1P EVIDENCE THAT NO MAY CONTRIBUTE TO "EDHF-LIKE" RESPONSES IN RAT MESENTERIC AND HEPATIC SMALL ARTERIES

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Endothelium-dependent dilatation, in the presence of nitric oxide synthase (NOS) and cyclooxygenase (COX) inhibition, has been attributed to endothelium-derived hyperpolarizing factor (EDHF). The identity of EDHF is controversial, but it is thought that the release and effects of EDHF involve modulation of K^+ flux. Release of EDHF appears to be charybdotoxin (ChTx) and apamin-sensitive (blockers of large and small conductance calcium-dependent potassium channels, respectively). It is thought that the effects of EDHF on smooth muscle are sensitive to Ba^{2+} and ouabain (inhibitors of the inward rectifier potassium channel, and the Na^+/K^+ATPase pump, respectively) (Weston *et al.*, 1998). NO also modifies K^+ channel activity (Cohen *et al.*, 1997), and characterisation of the EDHF pathway is likely to be confounded by NO unless steps are taken to maximally inhibit NO activity. The aim of this study was to characterise EDHF responses in rat resistance vessels in the presence of maximal NO inhibition, achieved using the NOS inhibitor, L-NAME and the NO scavenger, oxyhaemoglobin (OxyHb).

Rat mesenteric and hepatic resistance arteries (150-250 μm i.d.) were mounted in a tension myograph for isometric tension measurements. Tissues were maintained at 37°C in Krebs solution and gassed with 5%CO_2/95%O_2. Vessels were contracted with thromboxane A_2 analogue, U46619 (1μM) until stable responses were obtained. Vessels were contracted sub-maximally (EC_{75}) and concentration-response curves constructed to acetylcholine (ACh, 1-3000nM) or the NO donor, sodium nitroprusside (SNP, 1-3000nM) in the presence or absence of a combination of L-NAME (300μM) + COX inhibitor indomethacin (INDO, 5μM), the soluble guanylate cyclase (sGC) inhibitor ODQ (1μM), ChTx (100nM) + apamin (100nM), Ba^{2+} (30μM) + ouabain (1mM) or OxyHb (20μM). Relaxation of pre-contracted vessels was expressed as pEC_{50} and max. relaxation (mean±SEM) and statistical analysis performed using ANOVA.

Responses to ACh in mesenteric arteries were unaffected by pretreatment with L-NAME+INDO in the absence or presence of ODQ. In contrast addition of OxyHb significantly attenuated the maximum response. The residual ACh response, in the presence of L-NAME+INDO+OxyHb, was abolished by either K^+ (30mM, n=11; $P<0.01$) or ChTx+apamin (n=11; $P<0.01$) but unaffected by Ba^{2+}+ouabain (see table 1.)

Table 1. Effect of inhibition of NO, prostanoid and K+ flux on ACh induced relaxation

Pre-treatment	pEC_{50}	Max Response (%)	n and *P* value cf control
(a) Control	6.70 ± 0.13	95.0 ± 1.95	11
(b) L-NAME+INDO	6.04 ± 0.21	83.1 ± 8.20	11, NS cf a
(c) L-NAME+INDO+ODQ	6.97 ± 0.46	79.0 ± 7.53	11, NS cf b
(d) L-NAME+INDO+OxyHb	6.70 ± 0.20	57.9 ± 14.9	11, $P<0.01$ cf c
(e) + Ba^{2+}+Ouabain	6.97 ± 0.15	60.1 ± 10.10	9, NS cf d

In hepatic artery ACh responses (pEC_{50}=6.94±0.08, max=90.7±0.5, n=10) in the presence of L-NAME+INDO were significantly attenuated by treatment with either OxyHb (pEC_{50}=6.9±0.10, max=40.7±9.2% n=10, $P<0.001$) or Ba^{2+}+ouabain (pEC_{50}=6.30±0.45, max=17.1± 5.0%, n=10, $P<0.001$). However, addition of OxyHb to Ba^{2+}+ ouabain treated tissues had no greater effect than Ba^{2+}+ ouabain alone (pEC_{50}=6.99 ± 0.02, max=24.0 ± 11%, n=10). Similarly responses to SNP (max=88.7±0.2%, n=4) were suppressed by Ba^{2+}+ouabain (max=55.1±13.7%, n=4 $P<0.05$) confirming that Ba^{2+}+ouabain inhibited NO-mediated responses.

These studies show that in mesenteric and hepatic rat arteries a significant component of endothelium-dependent relaxation, after NOS and COX inhibition, is due to NO. This may be due to incomplete inhibition of NOS or release of NO from an intracellular store. The lack of effect of ODQ suggests that NO causes relaxation independent of sGC. The effect of Ba^{2+}+ouabain on SNP-induced relaxations imply that NO might also modulate K^+ channel activity. Therefore to specifically study EDHF in an NO-free environment it is necessary to use NO scavengers.

SDC is an BHF PhD Student, AA a BHF Intermediate Fellow, and LHC an MRC Senior Fellow.

Weston, A.H. *et al.*, (1998) *Nature* **396** 269-272
Cohen, R.A. *et al.*, (1997) *Proc. Natl. Acad. Sci* **94** 4193-4198

2P REGULATION OF cGMP-MEDIATED VASORELAXATION IN MOUSE THORACIC AORTA: INTERACTIONS OF THE SOLUBLE AND PARTICULATE GUANYLATE CYCLASES

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Nitric oxide (NO) and atrial natriuretic peptide (ANP) cause cyclic GMP-mediated vasorelaxation via activation of soluble (sGC) and particulate (pGC) guanylate cyclases, respectively. We have shown previously that NO causes functional down-regulation of sGC-mediated responses (Hussain *et al.*, 1999). In the present study we investigated whether NO might also modify vasorelaxation mediated by the activity of pGC.

Studies were carried out using aortic rings (2 mm wide) from male wild type (WT) and NO-synthase (NOS) knockout (KO) mice (Jackson Laboratories, USA). Endothelium-intact rings were mounted in Krebs solution under 0.3g tension and gassed with 95%O_2/5%CO_2 at 37°C. All tissues were contracted to 75-90 % of the maximum phenylephrine (PE) response (0.83±0.04 g in WT and 0.91±0.03 g in eNOS KO; p>0.05) and concentration-response curves to ANP (10^{-10}M-10^{-7}M) or the NO-donor spermine-NONOate (SPER-NO; 10^{-9}M-10^{-5}M), were constructed. The effect of NO/cGMP deficiency on the sensitivity of vessels to ANP was investigated by incubation of vessels with the NOS inhibitor L-NAME ($3x10^{-4}$M; 30 min) or sGC inhibitor ODQ ($5x10^{-6}$M; 30 min) or by using vessels from eNOS KO animals. The effect of NO/cGMP excess was investigated by examining the effect of incubating vessels from eNOS KO animals with the NO-donor GTN ($3x10^{-5}$M; 30 min). Data are presented as % relaxation of the PE response. pEC_{50} values are used to compare the relaxant effects of the drugs before and after treatment. Statistical significance was established using unpaired Student's *t*-test.

ANP was more potent on the eNOS KO than on the WT aorta (pEC_{50} 8.85±0.01 and 8.41±0.02, respectively; n=6; P<0.05) but had similar potency on nNOS WT and KO (pEC_{50} 8.43±0.02 and 8.39±0.03, respectively; n=8; P>0.05) and iNOS WT and KO (pEC_{50} 8.47±0.03 and 8.45±0.04, respectively; n=6; P>0.05). In WT aorta, the potency of ANP was increased following pretreatment with either L-NAME (pEC_{50} 8.74±0.09 and 9.15±0.09 in the absence and presence of L-NAME, respectively) or ODQ (pEC_{50} 8.56±0.05 and 9.26±0.03 in the absence and presence of ODQ, respectively; both n=4, P<0.05).

Following incubation with GTN vessels from eNOS KO animals were less sensitive to both SPER-NO (pEC_{50} 7.39±0.04 and 6.40±0.05 in the absence and presence of GTN, respectively; n=4; p<0.05) and ANP (pEC_{50} 9.24±0.05 and 8.43±0.04 in the absence and presence of GTN, respectively; n=4; p<0.05). To examine if cGMP -dependent or -independent actions of NO were responsible for the decreased response to ANP in the presence of GTN, similar experiments were conducted in the presence of ODQ and GTN. In this case, the hyporesponsiveness to ANP following pretreatment with GTN was partially reversed by ODQ.

