Control tissues were exposed to U46619 to achieve a stable contraction equal to 30% of its maximal effect ([A_{30}]: 3-10nM) prior to the construction of a 5-HT concentration-effect (E/[A]) curve. Results are expressed as a percentage of the maximum response to 5-HT, and significance is judged if $p<0.05$ from a Student's unpaired t-test. Under basal conditions 0.3 µM forskolin virtually abolished the 5-HT E/[A] curve. The addition of U46619 increased the p[A_{50}] value (7.31±0.13 to 7.72±0.24, n=4), although this was not significant, and also did not significantly increase the asymptote (to 119.4±15.7%, n=4) of the 5-HT control curves. To investigate the effects of pre-contraction in combination with forskolin two experimental protocols were employed:

Protocol 1: tissues were exposed to [A_{30}] U46619 for 15 min followed by the addition of 0.3 µM forskolin for 15 min.

Protocol 2: 0.3 µM forskolin was incubated for 15 min before a 15 min exposure to [A_{30}] U46619 (estimated from control tissues).

Using protocol 1, forskolin relaxed pre-contracted tissues to below resting basal tension (-6.6±1.8%, n=4). Furthermore, this treatment significantly reduced both the 5-HT E/[A] curve asymptote and the p[A_{50}] value compared to U46619 alone treated tissues (119.4±15.7% to 56.1±1.0% and 7.72±0.24 to 6.81±0.04 (n=4), respectively). Using protocol 2, U46619 failed to elicit a contraction in the presence of 0.3 µM forskolin. Under these conditions 5-HT was more potent (p[A_{50}] 7.17±0.13, n=4) and more effective (aymptote 87.1±9.8%) than observed under protocol 1.

These results indicate that forskolin practically abolished 5-HT_1-like and [A_{30}] U46619-induced contractions of the rabbit isolated saphenous vein, yet a combination of these two agents partially overcomes this inhibitory effect. Surprisingly, vessel contraction by U46619 prior to forskolin (protocol 1) was less able to facilitate these 5-HT_1-like recepor-mediated contractions compared to protocol 2 (U46619 subsequent to forskolin). The biochemical basis underlying these differences is currently under investigation.

V. A. R. is a BBRSC student in collaboration with Wellcome Research Laboratories.
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39P EVIDENCE INDICATING THAT ACTIVATION OF CENTRAL 5-$HT_{2B/2C}$ RECEPTORS CAN CAUSE SYMPATHOEXCITATION IN ANAESTHETIZED RATS

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5-HT administered i.c.v. in both conscious and anaesthetized rats releases vasopressin by activating 5-HT_2 receptors (Brownfield et al, 1988; Pergola & Alper, 1991) resulting in an increase in blood pressure. The aim of the present study was to determine which subtypes of the 5-HT_2 receptor are involved in this response using the selective 5-$HT_{2B/2C}$ antagonist SB200646A (Kennett et al., 1994). The ability of 5-HT to release vasopressin was measured indirectly by monitoring the haemodynamic changes caused by 5-HT(i.c.v.) in the presence and absence of a vasopressin V_1 antagonist. These changes were compared those caused by 5-HT in the presence of SB200646A dissolved in 10% polyethylene glycol 400 (PEG).

In male Sprague-Dawley rats (250-350g) anaesthesia was induced with halothane and maintained with α-chloralose (80 mg kg^{-1}). The carotid artery and jugular vein were cannulated for the recording of mean blood pressure and heart rate and for the i.v. administration of drugs. Rats were artificially ventilated following neuromuscular blockade with decamethonium (3 mg kg^{-1}) and placed in a stereotaxic frame for i.c.v. injections (5 µl over 20s). Using a retroperitoneal approach pulse Doppler flow probes were placed around the mesenteric and renal arteries and the abdominal aorta below the ileocaecal artery (a measurement of hindquarters flow). Conductances were calculated from these measurements of flow. Drug induced changes (%) in conductance were compared by two-way ANOVA with time-matched PEG (5 µl, i.c.v.) controls. Changes were considered significant if $P < 0.05$.

5-HT (4 nmol kg^{-1}, n=5) in the presence of PEG caused an immediate and significant increase in blood pressure (17 ± 3 mmHg; mean ± s.e.mean) and significant decreases in mesenteric (-18 ± 2 %), renal (-17 ± 3 %) and hindquarters conductance (-26 ± 3 %) which were maintained for at least 5 min. Pretreatment with the V_1 antagonist [ß-mercapto-ß,ß-cyclopentamethylenepropionyl^1, O-Me-Tyr^2,Arg^8]-vasopressin, (10 µg kg^{-1}, i.v., n=6) significantly reduced the immediate rise in blood pressure (10 ± 2 mmHg) and the decrease mesenteric conductance (-11 ± 2 %). However, after 2 min the effects were similar to 5-HT alone. In the presence of SB200646A (300 nmol kg^{-1}, i.c.v., n=6) the pressor effect (8 ± 2 mmHg) and the decreases in mesenteric (-9 ± 3 %) and hindquarters (-11 ± 3 %) conductance were also significantly attenuated over the complete 5 min period. The decrease in renal conductance (-5 ± 2 %) was also significantly attenuated in the first min. PEG alone had the same effect as SB200646A alone which caused an immediate and significant increase in blood pressure (5 ± 2 mmHg) and significant decreases in renal (-9 ± 3%) and hindquarters conductance (-6 ± 3%).