The greater potency of ANP on vessels from eNOS KO mice is consistent with up-regulation of pGC-mediated responses. This effect is specific to the eNOS KO animals because ANP was equipotent on the nNOS and iNOS WT and KO aorta. In the WT mice, a reduction in the basal levels of NO and cGMP (using L-NAME and ODQ, respectively) enhanced the responses to ANP. Conversely, pre-incubation of vessels with GTN, at a concentration that shifted SPER-NO curves to the right, decreased the potency of ANP. ODQ partially reversed the inhibitory effects of GTN on ANP responses. These results suggest that in the mouse thoracic aorta the ambient NO concentration can modulate responses to pGC activation (by ANP) and that this effect is mediated partly by cGMP.

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3P CAPSAICIN AND RUTHENIUM RED ANTAGONISM OF ANANDAMIDE-INDUCED RELAXATION IS BLOCKED BY L-NAME IN THE RAT MESENTERIC ARTERIAL BED

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The endogenous cannabinoid anandamide is a potent vasorelaxant (Randall & Kendall, 1998). It has recently been suggested that anandamide induces vasorelaxation via activation of vanilloid receptors on sensory nerves, leading to release of calcitonin gene-related peptide (Zygmunt *et al.*, 1999). In addition, anandamide may mediate vasorelaxation by actions at smooth muscle cannabinoid receptors (Randall & Kendall, 1998). Both proposed pathways may involve activation of K^+ channels (Randall & Kendall, 1998). As K^+ activity may be upregulated on inhibition of NO production, we have investigated the possible involvement of NO as a modulator of vasorelaxant responses to anandamide.

Male Wistar rats (250-350g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1}, i.p.) and exsanguinated. The mesenteric arterial bed was isolated and perfused with oxygenated Krebs-Henseleit solution containing both indomethacin (10μM) and L-NAME (300μM). Following 20min equilibration, methoxamine was added to increase perfusion pressure (100-150mmHg). The vasorelaxant effects of anandamide (10nM-10μM) or capsaicin (1nM-10μM) were then assessed either prior to or following 10μM capsaicin treatment (1h incubation with 20min washout) or in the absence and presence of 1μM ruthenium red. All data were compared by ANOVA.

The vanilloid agonist capsaicin induced concentration-dependent relaxations (EC_{50}=0.06±0.03μM; R_{max}=88.2±3.7%; n=6). Pretreatment with capsaicin caused a 63-fold rightward shift of the concentration-response curve (CRC) for capsaicin (EC_{50}=3.8±0.8μM, P<0.01; R_{max}=52.2±5.5%, P<0.001; n=8). Addition of ruthenium red caused a 25-fold rightward shift of the CRC for capsaicin (EC_{50}=1.5±1.0μM, P<0.01; R_{max}=61.8±6.2%, P<0.001; n=5).

Anandamide caused concentration-dependent relaxations (EC_{50}=0.67±0.14μM; R_{max}=91.1±1.5%; n=6), whilst capsaicin treatment had no effect on its potency but caused a slight reduction of the maximum response (EC_{50}=0.70±0.09μM; R_{max}=81.9±2.8%, P<0.05; n=7). Ruthenium red also reduced the maximum response without affecting potency (EC_{50}=0.50±0.04μM; R_{max}=79.8±4.5%, P<0.001; n=7). In the absence of L-NAME, anandamide caused concentration-dependent vasorelaxation (EC_{50}=0.70±0.07μM; R_{max}=89.4±2.2%; n=8). Pretreatment with capsaicin caused a 3-fold rightward shift of the CRC for anandamide (EC_{50}=2.0±0.8μM, P<0.001; R_{max}=56.3±5.2%, P<0.001; n=6) and ruthenium red caused a 3.7-fold rightward shift of the CRC (EC_{50}=2.5±0.6μM, P<0.001; R_{max}=33.7±3.9%, P<0.001; n=5).

The present study shows that, in the absence of L-NAME, vasorelaxation to anandamide is attenuated by both capsaicin pre-treatment and ruthenium red. Thus, as in rat isolated arterial segments (Zygmunt *et al.*, 1999), vasorelaxation to anandamide is mediated, at least in part, via vanilloid receptors on sensory nerves in the rat mesenteric arterial bed. However, in the presence of L-NAME, whilst capsaicin pre-treatment and ruthenium red inhibited capsaicin-induced relaxation, neither agent affected the potency of anandamide, although the maximal responses were slightly reduced. The clear difference in the potency of capsaicin and ruthenium red as antagonists of capsaicin and anandamide relaxations in the presence of L-NAME suggests that in the absence of a functional NO system, anandamide mediates vasorelaxation preferentially via a vanilloid receptor-independent mechanism.

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Randall,M.D. & Kendall,D.A.(1998)*Trends Pharmacol. Sci.*19, 55-58.
Zygmunt, P.M. *et al.* (1999). *Nature*, 400, 452-457.

4P VANILLOID RECEPTORS ON CAPSAICIN-SENSITIVE SENSORY NERVES MEDIATE RELAXATION TO METHANANDAMIDE IN THE RAT ISOLATED MESENTERIC BED

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In isolated small arteries the endogenous cannabinoid anandamide mediates vasorelaxation by actions at vanilloid receptors on sensory nerves (Zygmunt *et al.*, 1999). However, differences between vasorelaxant responses to cannabinoids in arterial segments versus isolated arterial beds have been observed. Therefore, this study investigated whether capsaicin-sensitive sensory nerves mediate vasorelaxation to methanandamide also in the rat isolated mesenteric arterial bed.

Wistar rats of either sex (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused via the superior mesenteric artery with oxygenated Krebs' solution at 5 ml min^{-1} (Ralevic *et al.*, 1995), whereas arterial segments were suspended in tissue baths (Zygmunt *et al.*, 1999). After 30min equilibration, preparations were pre-constricted with methoxamine (10-50μM, beds) or phenylephrine (1-10μM, segments) and relaxant responses to methanandamide (0.01-10μM) and capsaicin (0.005-50 nmol) were investigated. A group of preparations was pre-treated with capsaicin (10μM for 1h) in order to cause desensitization and/or neurotransmitter depletion in sensory nerves. The concentration of methanandamide and the dose of capsaicin required to elicit 50% relaxation are given as -logEC_{50} (pEC_{50}) and -logED_{50} (pED_{50}) values, respectively. E_{max} denotes the maximal relaxation achieved. Data are expressed as mean±s.e.m. and analysed by Student's t test.

Methanandamide elicited concentration-dependent relaxation of the pre-constricted mesenteric artery. In arterial beds the pEC_{50} and E_{max} values were 5.9±0.04 and 86.6±2.7% (n=6), respectively, whereas in arterial segments the corresponding values were 6.4±0.1 and 93±3% (n=11), respectively. In arterial beds pre-treated with capsaicin, the relaxation response curve to methanandamide was shifted to the right; the pEC_{50} value was 5.2±0.2 (n=4; P<0.01). After capsaicin pre-treatment, dose-dependent relaxant responses to capsaicin were also attenuated (control: pED_{50} = 10.6±0.1; E_{max} = 98.5±0.9%; treatment: pED_{50} = 7.0±0.1, P<0.001; E_{max}= 98.6±0.7%; n=4-6). In arterial segments, capsaicin pre-treatment abolished methanandamide relaxations (n=6). Ruthenium red (0.1μM), an antagonist of vanilloid receptors, attenuated significantly the relaxant responses to methanandamide in arterial beds. The pEC_{50} and E_{max} values were 5.6±0.04 (P<0.001) and 89.2±1.4%, respectively (n=6). Ruthenium red (0.1μM) also antagonised responses to capsaicin (pED_{50} 9.99±0.1, P<0.01; E_{max} 98.2±0.7%) (n=6). At 1μM ruthenium red responses to both methandamide and capsaicin were were virtually abolished (n=4). Likewise, ruthenium red (0.15μM) almost abolished relaxations to methanandamide in arterial segments (n=6). Calcitonin gene-related peptide (CGRP) is the principal sensory neurotransmitter in rat mesenteric arteries. Ruthenium red had no significant effect on relaxations evoked by exogenous CGRP in arterial beds and in arterial segments (n=4-6).

These data show that both the sensory neurotoxin capsaicin, and the vanilloid receptor antagonist ruthenium red, attenuate vasorelaxant responses to methanandamide in the rat isolated mesenteric arterial bed and mesenteric arterial segments. We conclude that, as in isolated vessels, vanilloid receptors on capsaicin-sensitive sensory nerves mediate the vasorelaxant action of methanandamide in the rat mesenteric arterial bed.

Ralevic, V. *et al.* (1995) *J. Pharmacol. Exp. Ther.* **274,** 64-71
Zygmunt, P.M. *et al.* (1999) *Nature*, **400,** 452-457.

5P COMPARATIVE HAEMODYNAMIC EFFECTS OF i.p. ADMINISTRATION OF ANANDAMIDE OR CGRP IN CONSCIOUS RATS

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Zygmunt *et al.* (1999) recently reported that release of CGRP from perivascular sensory nerves was responsible for the vasodilator effect of anandamide in mesenteric arterioles isolated from rats. Previously, we (Gardiner *et al.* 1989) have assessed *in vivo* haemodynamic effects of exogenous CGRP administered i.v., and have commented upon the absence of a mesenteric vasodilator effect. On the basis of the data of Zygmunt *et al.* (1999), we hypothesised that CGRP or anandamide, given i.p., would cause mesenteric vasodilatation; here, we tested that hypothesis.

Male, Long-Evans rats (350 - 450g) were instrumented with renal, mesenteric and hindquarters Doppler probes and intravascular and i.p. catheters (all surgery under sodium methohexitone anaesthesia 60 mg kg^{-1} i.p., supplemented as required). Rats (n=8) were given randomised, i.p. bolus doses of anandamide (0.2 - 7 μmol kg^{-1}) or human α-CGRP (0.1 nmol - 1 nmol kg^{-1}).

Over the first 3 min following administration, anandamide caused pressor effects accompanied by renal and mesenteric vasoconstriction, but hindquarters vasodilatation. In contrast, CGRP caused clear hypotension together with renal, mesenteric and hindquarters vasodilatation (Table 1).

Table 1. Integrated (areas under (AUC) or over (AOC) curves) cardiovascular responses following (0-3 min) i.p. anandamide (0.2 μmol kg^{-1}) or CGRP (1 nmol kg^{-1}) in the same, conscious Long Evans rats (n=8). Values are mean ± s.e. mean; * CGRP vs anandamide response (Wilcoxon test).

	Anandamide	**CGRP**
Heart rate (AUC, beats)	194±38	131±31
Mean blood pressure (AUC, AOC, mmHg min)	56±8	−37±17*
Renal conductance (AOC, AUC, % min)	−52±9	82±13*
Mesenteric conductance (AOC, AUC, % min)	−107±15	33±6*
Hindquarters conductance (AUC, % min)	61±16	49±8

Thus, although CGRP administered i.p. does cause mesenteric vasodilatation (in contrast to the vasoconstriction seen with i.v. administration), i.p. anandamide causes mesenteric vasoconstriction. While these results do not directly contravert the findings of Zygmunt *et al.* (1999), they do indicate that any putative role for endogenous anandamide in control of regional haemodynamics probably involves mechanisms in addition to CGRP release.

Gardiner, S.M. *et al.* (1989). Br.J.Pharmacol., **98**, 1225-1232.
Zygmunt, P.M. *et al.* (1999), Nature, **400**, 452-457.

6P DIRECT COUPLING BETWEEN NITRERGIC NERVE TERMINALS AND Ca^{2+}_i IN SMOOTH MUSCLE CELLS OF RABBIT CEREBRAL CORTEX ARTERIOLES

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Nitrergic nerves appear to be involved in the coupling process between neuronal activity and blood flow in the brain (Lovick *et al.*, 1999). This process may involve direct interaction between nitric oxide synthase (NOS)-containing nerve terminals and arteriolar smooth muscle cells because electron micrographs, at least from the major anterior cerebral arteries, reveal close localisation of these structures (Barroso *et al.*, 1996). We thus aimed to investigate the presence and the localisation of neuronal NOS (nNOS) as well as the effect of neuronal NO on the contraction and the $[Ca^{2+}]_i$ of cerebral precapillary arterioles.

Male rabbits (1-1.5 kg) were given an intravenous overdose of 70 $mg.kg^{-1}$ sodium pentobarbitone and small arteriolar fragments were mechanically and enzymatically isolated from the pial membrane (Guibert *et al.*, 1999). Arterioles were loaded with fura-PE3 (1 μM) for $[Ca^{2+}]_i$ measurements and external arteriolar diameter was recorded with a video-edge detection system. Monoclonal antibodies were used for immunofluorescence labelling of endothelial NOS (eNOS), inducible NOS (iNOS), nNOS (Transduction Laboratories), vasoactive intestinal peptide (VIP) (Chemicon) and smooth muscle α actin (α-SMA) (Sigma) in order to identify and localise the source of NO release. Values are given as mean ± s.e.mean and statistical significance ($p<0.05$) was assessed by Student's *t*-test.

Changing from Ca^{2+}-free (0.4 mM EGTA) to Ca^{2+}-containing bath solution evoked a significant $[Ca^{2+}]_i$ increase in L-Name 0.3 mM (change in 350/380 ratio, $\Delta R=0.057 \pm 0.01$, n=19) but not in control arterioles (n=10). Arteriolar diameter was not modified (35.0 ± 3.0 vs 33.5 ± 5.0 μm in control conditions, n=49 and 34 respectively). The L-Name-induced $[Ca^{2+}]_i$ was significantly decreased by L-arginine 1 mM, a substrate for NOS ($\Delta R=0.01 \pm 0.007$ vs 0.036 ± 0.004 in control conditions, n=5 and 7 respectively) and diethylamine NONOate 0.3 mM, a NO donor ($\Delta R=0.021 \pm 0.01$ vs 0.047 ± 0.01 in control conditions, n=8 and 10 respectively). S-methyl-L-thiocitrulline (MTC, 1 μM), a preferential inhibitor of nNOS, induced a Ca^{2+} increase of 0.037 ± 0.005 (n=14). iNOS staining was negative (n=3). eNOS was detected in the endothelium of 7 arteriolar fragments but was undetectable in 31 arterioles. By contrast nNOS staining was detected in 11/11 arterioles. Furthermore, follow-up labelling of arterioles used for Ca^{2+} measurements showed that MTC or L-Name responses were associated with nNOS (n=3) but not eNOS staining (n=2). nNOS staining was punctate and partially co-localised with VIP-containing nerve terminals (n=4).

The data provide evidence for direct coupling between NO generated by nitrergic nerve terminals and $[Ca^{2+}]_i$ in cerebral arteriolar smooth muscle cells.

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Barroso C.P., Edvinsson L., Zhang W. *et al.* (1996). *J Auton Nerv Syst* **58**, 108-114.
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Lovick T.A., Brown L.A., Key B.J. (1999). *Neuroscience* **92**, 47-60.

7P EFFECTS OF OXIDATIVE STRESS ON THE SPONTANEOUS AND CARBACHOL-STIMULATED MYOGENIC ACTIVITY OF THE RAT ISOLATED PORTAL VEIN

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Oxidative stress has been shown to alter the function of autonomic receptors (Peters *et al*, 1998). In isolated rat left atria, exposed to electrically generated oxygen free radicals, we found an enhanced negative inotropic effect of muscarinic receptor stimulation. In the present study we want to investigate, whether this effect can be observed in another, non-cardiac tissue as well.

The rat portal vein is an often used model to investigate drug effects on spontaneous and stimulated vascular myogenic activity. Although located in the venous part of the circulation, this vessel shows properties quite untypical for a vein, such as a vasoconstrictive effect to muscarinic receptor stimulation, mediated by the M_3-receptor subtype, even in the presence of an intact endothelium (Pfaffendorf & van Zwieten, 1993).

Male Wistar rats of 250-300 g were killed and the portal veins excised and mounted in 5 ml organ baths. The isometric force of contraction was recorded under a constant pre-tension of 5 mN. The Tris buffered Krebs solution (37°C, pH 7.4) used contained 2.5 mM calcium for all experiments. Oxygen free radicals were generated by a constant current of 30 mA for 75 sec via electrodes at the bottom of the organ bath (Peters *et al.*, 1998). The spontaneous myogenic activity was recorded for half an hour before the electrolysis was applied. After another 30 minutes of equilibration in the presence or absence of 3 mM glutathione (GSH), a cumulative carbachol concentration-response curve was constructed (1 nM - 0.3 mM) and the phasic and tonic part of the response analysed seperately. At the end of each experiment, the preparations were exposed to an isotonic 60 mM KCl solution to test the maximal, depolarization-induced contration. All results are given as mean values ± standard deviation of the mean of at least 6 experiments.