These results show that both SB2000646A and the V_1 receptor antagonist attenuate the mesenteric vasoconstriction whilst SB200646A also attenuates the hindquarters vasoconstriction evoked by i.c.v. 5-HT. This latter observation suggests that activation of central 5-$HT_{2B/2C}$ receptors causes central sympathoexcitation. Therefore in these experiments attenuation of mesenteric vasoconstriction by SB2000646A could be due to blockade of this sympathoexcitatory effect rather than the release of vasopressin.

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40P THE EFFECTS OF CHRONIC NITRIC OXIDE SYNTHASE INHIBITION ON VASOREACTIVITY OF RAT MESENTERIC RESISTANCE ARTERIES

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Chronic inhibition of nitric oxide synthase (NOS) by N^G-nitro-L-arginine methyl ester (L-NAME) induces a sustained hypertension in normotensive rats. This study was conducted to investigate the effects of chronic L-NAME induced hypertension on vascular reactivity in mesenteric resistance arteries.

Male Wistar rats (120 to 130 g) were given L-NAME (50 mg/ 100 ml) in the drinking water for 3 weeks. This insured a daily intake of L-NAME of 50 mg/kg (Arnal et al., 1993). In the control group rats were given tap water. At the end of the treatment period rats were anaesthetised (pentobarbital 50 mg/kg i.p.) and the carotid artery was cannulated in order to measure blood pressure. After the blood pressure measurement, heparin 1000 i.u./kg was injected through the cannulae and mesenteric artery segments, approximately 200 µm in external diameter were then isolated. Two mm segments of mesenteric artery were mounted for isometric tension recording in a myograph. Vascular reactivity was assessed in response to KCl (125mM), phenylephrine (PE)(10^{-6}M-3x10^{-5}M), acetylcholine (ACh)(10^{-9}M-10^{-6}M) or sodium nitroprusside (SNP)(10^{-9}M-10^{-7}M). Responses to ACh and SNP were examined following preconstriction with an EC_{50} concentration of PE.
Contractile responses are expressed in mN/mm3, relaxation responses are expressed as a percentage of the PE induced tone. The results are presented as mean ± s.e. mean, n= number of rats. EC_{50} and E_{MAX} were calculated individually for each concentration response curve. Statistical analysis was performed by ANOVA, differences were considered to be significant when $p<0.05$. Dunnets test was used for comparisons where appropriate.

Mean arterial blood pressure was significantly elevated in the L-NAME treated group (173±2.6mmHg (n=20) vs. control 121± 2.6mmHg (n=15)). Vascular reactivity of mesenteric arteries was significantly affected following chronic treatment with L-NAME. Maximum contractile responses to KCl (L-NAME : 170±12.5, n=10; control : 257±21, n=13) and PE (L-NAME : 168±24, n=10; control : 295±19, n=13) were significantly reduced, the EC50 for PE was not significantly affected (L-NAME : 2.4± 0.5µM, n=10; control : 2.1±0.3µM, n=13). A significant decrease in sensitivity to ACh was observed following chronic treatment with L-NAME (EC_{50} - L-NAME : 98± 21nM, n=10; control : 45± 10nM, n=13) with the maximum relaxation unaffected, the magnitude of this effect is similar to the acute effect of Nω-nitro-L-arginine (L-NNA) in this tissue. Addition of indomethacin (10^{-5}M) did not affect the response to ACh. Sensitivity to SNP was significantly enhanced following chronic treatment with L-NAME (EC_{50} - L-NAME : 2.2± 0.6nM, n=6; control : 37± 8nM, n=8).

These results demonstrate that continuous NO blockade, which suppresses a continuous relaxing stimulus inducing an increase in vasoconstrictor tone, leads to a compensatory down-regulation of the contractile apparatus. The relatively small reduction in ACh sensitivity confirms the role of non-NO dependent relaxation in small vessels. The increase in sensitivity to SNP probably reflects the hypo-stimulated state of the NO/cGMP pathway.

FJD is a Wellcome Travelling Research Fellow.

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41P INDUCTION OF NITRIC OXIDE RELEASE BY DIFFERENT GRAM-POSITIVE BACTERIA IN MURINE MACROPHAGES AND RAT AORTIC SMOOTH MUSCLE CELLS

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There is little information regarding the mechanism by which gram-positive organisms induce septic shock. Like endotoxin, lipoteichoic acid from the cell wall of *Staphylococcus aureus* induces the release of cytokines and nitric oxide *in vitro* (Cunha *et al.*, 1993) and *in vivo* (De Kimpe *et al.*, 1995). However, the cell wall of gram-positive organisms is very heterogeneous and contains more components able to innitiate an inflammatory response (see: Bone, 1993). Moreover, the chemical structure of these cell wall components can vary considerably among different species. Here, we investigate the ability of different gram-positive organisms (Group A Streptococcus, *Staphylococcus aureus, Staphylococcus epidermis, Streptococcus pneumoniae*) to induce the release of nitric oxide in cultured macrophages (J774.2) and rat aortic smooth muscle cells (RASM).

The gram-positive organisms (isolates from septic patients) were grown to stationary phase, washed in saline and killed by boiling for 15 min followed by sonication for 1 min. J774 macrophages and RASM were cultured to confluency in 96-well plates with 200 µl of culture medium (DMEM or RPMI 1640, respectively). Cells were stimulated with the different killed gram-positive bacteria in the presence of interferon-γ (IFNγ, 10U/ml). After 24 h (J774.2) or 40 h (RASM) nitrite accumulation, as an indicator of nitric oxide synthesis, was assayed using the Griess reaction.