The electrolysis resulted in an immediate decrease of the spontaneous myogenic activity of 29.6 ± 9.9% (n=8). The potency (-logEC_{50}, M) of carbachol in inducing an increase in the phasic was slightly but significantly reduced, whereas for the tonic response it was unaltered (Phasic: 6.8 ± 0.4, control n=6 vs. 5.4 ± 0.4, n=8 electrolysis; tonic 4.9 ± 0.2 control vs. 4.8 ± 0.1 electrolysis). However, the maximal effects were significantly reduced for both types of responses (Phasic: 8.5 ± 0.8 mN control *vs.* 5.7 ± 0.34 mN electrolysis; tonic: 7.5 ± 1.2 mN control *vs.* 2.7 ± 0.6 mN electrolysis). In the presence of the scavenger glutathion (3 mM) both the spontaneous and the maximal carbachol-stimulated phasic response (-3.6 ± 5.8 %; 6.7 ± 0.8 mN, n=6), as well as the potency of carbachol (-log EC_{50}:6.7 ± 0.8) remained uninfluenced by electrolysis. The KCl-induced contracture, as a measure for the ability of the preparation to react to a maximal contractile stimulus, was not different in the three groups (9.3 ± 1.0 mN control, 7.2 ± 0.6 mN electrolysis, 7.8 ± 1.2 mN electrolysis + GSH).

From the presented data we conclude that oxygen free radicals, generated by electrolysis, impair the spontaneous myogenic activity as well as the reaction to muscarinic stimulation in the isolated rat portal vein. Incubation with the oxygen free radical scavenger glutathione prevented the observed alterations.

Peters S.L.M. et al. (1998) *Br.J.Pharmacol.*, **123**, 952-958

Pfaffendorf M., & van Zwieten P.A. (1993), *Br.J.Pharmacol.*, **108**, 132-138

8P THE EFFECT OF ENDOTOXAEMIA ON THE INOTROPIC RESPONSE TO ISOPRENALINE: THE ROLE OF CYCLIC AMP

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Nitric oxide (NO) has been implicated in the control of myocardial function under physiological conditions (Brutsaert *et al*, 1988). Controversy exists regarding the mechanisms of the myocardial dysfunction characteristic of septic shock. We have previously shown that in endotoxaemia, L-arginine becomes rate-limiting for the production of NO in atria, and that this tissue has a reduced inotropic response to the beta agonist isoprenaline (Price *et al* 1999). Moreover, when L-arginine is added, the increased NO produced restores the diminished response to isoprenaline. In order to further understand the processes involved in this phenomenon, we have assessed the effect of the phosphodiesterase (PDE) III inhibitor, milrinone on the inotropic response to isoprenaline in control and endotoxaemic atria. In addition we have studied the response to the cell-permeable cyclic AMP analogue, dibutyryl-cyclic AMP (DcAMP).

Control and endotoxaemic (LPS 20mg^{-1}kg^{-1} i.p 4h) male Wistar rats (250-300g) were anaesthetised with sodium pentabarbitone (100mg^{-1}kg i.p) and killed by cervical dislocation. Hearts were removed and left atria attached to a force-transducer in gassed (95%O_2:5%CO_2) Krebs' buffer. Preparations were maintained at 37°C and paced at 0.5Hz (100% above the threshold voltage) and set at Lmax. Changes in generated tension were measured in response to increasing concentrations of milrinone (10^{-7} to10^{-4}M). At the end of each experiment, a single dose of isoprenaline (10^{-5}M) was added. In a separate group of experiments, concentration-response curves to DcAMP (10^{-6} to $3x10^{-4}$M) were performed. All data are shown as mean ± S.E.M unless otherwise stated. A p value <0.05 was taken as significant and denoted by *.

Endotoxaemia reduced the inotropic response to isoprenaline and milrinone *(Fig 1)*. Moreover, milrinone restored the inotropic response to isoprenaline in atria from endotoxaemic rats to that of controls *(Fig 1)*. However, milrinone further increased the inotropic response to isoprenaline in atria from control animals. In contrast to the findings with milrinone and isoprenaline ,there was no difference in the inotropic response to DcAMP observed in atria from control and endotoxaemic animals.

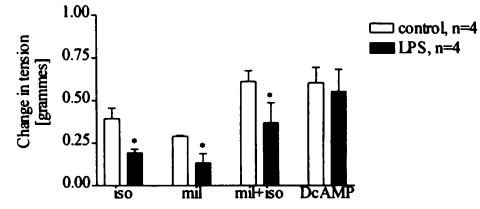

Fig 1

Maximal inotropic responses [change in generated tension] in atria from control and endotoxaemic rats treated with isoprenaline 10^{-4M}, milrinone 10^{-4}M or DcAMP $3x10^{-4}$M

Thus, in endotoxaemia atria are hyporesponsive to both isoprenaline and milrinone, but not to DcAMP. These data suggest that sepsis causes a dysfunction in atrial cAMP generation (i.e. adenyl cyclase activity) or survival (i.e. PDE activity). These observations suggest that inhibition of cAMP-metabolising PDE's may reverse the myocardial dysfunction in sepsis.

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

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9P EFFECT OF ENDOTOXAEMIA ON THE INOTROPIC AND CHRONOTROPIC EFFECTS OF ENDOTHELIN: ROLE OF CYCLO-OXYGENASE

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The pathogenesis of the myocardial dysfunction that characterises septic shock remains unclear, although the changes that result have been well documented (Parker *et al* 1984). Endothelin-1 (ET-1) is known to have inotropic effects, although its action on heart rate (chronotropy) is more controversial (Moravec *et al* 1989; Reid *et al* 1991). Furthermore, ET-1 is known to be elevated in endotoxaemia (Warner *et al* 1996) but little evidence exists regarding the role of ET-1 in modulating the changes in myocardial function in septic shock.
In this study we have characterised the effect of endotoxaemia on both the inotropic and chronotropic effects of ET-1 in the presence and absence of the cyclo-oxygenase (COX) inhibitor, ibuprofen.

Control and endotoxaemic (LPS $20mg^{-1}kg^{-1}$ i.p 4h) male Wistar rats (250-300g) were anaesthetised with sodium pentabarbitone ($100mg^{-1}kg$ i.p) and killed by cervical dislocation. Hearts were removed and right and left atria attached to a force-transducer in gassed (95%O_2:5%CO_2) Krebs' buffer. Preparations were maintained at 37°C. Right atria were allowed to beat spontaneously, and left atria paced at 0.5Hz (100% above the threshold voltage). Change in right atrial rate and left atrial generated tension were measured in response to increasing concentrations of ET-1 (10^{-15} to$3x10^{-7}$M), in the presence or absence of the ibuprofen (10^{-5}M). All data are shown as mean ± S.E.M unless otherwise stated. *n=4-6* in each group.

There was no significant difference between baseline right atrial rate (beats per min; bpm) in control and endotoxaemic animals (control 308±12; LPS 296±9). ET-1 had no significant chronotropic effect in atria from control or endotoxaemic animals. By contrast, ET-1 produced a clear concentration-related increase in inotropic response in left atria from both control and endotoxaemic animals *(Table 1)*. The inotropic response to ET-1 was reduced in left atria from endotoxaemic rats *(Table 1, $p<0.01$)*. In the presence of ibuprofen, atria from control (but not endotoxaemic) animals exhibited a significant positive chronotropic response to ET-1 *(Table 1, $p<0.0001$, Two way ANOVA)*. Ibuprofen however had no effect on the inotropic response to ET-1 in atria from control or endotoxaemic animals *(Table 1)*.

Table 1
Maximal chronotropic and inotropic responses to ET-1 (10^{-7}M) in atria from control and endotoxaemic animals

	chronotropy (bpm)		inotropy (g)	
treatment	control	LPS	control	LPS
nil	-10±9	10±2	0.2±0.03	0.07±0.02
ibuprofen	48±7	12±9	0.21±0.02	0.09±0.02

These data show that in the absence of ibuprofen, endothelin has no significant chronotropic effect on isolated right atria. In the presence of ibuprofen, however, right atria from control animals have a significant positive chronotropic response to endothelin, a response that was not present in atria from endotoxaemic animals.
These observations suggest that COX activity limits the chronotropic effects of endothelin-1 under physiological conditions, an effect that appears to be lost in endotoxaemia.