Gram-positive organisms induced the release of nitrite by J774.2 in the following potency order: Group A streptococcus > *S. aureus* = *S. epidermis* > *S. pneumoniae* (Table 1). Costimulation with IFNγ, greatly enhanced the release of nitrite caused by these micro-organisms. In RASM, gram-positive organisms (in the presence of IFN-γ) also induced a significant release of nitrite, whereby the staphylococci were more potent than the streptococci (Table 1).

In summary, different species of gram-positive organisms (without endotoxin) induce the release of nitrite in J774.2 and RASM, presumably due to induction of NO synthase.

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Table 1. Nitrite release (µM) in cultured J774.2 macrophages or rat aortic smooth muscle cells (RASM) activated by gram-positive organisms.

Cells	Number of bacteria	IFNγ	Group A Streptococcus	*S. aureus*	*S. epidermis*	*S. pneumoniae*
J774.2	10^8/ml	-	29 ± 4	15 ± 4	16 ± 4	5 ± 3
	10^7/ml	+	45 ± 4	36 ± 2	35 ± 3	25 ± 3
	10^8/ml	+	54 ± 5	54 ± 4	50 ± 3	50 ± 6
RASM	10^8/ml	+	27 ± 6	49 ± 4	43 ± 6	27 ± 7

mean ± sem of at least three experiments in triplicate.

42P THE LONG-LASTING VASODILATOR ACTION OF CORTICOTROPHIN RELEASING HORMONE IS ENDOTHELIUM-DEPENDENT IN THE RAT PERFUSED MESENTERY

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Systemic administration of corticotrophin releasing hormone (CRH) or the structurally homologous peptides, urotensin I and sauvagine, produce a long lasting fall in blood pressure (Corder *et al.*, 1992; Gardiner *et al.*, 1988). This appears to be a direct vascular effect as CRH has a long lasting vasodilator action in the isolated rat heart (Grunt *et al.*, 1993). But the mechanism of this effect is unclear. L-NAME and indomethacin reduced the response in the coronary circulation (Grunt *et al.*, 1993), and receptor autoradiography of the rabbit aorta has shown ^{125}I-CRH to bind mainly to the endothelium (Dashwood *et al.*, 1987), but other studies have reported CRH-induced vasodilatations to be endothelium-independent (Lei *et al.*, 1993). Here we have studied the mechanism of action in the perfused rat mesentery as this is a major site of action *in vivo* (Gardiner *et al.*, 1988).

Male Wistar rats (200-400 g) were anaesthetised with pentobarbitone. The mesenteric circulation was isolated and perfused at 5 ml. min^{-1} with warmed (37°C) and gassed (95% O_2/5% CO_2) Krebs' solution. The mesenteric vessels were preconstricted with methoxamine (50 µM), and endothelium-dependent and independent vasodilator responses were tested by bolus injections of acetylcholine (ACh, 100 pmol) and sodium nitroprusside (SNP, 1 nmol) respectively. Dose response curves to CRH were constructed by bolus injection (1 pmol-1 nmol). Subsequently a single dose of CRH (100 pmol) was used to compare responses in control preparations with those in which the endothelium was removed (10-15 s infusion with deoxycholic acid, DOA, 5 mM), or NO synthesis was inhibited with L-NAME (100 µM).

Both DOA and L-NAME caused significant reductions in the vasodilator responses to ACh, and increases in those to SNP. The size of the response to CRH was reduced by both treatments, and the prolonged phase (5-20 min) was largely abolished (Table 1). Thus, CRH acts both directly and indirectly on vascular smooth muscle to cause vasodilatation, with the sustained phase being mediated by NO release from the endothelium.

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Table 1 % reduction in perfusion pressure (mean ± sem, n = 7 - 9 per group, * = $p < 0.05$ compared to control)

			Time after CRH injection (min)							
	ACh	SNP	1	2	3	4	5	10	15	20
Control	48.3±4.0	32.9±4.1	22.1±5.4	28.3±4.8	27.5±3.7	25.9±3.3	24.6±3.2	19.2±4.1	19.5±4.0	19.0±4.8
DOA	2.6±2.6*	70.6±6.4*	12.8±4.6	13.8±3.9*	11.1±3.4*	10.1±2.9*	6.5±1.9*	3.0±0.9*	1.6±0.9*	2.2±1.1*
L-NAME	15.7±4.4*	50.1±8.9	19.0±3.2	17.0±4.0	13.7±4.2*	10.2±3.8*	7.8±3.3*	0.2±2.1*	-1.9±1.2*	-2.2±2.0*

43P ATTENUATION OF HYPERTENSION AND *IN VITRO* NORADRENERGIC VASCULAR REACTIVITY BY NITRIC OXIDE SYNTHASE INHIBITION IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Altered reactivity of vascular smooth muscle and impaired endothelium-dependent relaxation may be involved in development of diabetic vascular complications (Tomlinson et al, 1992). This study examined the effects of (i) streptozotocin (STZ)-induced diabetes (insulin-treated and untreated) and (ii) *in vivo* administration of the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) on mean arterial pressure (MAP) and vascular reactivity to noradrenaline (NA).

Six groups of male Wistar Han rats were studied: untreated and L-NAME treated non-diabetic (ND), STZ-diabetic (DIA) and insulin-treated DIA (DIA-I). Diabetes was induced by intravenous injection of STZ (70 mg/kg). Insulin (2 U/d s.c.) and L-NAME (23±1 mg/kg/d p.o.) treatments were commenced after confirmation of diabetes and continued for 4 weeks. The carotid artery was then cannulated for direct measurement of MAP. Mesenteric arteries were isolated under anaesthesia and intralumenally perfused as described by McGregor (1965) and modified by Douglas and Hiley (1990). Dose-responses to NA (0.2 - 20 μM) were performed.

Mean plasma glucose and glycated haemoglobin levels were elevated ($p<0.001$) in DIA and DIA[L-NAME] rats. Insulin treatment reduced ($p<0.01$) but did not normalise ($p<0.01$) these parameters (Table 1). MAP was not significantly different between ND, DIA and DIA-I groups. L-NAME increased MAP in all three groups ($p<0.01$) although the degree of hypertension was lower ($p<0.01$) in the two diabetic groups. There was no difference in the maximal response above basal perfusion pressure (MAX) to NA in the three untreated groups. DIA rats demonstrated a significant increase ($p<0.05$) in sensitivity to NA which was absent in the DIA-I group. The increased MAX following L-NAME treatment was significantly greater for non-diabetic ($p<0.001$) than diabetic rats, irrespective of insulin treatment ($p<0.01$). L-NAME increased NA sensitivity in ND rats ($p<0.001$) but had no effect on sensitivity in the diabetic groups.

This study demonstrates an increased sensitivity to NA in DIA rats. The lack of effect on vascular sensitivity to NA, the smaller increase in maximal response, and reduced hypertension as a result of L-NAME treatment in the DIA groups in comparison to ND rats suggests that NO production and/or activity is reduced in diabetes. These factors may be important in the development of diabetic vascular complications.

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Table 1. Biochemical, physiological and pharmacological characteristics of animal groups studied. Values represent mean ± SEM.

Group	n	Plasma glucose conc. (mmol/l)	Glycated haemoglobin (%)	MAP (mmHg)	MAX (mmHg)	EC50 (μmol/l)
ND	14	9.3±0.5	3.0±0.1	125±4	127±9	1.53±0.15
DIA	11	40.4±3.4	15.9±0.9	120±4	140±13	1.12±0.11
DIA-I	13	26.2±3.4	10.1±0.8	120±5	142±10	1.82±0.36
ND[L-NAME]	9	9.1±0.7	3.2±0.2	172±4	198±9	0.78±0.13
DIA[L-NAME]	9	39.6±2.0	18.6±0.7	150±6	188±8	0.96±0.16
DIA-I[L-NAME]	10	21.8±3.8	9.4±1.0	148±4	185±11	1.08±0.13

44P THE DILATOR ACTION OF CAPSAICIN IN THE CORONARY CIRCULATION OF THE RABBIT IS MEDIATED BY NITRIC OXIDE

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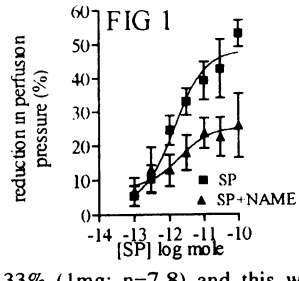
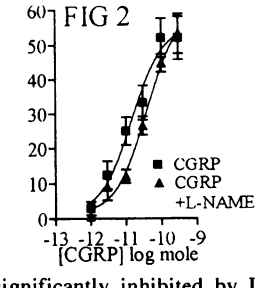
Calcitonin gene-related peptide (CGRP) and substance P (SP) are located in perivascular sensory neurones and are potent dilators of the human coronary circulation *in vivo* (McEwan *et al* ,1986. Crossman *et al*, 1989). SP acts via the release of nitric oxide (NO)from the endothelium, but CGRP-induced vasodilation has been described as both dependent and independent of NO.

In this study we used isolated, perfused rabbit hearts to determine the involvement of NO in the coronary vascular responses to SP, CGRP, and the sensory nerve stimulant capsaicin. Hearts removed from male or female New Zealand White rabbits were immediately suspended and perfused via the aorta with a constant flow (35ml/min) of warmed (37°C), and gassed (95% O_2, 5% CO_2) Kreb's buffer. Coronary perfusion pressure (CPP) and left ventricular pressure (LVP) were recorded using pressure transducers connected to the aortic cannula and a latex balloon in the left ventricle. Following equilibration, CPP was raised using a constant infusion (1-10nM) of the thromboxane mimetic U46619. In some experiments the specific NO synthase inhibitor L-NAME (N^Gnitro-L-arginine methyl ester;100μM) or the CGRP antagonist $CGRP_{8-37}$ (100nM) were included in the perfusate. The CPP was then adjusted to ~120mmHg by titration of U46619. The effects of saline vehicle, human CGRP ($1x10^{-13}$-$1x10^{-10}$moles), SP ($1x10^{-13}$-$1x10^{-10}$), sodium nitroprusside (SNP; $1x10^{-10}$-$1x10^{-7}$) and capsaicin (CAPS; 0.01-1mg in ethanol) on CPP were recorded after injection (10-30μl) via the aortic cannula.

U46619 infusion increased basal CPP from 49±3mmHg (mean ±SEM;n=16) to 123±6 mmHg and altered the maximum SNP-induced reduction in CPP from 17±2% to 49±10%. CGRP and SP produced similar dose-dependent reductions in CPP (Figures 1&2) with maximum effects of 52±2% and 55±3% respectively. L-NAME infusion increased basal CPP from 49±3 to 74±8mmHg ($p\leq0.003$, n=10-16; t-test). L-NAME did not alter dilator responses to SNP but significantly inhibited the effect of SP (Figure 1) and partially inhibited the response to CGRP at the $1x10^{-11}$ mole dose (Figure 2; $p\leq0.01$). CAPS reduced CPP by





33% (1mg; n=7-8) and this was significantly inhibited by L-NAME to a 11% reduction in CPP ($p\leq0.01$). At concentrations which caused the same reduction in CPP, SP ($3x10^{-12}$ moles;n=6) but not CGRP ($1x10^{-11}$ moles;n=7) was significantly inhibited by L-NAME. The dilator effects of CAPS and CGRP were reduced by 46±10% and 54±8% respectively by infusions of $CGRP_{8-37}$ (n=3) whereas the response to SP was unaffected.