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10P CYTOKINE-STIMULATION OF ET-1 RELEASE FROM HUMAN VASCULAR SMOOTH MUSCLE CELLS (HVSMCs) IS MODULATED BY cAMP

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As cyclic AMP is a key modulator of many physiological processes (Delmas *et al.*, 1994) we have attempted to determine whether an elevation of intracellular cAMP may affect cytokine-induced production of ET-1 in HVSMCs that we have previously reported (Woods *et al.*, 1999).

Saphenous vein (SV) was obtained from patients undergoing coronary artery bypass graft surgery. Explants of HVSMCs were grown in DMEM supplemented with 2mM glutamine and 15% foetal calf serum (37°C; 5% CO_2; 95% air). HVSMCs were identified by α-actin staining. Once confluent, HVSMCs were incubated with TNF-α (10ng/ml) and IFN-γ (1000U/ml) for 24-48 h in the presence or absence of cicaprost, forskolin or Ro-20-1724. ET-1 levels were measured by specific sandwich ELISA (R&D Systems). Total RNA was isolated by a guanidinium thiocyanate/isopropanol method with minor modifications (Chomczynski and Sacchi, 1987). Reverse transcription coupled with polymerase chain reaction (RT-PCR) was performed using standard methods.

Cicaprost, a prostacyclin analogue, maximally inhibited cytokine-stimulated ET-1 production by 49.1±4.4% at a concentration of 1μM with half maximal inhibition occurring at 3.5nM. Forskolin, an activator of adenylate cyclase, similarly inhibited cytokine-stimulated ET-1 with an IC_{50} of 15.8μM, as did Ro-20-1724, a type IV phosphodiesterase inhibitor, with an IC_{50} of 43.7μM. Semi-quantitative RT-PCR showed a marked decrease (26.3%±3.2%) in cytokine-induced expression of prepro-ET-1 in SV after incubation with Ro-20-1724 for 48 h.

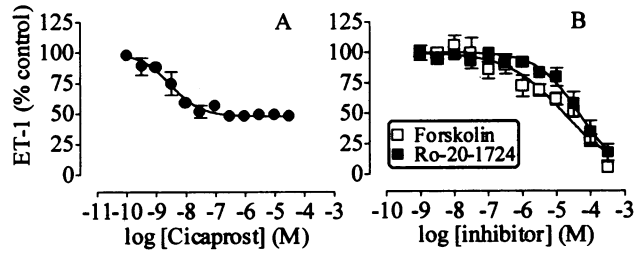

Figure 1. Effect of Cicaprost (A), forskolin or Ro 20-1724 (B) on cytokine-induced ET-1 production in SV HVSMCs. Data represents mean ± s.e.m of cells from n=5 donor patients.

These findings demonstrate that cytokine-induced ET-1 mRNA expression and peptide production is reduced by elevations in intracellular cAMP. This may well provide a mechanism to explain some of the well-known beneficial vascular effects of prostacyclin and related compounds.

This work was supported by grants from The British Heart Foundation (FS/951003, PG/99001). Cicaprost was kindly provided by Schering AG (Berlin, Germany).

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11P EFFECTS OF TREATMENT WITH ALPRENOLOL AND CARVEDILOL IN A MOUSE MYOCARDIAL INFARCT MODEL OF HEART FAILURE

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In previous studies in transgenic mice cardiac-specifically over-expressing the human β_2-adrenoceptor, chronic infusions of inverse agonists (e.g. carvedilol), but not a neutral antagonist (alprenolol), resulted in increased baseline atrial contractility (Nagaraja *et al.*, 1999). In the present study, we examine whether treatment with carvedilol or alprenolol produces differential effects in a mouse myocardial infarct model of heart failure (MI-HF).

In 12-15 week old, male C57BL/6 mice with a mean bodyweight of 26.5±0.27g myocardial infarction was induced by permanent occlusion of the left anterior descending coronary artery (Michael et al., 1995). They were randomised into three different groups. The mice were weighed weekly. After a two week recovery period oral drug treatment was started, with 400 ppm carvedilol (CAR), 2400 ppm alprenolol (ALP) and control chow (CO). After one week, doses were raised to 1200 ppm CAR and 7200 ppm ALP. After another week CAR was increased to 2400 ppm, whereas ALP was not further elevated, since the condition of these mice did not allow a further increase. Cardiac *in vivo* parameters were determined pre and post occlusion (PO) (2,3,4,5 and 6 weeks) using Doppler technique (Hartley et al., 1995). Subsequently, the mice were killed and left atria were isolated for isometric tension recordings as described by Bond *et al.*, 1995. Sodium pentobarbital (40mg.kg^{-1}) was used as an anaesthetic.

As an index of cardiac diastolic function, mitral E-peak velocity (MEPV) was measured and expressed as percentage of preocclusion values. The differences (Δ) in MEPV between week 2 (start of treatment) and 5 (3 weeks of treatment) were calculated (table 1). The CAR group was increased significantly compared to the ALP group. Aortic peak velocity, an index for cardiac systolic function, was not different among the groups (data not shown). Percent weight changes between pre occl. and five weeks PO were calculated (table 1). The ALP group gained significantly less weight than the CAR and CO PO group. Maximum left atrial tension (mLAT; maximal response to isoprenaline) was measured and normalised to control. The CAR group was significantly greater than the ALP and CO PO groups (table 1). Data are expressed as mean±s.e.mean.

Table 1	CAR (n=9)	ALP (n=9)	CO PO (n=6)	CO sham (n=5)
MEPV pre occl (cm/s)	75.4±2.9	80.3±1.9	83.1±2.6	69.9±3.1
Δ MEPV (%)	7.3±4.4[1]	-9.0±4.9	-1.2±1.8	3.0±5.0
weight pre occl (g)	27.7±0.6	26.2±0.5	25.5±0.4	25.0±0.4
Δ weight (%)	1.0±1.5	-6.1±1.7[3]	0.53±1.8	2.0±0.3
mLAT (%)	49.9±9.4[2]	25.1±5.5	26.9±5.9	93.7±10.3[3]

$p<0.05$ vs: [1] ALP; [2] ALP and CO PO; [3] all other groups (ANOVA)

These results suggest that antagonists and inverse agonists have differential effects in a mouse MI-HF model. Carvedilol showed beneficial effects on two cardiac parameters and no adverse effects in post infarct recovery. However, other pharmacological differences between alprenolol and carvedilol have to be excluded as the possible cause before determining that the inverse agonist property is responsible for the beneficial effects.

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12P LIPOTEICHOIC ACID INDUCES DELAYED PROTECTION IN THE RAT HEART VIA INHIBITION OF ENDOTHELIUM/LEUCOCYTE INTERACTIONS

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Pretreatment of animals for 8-24 h with wall fragments of Gram-negative (lipopolysaccaride, Brown *et al.*, 1989; Zacharowski *et al.*, 1999a) or Gram-positive (lipoteichoic acid, LTA, Zacharowski *et al.*, 1999b) bacteria protects the heart against subsequent ischaemia-reperfusion injury. This phenomenom has been termed second window of protection (SWOP, Marber *et al.*, 1993). Numerous studies suggest that polymorphonuclear granulocytes (PMNs) may play a major role in determining ultimate infarct size in models of myocardial ischaemia followed by prolonged periods of reperfusion (Hansen, 1995). This study was designed to investigate if LTA induces a SWOP via inhibition of endothelium/PMN interactions.

Eighteen male Wistar rats (250-300 g) were anaesthetised (thiopentone sodium, 120 mg kg^{-1} i.p.), tracheotomised and ventilated (tidal volume: 10 ml kg^{-1}, 70 strokes min^{-1}, inspiratory oxygen-concentration: 30%, positive end-exspiratory pressure: 1-2 mmHg). The carotid artery was cannulated to measure mean arterial blood pressure. Following a left-sided thoracotomy, a ligature was placed around the left anterior descending coronary artery (LAD). The animals were allowed to stabilise for 30 min and subsequently the LAD was occluded for 25 min and then reperfused for 2 h. At the end of the experiment, the LAD was re-occluded and 1 ml of Evans Blue dye (2% w/v) was injected to determine the perfused and the non-perfused (area at risk, AR) myocardium. Infarct size (IS) was determined by incubation of the slices of the heart with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1}). The number of PMNs, intercellular adhesion molecule-1 (ICAM-1) and P-selectin expression in the border zone of the infarcted tissue in slices were determined as described previously (Gauthier *et al.*, 1994; Zacharowski *et al.*, 1999c). Rats were randomized to the following groups: (1) saline (16 h pretreatment, 1 ml kg^{-1}; n=6) or (2) LTA (16 h pretreatment, 1 mg kg^{-1}; n=6) followed by LAD-occlusion-reperfusion or (3) sham-operation, no LAD-occlusion (16 h pretreatment with saline, 1 ml kg^{-1}; n=6).