These results suggest that NO contributes significantly to coronary dilation following capsaicin stimulation of sensory nerves. Substance P-induced dilation is mediated post-synaptically by NO. CGRP appears to act mainly through NO-independent mechanisms, although NO may modulate sensory neuropeptide release (Hughes & Brain, 1994).

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45P HAEMODYNAMIC EFFECTS OF THE NON-SELECTIVE ENDOTHELIN ANTAGONIST, SB 209670, IN CONSCIOUS, TRANSGENIC (TGR (mRen-2) 27) HYPERTENSIVE RATS

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Introduction of the mouse Ren-2 gene into the rat genome produces animals (TGR (mRen-2) 27) with hypertension (Mullins *et al.*, 1990). Since there is evidence for increased vascular formation of AII in these transgenic rats (Hilgers *et al.*, 1992), and since AII up-regulates endothelin gene expression and endothelin release (Imai *et al.*, 1992), we investigated the effects of the endothelin antagonist, SB 209670 (Ohlstein *et al.*, 1994), in male, heterozygous transgenic rats. Rats (4-5 months old; 400-500g) were instrumented with pulsed Doppler flow probes and intravascular catheters (all surgery under sodium methohexitone anaesthesia, 40 mg kg^{-1} i.p., supplemented as required. Animals (n=7) received a continuous i.v. infusion of saline (0.4 ml h^{-1}) for 8 h or SB 209670 (0.6 mg kg^{-1} h^{-1}) for 8 h (n=7; 5 of these animals had received saline on the previous day); we measured heart rate (HR), mean blood pressure (BP), renal, mesenteric and hindquarters flow (RF, MF, HF, respectively), and calculated renal, mesenteric and hindquarters vascular conductance (RC, MC, HC, respectively). Some of the results are summarised in Table 1.

After infusion of saline for 8 h there were no significant changes in cardiovascular variables. However, during infusion of SB 209670 there was progressive hypotension and increases in renal, mesenteric and hindquarter flows and vascular conductances. These observations raise the possibility that endothelin is involved in the maintenance of hypertension in heterozygote TGR (Ren-2) 27 animals.

Thanks to Dr E. Ohlstein for the gift of SB 209670.

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Table 1. Cardiovascular variables during infusion of saline or SB 209670 in transgenic rats. Values are mean ± s.e. mean; * P<0.05 versus baseline (Friedman's test).

	Baseline		1 h		4 h		8 h	
	Saline	SB 209670	Saline	SB 209670	Saline	SB 209670	Saline	SB 209670
HR (beats min^{-1})	341 ± 10	339 ± 12	339 ± 9	360 ± 14	354 ± 9	347 ± 13	346 ± 12	359 ± 14
BP (mm Hg)	156 ± 9	159 ± 3	160 ± 7	152 ± 5	153 ± 7	137 ± 3*	151 ± 8	126 ± 2*
RF (kHz)	5.2 ± 0.2	5.8 ± 0.4	5.3 ± 0.3	6.7 ± 0.4*	5.4 ± 0.3	6.8 ± 0.4*	5.7 ± 0.4	6.7 ± 0.4 *
MF (kHz)	6.2 ± 0.8	5.5 ± 0.4	5.4 ± 0.5	5.5 ± 0.6	5.6 ± 0.6	5.7 ± 0.5	5.6 ± 0.5	6.4 ± 0.5*
HF (kHz)	3.9 ± 0.4	3.6 ± 0.2	3.9 ± 0.4	4.3 ± 0.4*	3.9 ± 0.3	4.5 ± 0.4*	4.0 ± 0.4	4.9 ± 0.4*
RC (kHz mm Hg^{-1} 10^3)	34 ± 3	37 ± 3	34 ± 3	45 ± 4*	36 ± 3	50 ± 3*	39 ± 4	53 ± 4*
MC (kHz mm Hg^{-1} 10^3)	42 ± 7	35 ± 3	35 ± 4	36 ± 4	38 ± 5	42 ± 3	38 ± 4	51 ± 4*
HC (kHz mm Hg^{-1} 10^3)	27 ± 4	23 ± 1	25 ± 4	29 ± 3*	27 ± 4	33 ± 3*	27 ± 4	40 ± 4*

46P NORADRENALINE RELAXES PRECONTRACTED RAT SMALL MESENTERIC ARTERIES IN THE PRESENCE OF α_1-ADRENOCEPTOR BLOCKADE VIA AN ENDOTHELIUM-INDEPENDENT PATHWAY

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Noradrenaline (NA) is generally accepted to contract resistance arteries by acting at α-adrenoceptors. However, recently we showed that prazosin and other selective α_1-adrenoceptor antagonists do not produce the expected behaviour for simple competitive antagonism in the rat small mesenteric artery (s.m.a.; Van der Graaf *et al.*, 1995). Analysis of the steep Schild plots led us to propose that NA can also relax this tissue. We now report that NA does indeed inhibit the contraction of the s.m.a. to 5-HT and the thromboxane A_2 mimetic, U46619, via an endothelium-independent pathway.

S.m.a.'s (internal diameter 100-300μm) from male Wistar rats (225-300g) were mounted as 2mm ring segments in a myograph (37°C, gassed with 95%O_2/5%CO_2) as described by Mulvany & Halpern (1977). The endothelium was removed, as confirmed by the lack of relaxant response to 10μM of the acetylcholine M-receptor agonist, 5-methylfurmethide, after precontraction with 10μM NA. In the presence of 100nM prazosin, vessels were precontracted with 1μM 5-HT which produced a sustained response of 83±4% (n=6) compared to the response to 10μM NA. Cumulative addition of 100nM-10μM NA had no significant effect on this response and higher concentrations caused an increase in tension. The presence of a 10-fold higher concentration of prazosin (1μM) did not significantly change the response to 1μM 5-HT (80±3% compared to 10μM NA, n=6). However, 1-100μM NA now caused significant inhibition of the 5-HT response (Table 1). The relaxation appeared not to be mediated via β_1/β_2- or α_2-adrenoceptors or by stimulation of the synthesis of nitric oxide, because timolol (10μM), idazoxan (1μM) and L-N^G-nitro-arginine methyl ester (L-NAME, 100μM), respectively, had no significant effect on the location and maximum of the NA relaxation curve (ANOVA: P>0.1; Table 1). The response to 100nM U46619, which produced a contraction of 69%±7% compared to 10μM NA (n=5), was also inhibited by NA in the presence of 1μM prazosin, 10μM timolol, 1μM idazoxan and 100μM L-NAME (maximum relaxation=51± 7%, $p[A]_{50}$=5.2±0.1), suggesting that the inhibition of the 5-HT response was not due to blockade of 5-HT receptors by NA. In contrast, 10nM-100μM NA caused no significant relaxation after precontraction with 75mM KCl in the presence of 1μM prazosin, 10μM timolol, 1μM idazoxan and 100μM L-NAME (maximum relaxation =10±6% and 5±3% for NA and vehicle, respectively; n=3, P>0.5). We conclude that NA stimulates an endothelium-independent inhibitory pathway in the rat s.m.a. The mechanism of this action has still to be characterised.

Table 1
NA relaxation of s.m.a. after precontraction with 1μM 5-HT

treatment(μM)	$p[A]_{50}$	maximum relaxation (%)	n
control	4.9±0.1	53±12	6
timolol (10)	5.1±0.1	61±9	4
idazoxan (1)	5.1±0.1	65±10	4
L-NAME (100)	5.1±0.1	79±5	5

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47P MODULATION OF FACIAL SKIN BLOOD FLOW RESPONSES EVOKED BY TRIGEMINAL GANGLION STIMULATION IN ANAESTHETISED RATS

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Electrical stimulation of the trigeminal ganglion leads to an increase in rat facial skin blood flow, which is selectively inhibited by the calcitonin gene-related peptide (CGRP) antagonist, $CGRP_{8-37}$ (Escott *et al*, 1995). Recent research has suggested that several pharmacological agents can inhibit the release of neuropeptides from sensory nerves. In this study, we investigated the action of some of these agents on facial skin blood flow responses evoked by trigeminal ganglion stimulation. Male Wistar rats (180-200g) were pretreated 24 h prior to trigeminal ganglion stimulation with guanethidine 20 mg/kg, s.c. (Escott *et al*, 1995). In anaesthetised and paralysed rats, stimulation of the trigeminal ganglion (5 Hz, 10 V, 1 ms for 30 s) led to an increase in facial skin blood flow, measured as arbitrary units of flux by laser Doppler flowmetry (Moor MDF3D). Two responses to electrical stimulations were obtained with a 30 min interval between stimulations (1st increase 106.7 ± 14.8 flux and 2nd 112.3 ± 15.8 flux, n = 6). Vehicle or test agents were administered i.v. 10 - 15 min prior to the second stimulation. Results are expressed as % inhibition (mean ± s.e.mean) of the peak increase in flux, of the second compared to the first stimulation. Comparisons between treatments were made using Student's t-test or ANOVA followed by Bonferroni's modified t-tests and statistical significance was accepted when $p < 0.05$.

[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-Enkephalin (DAGO), a μ-opioid receptor agonist, modulates sensory nerve responses in the rat hind paw (Barber, 1993) and in this study caused a dose dependent inhibition of the blood flow response to trigeminal ganglion stimulation (0.058 to 2 μmol/kg, 1.3 ± 6.5 to 42.9 ± 8.8 %). The inhibitory effect of DAGO at 2 μmol/kg was significantly reversed by naloxone, 2.7 μmol/kg, from 42.9 ± 8.8 % inhibition (n = 6) to 3.6 ± 5.7 % (n = 4, $p < 0.01$). Neither saline nor naloxone alone had any effect on the second response when compared to the first (-5.3 ± 2.2 % and -0.9 ± 3.3 % inhibition respectively, n = 4 - 6, $p > 0.05$). Furthermore, the H_3-histamine receptor agonist α-methylhistamine (up to 35 μmol/kg, Matsubara *et al*, 1992), the $5\text{-}HT_{1D}$ receptor agonists sumatriptan (up to 2.4 μmol/kg) and CP122,288 (up to 3 μmol/kg, Lee & Moskowitz, 1993), nor the appropriate vehicles had any significant effect on the increase in facial blood flow induced by trigeminal ganglion stimulation (n = 4 - 6 in each group, $p > 0.05$). In conclusion, facial skin vasodilatation evoked by trigeminal ganglion stimulation is attenuated by a μ-opioid receptor agonist, but not by H_3 or $5\text{-}HT_{1D}$ receptor agonists.