The AR was similar in all groups studied (Table 1). LTA pretreatment caused a significant reduction in (i) IS, (ii) accumulation of PMNs, (iii) expression of P-selectin and (iv) expression of ICAM-1 compared to control. There were no haemodynamic differences between any of the groups studied (data not shown).

Table 1: Infarct size, area at risk, PMN and P-selectin/ICAM-1 (expressed as % of total tissue area, % TTA).
(* $p<0.05$ vs. control, ANOVA followed by Bonferroni's test).

	IS (%)	AR (%)	PMNs (per mm^2)	P-selectin (% TTA)	ICAM-1 (% TTA)
sham	2±1*	47±5	10±5*	0.01±0.01*	0.5±0.1*
control	57±4	49±4	450±107	0.80±0.10	1.0±0.1
LTA	19±7 *	54±2	19±7 *	0.02±0.01 *	0.3±0.1 *

Pretreatment of rats with LTA causes a substantial protection against a subsequent period of myocardial ischaemia and reperfusion. The cardioprotective effects of LTA may be due to inhibiton of the endothelium/PMN system.

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13P PRETREATMENT WITH LIPOTEICHOIC ACID REDUCES MYOCARDIAL INFARCT SIZE IN THE ANAESTHETISED RAT

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Classical ischaemic preconditioning (IPC) transiently (30-120 min) protects the myocardium against subsequent lethal ischaemia-reperfusion injury (Murry *et al.*, 1991). Following dissipation of this acute protection, a second window of protection (SWOP) appears 12-24 h later, which lasts up to 3 days (Marber *et al.*, 1993). Several triggers are known to induce a SWOP including brief repetitive cycles of coronary artery occlusion (Marber *et al.*, 1993), stimulation of adenosine A_1 receptors (Baxter *et al.*, 1994) or administration of wall fragments of Gram-negative bacteria including lipopolysaccharide (Brown *et al.*, 1989; Zacharowski *et al.*, 1999). The aim of this study was to investigate whether lipoteichoic acid (LTA), a cell wall fragment of Gram-positive bacteria, induces SWOP beyond 2 h in a rat model of regional myocardial ischaemia and reperfusion.

Fourty-one male Wistar rats (240-300 g) were pretreated with i.p. boluses of either LTA (*Staphylococcus aureus*, 1 mg kg^{-1}) or saline. At 2, 4, 8, 16 or 24 h after LTA/saline administration, rats were anaesthetised (thiopentone sodium, 120 mg kg^{-1} i.p.), tracheotomised and ventilated (tidal volume: 10 ml kg^{-1}, 70 strokes min^{-1}, inspiratory oxygen-concentration: 30%, positive end-exspiratory pressure: 1-2 mmHg). The carotid artery was cannulated to measure mean arterial blood pressure (MAP) and the jugular vein was cannulated for the administration of drugs. The chest was opened by a left-sided thoracotomy, the pericardium incised and a suture was placed around the left anterior descending coronary artery (LAD). The animals were allowed to stabilise for 30 min and subsequently the LAD was occluded for 25 min and then reperfused for 2 h. At the end of the experiment, the LAD was re-occluded and 1 ml of Evans Blue dye (2% w/v) was injected into the jugular vein to determine the perfused and the non-perfused (area at risk, AR) myocardium. Infarct size (IS) was determined by incubation of the slices of the heart with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1}).

The following groups were studied: LAD-occlusion and reperfusion of **(1)** vehicle (n=10); **(2)** LTA for 2 h (n=6); **(3)** LTA for 4 h (n=6); **(4)** LTA for 8 h (n=5); **(5)** LTA for 16 h (n=8) or **(6)** LTA for 24 h (n=6).

The mean AR were similar in all groups studied (Table 1). When compared to vehicle, pretreatment of rats with LTA for 8 h caused a significant reduction in IS of approximately 40 % (Table 1). The reduction in IS afforded by LTA was maximal at 16 h (Table 1). There were no haemodynamic differences between any of the groups studied (data not shown).

Table 1: Infarct size and area at risk data (* $p<0.05$ vs. saline, ANOVA followed by Bonferroni's test).

LTA	2 h	4 h	8 h	16 h	24 h	saline
IS (%)	56±6	33±7	27±11*	20±9*	25±6*	60±2
AR (%)	55±2	50±4	56±4	55±2	48±3	47±2

Thus, pretreatment of rats with LTA causes a time-dependent, substantial protection against a subsequent period of myocardial ischaemia and reperfusion.

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14P DELAYED PRECONDITIONING AND DIAZOXIDE-MEDIATED CYTOPROTECTION OCCUR INDEPENDENTLY OF A CHANGE IN MITOCHONDRIAL MEMBRANE POTENTIAL IN A HUMAN CARDIAC CELL LINE

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Evidence of delayed preconditioning or second window of protection (SWOP) in man is limited and obvious ethical considerations make it difficult to study . We investigated the mechanisms of SWOP in an adult human cardiac myoblast cell line (Girardi) model exposed to lethal simulated ischaemia. The mitochondrial K_{ATP} channel has been implicated by a number of investigators (Garlid, *et al* 1997; Gross, *et al* 1999) as important in the maintainance of mitochondrial integrity and function during ischaemia reperfusion injury. However the mechanisms by which this channel is protective remain speculative. This study was performed with particular reference to the role of this channel in altering mitochondrial membrane potential.

Group 1 cells were preconditioned (PC) by 1 hour of mild simulated ischaemia and allowed to recover for 24 hours before being subjected to 6 hours of more severe lethal simulated ischaemia (LSI), followed by 1 hour reperfusion. At the end of reperfusion, cell viability was assayed by propidium iodide (%PI) exclusion and lactate dehydrogenase efflux into medium. Group 2 cells were preconditioned as above but immediately prior to LSI were incubated in 5-hydroxydecanoate sodium (5-HD), 50 µM, a dose known to specifically inhibit the mitochondrial KATP channel. Groups 3 and 4 consisted of naive cells treated with the mitochondrial KATP channel opener diazoxide, 30 µM, alone, or following prior incubation with 5-HD.

In a parallel series of experiments, at the time point corresponding to the onset of LSI, representative groups of cells were loaded with the potential-sensitive ratiometric fluorophore 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), 10 µg/ml for 15 minutes, trypsinised, and analysed by flow cytometry. This agent normally exists as monomers which fluoresce at 527 nm (green, FL1)., When however the compound enters the negatively charged space of the mitochondrion, so-called J-aggregates form with a shift in fluorescesence to 590 nm (orange,FL2). The ratio of red to green fluorescence is linear in a range of mitochondrial membrane potential (mmp) from 10-180mV (Smiley, *et al*, 1991). This fluorophore can be used to monitor the effect of diazoxide or preconditioning on the mitochondrial membrane potential. As a control, a further group of cells were incubated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP),100µM, which is known to completely depolarise mitochondria with consequent collapse of mmp and hence represents the depolarised state. Data are expressed as median peak fluoresence intensity in channel FL2 in Table 1, which summarises the results of n=12 per group.

Group	% PI positive	Median FL2 value
Naive cells	<2	122±1
Control (LSI)	34.4±2.0	--
PC	14.6±1.4 *	124±1
Diazoxide	10.67±1.7*	123±1
Diazoxide+5-HD	33.3±3.6	122±3
CCCP	--	12.2±3#

*$p<0.01$ vs LSI; one way ANOVA.
#$p<0.01$ vs naive cells; one way ANOVA

Conclusion: Both delayed preconditioning and cytoprotection by the mitochondrial K_{ATP} channel opener diazoxide in this model appear to act by a mechanism that is independent of a change in mitochondrial transmembrane potential gradient as measured *in situ* by JC-1 ratiometric flow cytometry.

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15P SPHINGOLIPID-DEPENDENT CALCIUM MOBILISATION IN SH-SY5Y NEUROBLASTOMA CELLS

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The release of Ca^{2+} from intracellular stores via the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) has been extensively characterised. Less well described is the potential Ca^{2+} mobilising effects of intracellular sphingolipids (Meyer zu Heringdorf *et al.*, 1988). We have recently demonstrated that in the human SH-SY5Y neuroblastoma cell line lysophosphatidic acid (LPA) acting on an extracellular G-protein coupled receptor can mobilise intracellular Ca^{2+} via a mechanism that may involve the activation of sphingosine kinase (Young *et al.* 1999). We now present evidence that intracellular sphingolipids can themselves cause Ca^{2+} mobilisation in SH-SY5Y cells, and we examine the role of this Ca^{2+} release pathway in the elementary Ca^{2+} events observed upon stimulation of these cells with LPA.