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48P MULTIPLE ACTIONS OF ANGIOTENSIN II ON NORADRENERGIC NERVES OF CAUDAL ARTERIES OF NORMOTENSIVE AND HYPERTENSIVE RATS

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The receptor(s) subserving the facilitatory action of angiotensin II (AII) on transmitter noradrenaline (NA) release were investigated in caudal arteries from Sprague-Dawley (SD), Wistar-Kyoto (WKY) and spontaneously hypertensive (SH) rats. We attempted to characterise the receptors using the selective AII receptor antagonists losartan (AT_1) and PD 123319 (AT_2/AT_{1B}).

Caudal artery segments were labelled with [^{3}H]-NA and perfused and superfused with physiological salt solution (4 ml/min). Transmitter release was evoked by two periods of field stimulation (5 Hz, 30 s) given 30 min apart. The perfusate/superfusate was collected over 1-min periods and [^{3}H]-NA was chromatographically separated and measured by liquid scintillation counting. The stimulation-induced (S-I) efflux of [^{3}H]-NA for the second period of stimulation was expressed as a percentage of that with the first period (% S_2/S_1).

In control experiments, % S_2/S_1 was 96 ± 7 % (n = 7), 95 ± 7 % (n = 5) and 95 ± 7 % (n = 8) in preparations from SD, WKY and SH rats respectively. AII produced concentration-dependent increases in S-I efflux from preparations of all three strains. The maximal effects were observed with 1 μM AII, the mean values of % S_2/S_1 being increased to 152 ± 7 % (n = 6), 159 ± 7 % (n = 3) and 125 ± 10 % (n = 10) in preparations from SD, WKY and SH rats respectively. The enhancement by AII of S-I efflux from preparations of each strain was antagonised by losartan and PD 123319. Thus, with preparations from WKY and SH rats, the effect of 1 μM AII was abolished by 0.01 μM losartan and PD 123319. In arteries from SD rats, a 10-fold higher concentration of PD 123319 was required to abolish the effect of 1 μM AII. We have previously suggested that the facilitatory action of AII on NA release in caudal arteries from SD rats, involves AT_{1B} receptors (Cox *et al.*, 1995). The present findings indicate that the same receptor subtype subserves the facilitatory action of AII in caudal arteries from WKY and SH rats.

In arteries of all three strains there were additional and unexpected interactions between AII and the two receptor antagonists. Thus, in SD and WKY arteries, combination of 0.1 μM AII (which alone, was without effect on S-I efflux) and 0.01 μM losartan enhanced S-I efflux. The mean values of % S_2/S_1 were increased to 156 ± 5 % (n = 8) and 155 ± 4 % (n = 3) in SD and WKY artery preparations respectively. Combination of 0.1 μM AII and PD 123319 (either 0.01 or 0.1 μM) were without effect on S-I efflux from SD and WKY artery preparations.

In contrast to the findings with arteries from the two normotensive strains, in SH rats, combination of 0.1 μM AII with losartan (either 0.01 or 0.1 μM) did not alter S-I efflux. However, combination of 0.1 μM AII and PD 123319 enhanced S-I efflux, the mean value of % S_2/S_1 being increased to 128 ± 7 (n = 4).

The findings of interactions between the subthreshold concentration of AII with both losartan and PD 123319 suggest that, in addition to its well-known enhancing effect, AII exerts an inhibitory action on transmitter NA release, which is normally masked by the enhancing effect. Moreover, it appears that in SD and WKY caudal arteries, the putative inhibitory effect is subserved by AT_1 receptors, whereas in caudal arteries from SH rats, the inhibitory effect may be subserved by AT_2 receptors.

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49P VASCULAR RESPONSES OF AORTIC RINGS FROM WATANABE HERITABLE HYPERLIPIDAEMIC RABBITS AFTER EXPOSURE TO A FREE RADICAL GENERATING SYSTEM

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Atherosclerosis is likely to be a consequence of interaction between vascular endothelial and smooth muscle function with both environmental and genetic factors contributing to its development. We investigated the effects free radical (FR) exposure on vascular responses in an animal model of atherosclerosis - the heterozygous Watanabe heritable hyperlipidemic rabbit (WHHL) (Dowell et al, 1993a). Aortic rings from 3-4 months or 18-24 months old WHHL (plasma cholesterol 2.6 ± 0.8mM) and age matched New Zealand white rabbits (NZW) (plasma cholesterol 0.8 ± 0.4mM) were exposed to a xanthine oxidase/hypoxanthine free radical generating system (Dowell et al, 1993b). Vehicle/time control (V) rings from each animal were studied in parallel. Cumulative dose response curves to phenylephine (P) carbachol (C) and sodium nitroprusside (S) (10^{-8} - 10^{-5}M) were constructed before and after FR/V treatment. The effect of treatment (initial response - response after treatment) was calculated for each dose of each agonist in each ring and analysis of variance used to investigate genetic strain (WHHL vs NZW), treatment (V vs FR), dose and tissue effects; 2 and 3 way interactions between strains, treatment and dose were examined followed by Bonferroni multiple comparisons to identify specific differences. Differences between WHHL and NZW at selected doses of the agonists are shown in the table.

In general, treatment had a smaller effect on responses in WHHL as indicated by - sign in the table. A significant difference between WHHL and NZW in the vehicle/time effect was observed with P and C particularly at the upper end of the dose response curves. For S, any effect was smaller and occurred at the lower end of the dose response curve. FR treatment also appeared to have a smaller effect on responses in WHHL, but much of this could be accounted for by the vehicle/time effect. Overall, tissues from WHHL were more robust, maintaining responses better in the experimental setting. This did not appear to be related to development of atherosclerosis as similar observations were made in 3-4 month and 18-24 month animals. It is possible that differences in the L arginine/nitric oxide metabolising and/or free radical scavenging enzymes contribute to the better maintenance of responses in WHHL compared to NZW.

Dowell FJ, Hamilton CA, Lindop GBH, Jardine E, Reid JL. Br J Pharmac, 108: 39P; 1993a.
Dowell FJ, Hamilton CA, McMurray J, Reid JL. J Cardiovasc Pharmacol, 22(6): 792-797; 1993b.

Differences in the effect of treatment on agonist responses between WHHL and NZW

		3 - 4 months			18 - 24 months		
Dose (M)		$3x10^{-8}$	$3x10^{-7}$	$3x10^{-6}$	$3x10^{-8}$	$3x10^{-7}$	$3x10^{-6}$
Agonist	Treatment						
C	V	-0.7(2.8)	-18.8(4.0)*	-15.2(4.4)*	0.9(3.0)	6.0(4.5)	-3.2(3.9)
	FR	1.6(2.0)	-17.1(5.0)*	-6.0(6.0)	0.7(2.7)	-1.2(5.4)	-18.0(6.0)*
P	V	-6.9(3.8)	-15.0(5.2)*	-7.1(3.6)	-0.3(1.6	-13.4(3.4)*	-12.7(3.9)*
	FR	-7.1(3.5)	-11.1(4.7)	-5.9(4.4)	-1.6(2.3)	-4.1(3.6	-3.9(3.7)
S	V	-16.6(5.5)*	-7.5(3.3)	-0.1(1.4)	-6.8(5.1)	9.5(5.4)	8.3(2.6)
	FR	-10.2(6.0)	1.7(3.6)	-0.2(2.8)	-10.8(5.7)*	0.5(4.6)	6.8(2.9)

Standard deviations are given in brackets. * highlights significant differences between WHHL and NZW. (p=0.05).
Each group contains 5-10 vessels from 4-8 animals. C & S % relaxation, P g tension/100 mg tissue.

50P AGONIST-STIMULATED ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES AND DNA SYNTHESIS IN CULTURED PULMONARY ARTERY FIBROBLASTS

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Excess proliferation of pulmonary artery fibroblasts occurs in disease states such as pulmonary hypertension. Proliferation may be stimulated by various mediators released from cells in the blood or lungs (Stenmark *et al.*, 1988). A family of intracellular proteins called mitogen activated protein (MAP) kinases have been implicated in cellular responses in several cell types, including the regulation of proliferation and differentiation (Thomas, 1992). MAP kinases are activated by dual phosphorylation on tyrosine and threonine residues following stimulation (Anderson *et al.*, 1990). The present work assesses whether acute agonist-stimulated activation of the 42 and 44kDa MAP kinases (p42 and p44) correlates with DNA synthesis in cultured bovine pulmonary artery fibroblasts (BPAFBs).

BPAFBs cultured from primary explants were maintained in Delbeccos Modified Eagles Medium (DMEM) supplemented with 10% newborn calf serum (NCS) and utilised between passages 3-10. Cells prior to stimulation were quiesced in serum-free DMEM for 48h. Agonist-stimulated MAP kinase tyrosine phosphorylation and mobility shift bands were determined by Western blotting with anti-MAP kinase and anti-phosphotyrosine antibodies (Affiniti Research Products Ltd) respectively. MAP kinase activity was measured by *in vitro* [γ-^{32}P]ATP phosphorylation of an EGF receptor peptide pseudosubstrate (BIOTRAK MAP kinase assay kit, Amersham). DNA synthesis was assessed by incorporation of [^{3}H]thymidine during the final 4h of 24h exposure to agonist.

Platelet derived growth factor (PDGF), thrombin and the protein kinase C (PKC)-activator 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulated sustained tyrosine phosphorylation of both p42 and p44 MAP kinases in both a concentration and time-dependent manner. These responses correlated with *in vitro* MAP kinase activity (30ng ml^{-1} PDGF at 10min: 5.8±0.1 fold stimulation, 300nM thrombin at 30min: 4.8±0.6 fold, 100nM TPA at 10min: 3.6 ±0.4 fold). These agonists also stimulated concentration-dependent increases in [^{3}H]thymidine incorporation (control: 1426±328 dpm/well, 30ng ml^{-1} PDGF: 39617±8739 (28 fold stimulation), 300nM thrombin: 39860± 8219 (28 fold), 100nM TPA: 10098±2425 (7 fold)). Endothelin-1 (3nM-300nM) did not stimulate MAP kinase activation or [^{3}H]thymidine incorporation. Pretreatment of cells with 1μM TPA for 48h (48hPT) downregulated immunodetectable PKC α and ε isoforms, and abolished 100nM TPA-stimulated incorporation of [^{3}H]thymidine (control: 777±71 dpm/well, 48hPT: 1026±65, TPA: 13819±2877, TPA+48hPT: 1152±168). TPA pretreatment attenuated 30ng ml^{-1} PDGF-stimulated DNA synthesis (PDGF: 41260±5195, PDGF+48hPT: 27162±3388) but did not effect [^{3}H]thymidine incorporation induced by 100nM thrombin (thrombin: 23133±3806, thrombin+48hPT: 27088±4341). All values above represent mean± s.e.mean for at least 3 independent experiments.

These results suggest that activation of MAP kinases may be involved in mitogenic signalling by PDGF, thrombin and TPA in BPAFBs. The data does not provide evidence for a major role of PKC in regulating growth messages by PDGF and thrombin.

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