SH-SY5Y cells were cultured as described previously (Young *et al.* 1999). For experiments measuring the release of $^{45}Ca^{2+}$, SH-SY5Y cells were washed twice in cytosol-like buffer (CLB, in mM: KCl, 135; $MgCl_2$, 2.5; EGTA, 0.05; $CaCl_2$ 0.02; Hepes, 20; pH 7.1) before being resuspended in CLB supplemented with 100 µg/ml β-escin, 10 mM creatine phosphate, 10 U/ml creatine kinase, 2 mM ATP and 1.7 µCi/ml $^{45}Ca^{2+}$. Incubations were for 2 min at 37 °C, unless otherwise stated, and reactions were terminated by centrifugation. For fluorimetric measurements of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) fluo-3 loaded SH-SY5Y cells, grown on coverslips, were incubated with Krebs/Hepes buffer (KHB, in mM: Hepes, 10; $NaHCO_3$, 4.2; $MgSO_4.7H_2O$, 1.2; KH_2PO_4, 1.2; KCl, 4.7; NaCl, 118; pH 7.4) at room temperature, and imaged using a Noran Oz confocal laser scanning system at a sample rate of 7.5 Hz.

Incubation of β-escin permeabilised SH-SY5Y cells with 20 µM $InsP_3$ resulted in release of 78 ± 2% of the total pool of $^{45}Ca^{2+}$ as determined by the addition of 20 µM ionomycin. Sphingosylphosphorylcholine (SPC) and sphingosine (SPH) both at 50 µM released 25 ± 3% (n=11), and 31 ± 8% (n=5) of the ionomycin releasable pool. In contrast, neither sphingosine 1-phosphate (SPP), dimethylsphingosine (DMS), or n-acetylsphingosine (all at 50 µM) stimulated the release of $^{45}Ca^{2+}$. The effect of SPH was inhibited (52 ± 16 and 47 ± 6% inhibition of 30 and 50 µM SPH, respectively) by pretreating the cells for 10 min with 30µM DMS, an inhibitor of sphingosine kinase, suggesting that the Ca^{2+} releasing effect of SPH was due to it first being converted to SPP. The time-courses of SPH and SPC-induced $^{45}Ca^{2+}$ release were notably slower than that of $InsP_3$ with $t_{1/2}$ values of 21 and 18 s, respectively (n=3). Concentration-response data indicated that $^{45}Ca^{2+}$ release was only measurable at sphingolipid concentrations above 10 µM. Co-addition of 50 µM SPH to either 5 or 20 µM $InsP_3$ did not alter the $^{45}Ca^{2+}$ release response to $InsP_3$ alone, suggesting that these compounds utilise the same intracellular Ca^{2+} pool.

The addition of LPA (10^{-8} - 10^{-5} M) to fluo-3 loaded SH-SY5Y cells resulted in the production of intracellular Ca^{2+} "puffs" that were indistiguishable from concentration-matched applications of the muscarinic agonist methacholine (MCH). Incubation of SH-SY5Y cells with 1 mM MCH for 20 h, causes a marked down-regulation of the $InsP_3$ receptor ($InsP_3R$) (Young *et al.*, 1999) and prevented LPA from stimulating Ca^{2+} puffs, suggesting these events were $InsP_3R$ mediated. However, this pretreatment did not prevent the global intracellular Ca^{2+} response to LPA (which has previously been shown to be DMS-sensitive, Young *et al.*, 1999). This data suggests that LPA may increase $[Ca^{2+}]_i$ via the production of intracellular sphingolipids. This increase in $[Ca^{2+}]_i$ recruits $InsP_3Rs$ to produce elementary Ca^{2+} events, but does not require $InsP_3Rs$ for a global response.

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16P SOMATOSTATIN INHIBITS ELECTRICAL ACTIVITY IN THE MURINE PANCREATIC β-CELL LINE, MIN 6, BY ACTIVATION OF AN INWARDLY-RECTIFYING K^+ CHANNEL

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A rise in intracellular calcium is a key signal in the stimulation of glucose-induced insulin secretion from the pancreatic β-cell. This increase is primarily due to influx through voltage-gated Ca^{2+} channels activated during glucose-induced electrical activity (GIEA). Somatostatin (SRIF-14) is a potent inhibitor of insulin secretion, of which one mode of action is by inhibition of electrical activity (Sharp, 1996). Western blots of extracts from the murine pancreatic ß-cell line MIN-6, indicate the presence of all five somatostatin receptor subtypes: sst_1, $sst_{2a/2b}$, sst_3, sst_4 and sst_5. Using patch-clamp methods we have investigated the functional importance of the different receptor subtypes in the ability of somatostatin to inhibit GIEA, and insulin secretion, in MIN-6 cells.

Electrophysiological recordings were performed on single cells using the perforated-patch patch-clamp technique at 32°C (Dalle *et al.*, 1999). In current-clamp, 75% of cells tested responded to 10 mM glucose with continuous action potential firing. Within 30 s of its application, 100 nM SRIF-14 inhibited the action potential firing rate (APFR) by 56±8% (n=22). This was associated with hyperpolarization of the membrane potential by 10±2.1 mV (n=11) and a decrease of the input-resistance by 0.5±0.2 GΩ (n=6): data indicative of activation of an outward current. In 31% of cells tested, SRIF-14 abolished GIEA. Similar affects were observed with 100 nM SRIF-28. The effects of SRIF-14 were mimicked by 100 nM BIM-23056 and 100 nM L-362,855, partial agonists of sst_5 (Williams *et al.* 1997), which inhibited the APFR by 37±15% (n=6) and 38±10% (n=6) respectively. BIM-23027 and NNC-269,100 (100 nM), which show selective agonism towards sst_2 (Williams *et al.* 1997) and sst_4 respectively (Liu *et al.* 1998), were without effect.

In voltage-clamp, 100 nM SRIF-28 activated an inwardly rectifying K^+ current with a slope conductance of 93±23 pS (n=4; at –90 mV) and a reversal potential of -79±3.5 mV (n=5; 5.6 mM external K^+). Raising the extracellular concentration of K^+ to 56 mM increased the slope conductance to 1±0.2 nS (n=17; at –45 mV) and depolarized the reversal potential to -18±2.6 mV (n=12). Neither 1 µM BIM-23056, nor 1 µM L-362,855 activated a current, however at this concentration, both compounds markedly reduced the current activated by 0.1 µM SRIF-28 to 4±13% (n=3) and 11±14% (n=3) of control value respectively. Glibenclamide (0.1 µM), a specific blocker of the β-cell isoform of the ATP-sensitive K^+-channel (K-ATP, Gribble et al., 1998) did not inhibit currents activated by SRIF-28. In conclusion, somatostatin inhibits GIEA in MIN-6 through activation of an inwardly rectifying K^+ channel which is distinct from the K-ATP channel. This appears to be mediated by activation of sst_5 receptors.

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17P SOMATOSTATIN $sst_{2(a)}$ AND $sst_{2(b)}$ RECEPTORS MEDIATED OPPOSING PROLIFERATIVE EFFECT BY DIFFERENTIAL GBγ-ACTIVATION OF DISTINCT SIGNALLING PATHWAYS

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We have investigated the ability of somatostatin receptor splice variants to exhibit preferential coupling to distinct Gα protein pools to explain the opposing effects the receptors mediate on cell proliferation. Although the rat $sst_{2(a)}$ and $sst_{2(b)}$ receptor isoforms both potently inhibit adenylate cyclase activity when recombinantly expressed in Chinese hamster ovary (CHO-K1) cells (Schindler *et al.*, 1998), only the $sst_{2(b)}$ receptor mediates an increase in the re-population of denuded areas in an otherwise confluent cell monolayer (Alderton *et al.*, 1998). By contrast, somatostatin in $sst_{2(b)}$ expressing cells has no effect on the re-population induced by basic fibroblast growth factor (bFGF), which is potently inhibited following activation of $sst_{2(a)}$ receptors.

Somatostatin-stimulated labelling of receptor-coupled α-subunits with [^{35}S]-GTPγS was quantified using an immunoprecipitation strategy (Burford *et al.*, 1998). In the presence of 1μM GDP, somatostatin (300nM) increased total [^{35}S]-GTPγS (0.2nM) binding to $CHOsst_{2(a)}$ membranes by 644 ± 24% over basal (pEC_{50}: 8.9 ± 0.1) and for $CHOsst_{2(b)}$ membranes by 501 ± 17% (pEC_{50}: 8.7 ± 0.1) (n = 5, both data sets). Optimal agonist-stimulated [^{35}S]-GTPγS-binding following immunoprecipitation with $G\alpha_{i1/2}$, $G\alpha_{i3}$ or $G\alpha_o$ antibodies was resolved in the presence of 100μM GDP. Immunoprecipitation of $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{13}$ was performed with 1μM GDP using membranes from pertussis toxin-pretreated cells (100 ngml^{-1}, 18 h). Stimulated $sst_{2(a)}$ and $sst_{2(b)}$ receptors induced significant labelling of $G\alpha_{i2}$ (400 ± 67 and 360 ± 34% over basal, respectively), $G\alpha_{i3}$ (660 ± 113 and 448 ± 113%) and $G\alpha_o$ (120 ± 51 and 110 ± 25%) but not of $G\alpha_{q/11}$ (38 ± 35 and 3 ± 8%) or $G\alpha_{13}$ (12 ± 14 and 17 ± 17%). However, the preferred coupling for the receptor types appeared similar ($G\alpha_{i3}$ > $G\alpha_{i2}$ >> $G\alpha_o$) with the exception of a small but significant difference in the labelling of $G\alpha_s$ by $sst_{2(a)}$ (106 ± 37%) but not by $sst_{2(b)}$ receptors (4 ± 15%) (n = 3, for all data sets; P<0.01).

We have previously demonstrated that the proliferative effect of the sst_2 receptor is dependent on the release of Gβγ subunits (Sellers, 1999). Similarly, the proliferative effect mediated by $sst_{2(b)}$ receptors as well as the antiproliferative effect of the $sst_{2(a)}$ isoform against bFGF-induced growth were both inhibited by transient expression of the βγ-sequestrant, transducin (Table 1).

Table 1. Effect of transient expression of cDNA3 (Mock) or cDNA3 incorporating transducin on the mean number of cells (x10^3 ± SEM) harvested from a single coverslip following incubation with somatostatin (100nM), in the presence or absence of bFGF (10 ngml^{-1}), 24 h following application to partially denuded $CHOsst_{2(a)}$ or $CHOsst_{2(b)}$ monolayers.

	$sst_{2(a)}$		$sst_{2(b)}$	
Treatment	**Mock**	**Transducin**	**Mock**	**Transducin**
Basal	167 ± 2	161 ± 5	149 ± 4	142 ± 2
Somatostatin	155 ± 2	163 ± 2	[a]271 ± 1	[c]187 ± 7
bFGF	[a]280 ± 5	[a]279 ± 6	[a]281 ± 3	[a]277 ± 3
Som + bFGF	[b]168 ± 4	[c]217 ± 9	285 ± 4	279 ± 8

[a] P<0.001 *versus* Basal; [b] P<0.01 *versus* bFGF; [c] P<0.01 *versus* Mock; n = 4-6.

The proliferative function of $sst_{2(b)}$ receptors is dependent on the activation of Akt, whereas $sst_{2(a)}$ receptor-mediated inhibition of bFGF-induced growth requires p38 kinase (Sellers *et al.*, this meeting). Both the somatostatin-induced (100nM) phosphorylation of these kinases by Western detection following stimulation of the respective receptors, was markedly attenuated in cells transiently expressing transducin. In conclusion, the opposing proliferative responses of the somatostatin sst_2 receptor isoforms are mediated through distinct transduction pathways via the release of βγ subunits. It is possible that the sst_2 receptor splice variants which differ only in composition of their COOH termini (Schindler *et al.*, 1998) can preferentially select different βγ-pairings within the pertussis toxin-sensitive heterotrimeric G protein pool, thus enabling different transductional cascades to be activated.

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18P TYPE 3 INOSITOL TRISPHOSPHATE RECEPTORS IN RINm5F CELLS MEDIATE QUANTAL Ca^{2+} RELEASE AND ARE BIPHASICALLY REGULATED BY CYTOSOLIC Ca^{2+}

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Biphasic regulation of inositol trisphosphate ($Ins P_3$) receptors by cytosolic Ca^{2+} is well documented in cells expressing predominantly type 1 or type 2 $Ins P_3$ receptors. The effects of cytosolic Ca^{2+} on type 3 $Ins P_3$ receptors are less clear. A recent single channel analysis of RIN5F cells suggested that type 3 $Ins P_3$ receptors are stimulated, but not inhibited by cytosolic Ca^{2+} (Hagar *et al.*, 1998); this conflicts with results from another cell line that expresses largely type 3 $Ins P_3$ receptors (Missiaen *et al.*, 1998). Here, we examine the effects of cytosolic Ca^{2+} on $Ins P_3$-induced Ca^{2+} release in RINm5F cells, which express predominantly type 3 $Ins P_3$ receptors.

The effects of $Ins P_3$ on unidirectional $^{45}Ca^{2+}$ efflux from permeabilized RINm5F cells were examined in cytosol-like media (Marshall & Taylor, 1993) buffered at different free $[Ca^{2+}]$ ($[Ca^{2+}]_c$). Increasing $[Ca^{2+}]_c$ from ~4nM to 186nM caused a 3.4-fold increase in the sensitivity of the Ca^{2+} stores to $Ins P_3$: the EC_{50} for $Ins P_3$ fell from 281±15nM to 82±2nM (mean±sem, n≥3). Further increasing $[Ca^{2+}]_c$ caused a concentration-dependent inhibition of $Ins P_3$-evoked Ca^{2+} release resulting in a 10-fold decrease in the sensitivity of the stores when $[Ca^{2+}]_c$ was 2.4μM, and a more than 3000-fold decrease when $[Ca^{2+}]_c$ was 100μM. The inhibition caused by increasing $[Ca^{2+}]_c$ to 100μM reversed completely within 1min of restoring $[Ca^{2+}]_c$ to 186nM. Ruthenium red, which was included in the lipid bilayer analyses to inhibit ryanodine receptors (Hagar *et al.*, 1998), did not prevent the inhibition of $Ins P_3$ receptors by increased $[Ca^{2+}]_c$.

The time course of the $^{45}Ca^{2+}$ release established that submaximal concentrations of $Ins P_3$ stimulated $^{45}Ca^{2+}$ release from only a fraction of the $Ins P_3$-sensitive Ca^{2+} stores. After 5min, 60nM, 100nM and 200nM $Ins P_3$ released only 62±6%, 82±5% and 94±2 % (n=4), respectively, of the $Ins P_3$-sensitive Ca^{2+} stores. $Ins P_3$-evoked Ca^{2+} mobilization from RINm5F cells is therefore a quantal process, as it is in many other cell types.

In conclusion, the $Ins P_3$ receptors of RINm5F cells, which are largely type 3, are biphasically regulated by cytosolic Ca^{2+} and release Ca^{2+} in a quantal manner. The lack of inhibition observed during bilayer analyses of $Ins P_3$ receptors from RIN5F cells (Hagar *et al.*, 1998) may have resulted from loss of an accessory protein likely to be required for mediating the inhibitory effects of cytosolic Ca^{2+}.

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19P COMPARISON OF THE EFFECTS OF ACETYLCHOLINE AND VASOACTIVE INTESTINAL PEPTIDE ON INTRACELLULAR Ca^{2+} AND ZYMOGEN GRANULE EXOCYTOSIS IN RAT PANCREATIC ACINAR CELLS

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Stimulation of pancreatic acinar cells with acetylcholine (ACh) induces a rapid increase in cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$, that is an essential step in the signal transduction cascade for exocytosis in these cells (Thorn *et al.*, 1993). The cAMP-elevating secretagogue vasoactive intestinal polypeptide (VIP) also stimulates enzyme secretion in these cells (Collen *et al.*, 1982), but its effects on the $[Ca^{2+}]_i$ of isolated acinar cells are less clear. To compare these two agonists, we have used digital imaging to measure exocytosis, $[Ca^{2+}]_i$ and cell swelling in dispersed, rat pancreatic acinar cells.

Acinar cells were isolated by collagenase digestion of pancreata obtained from male Wistar rats (175-225 g). We quantitated exocytosis of individual zymogen granules using continuous time-differential analysis (Terakawa *et al.*, 1991) of brightfield digital images (collected every 1.25 s). Swelling of single cells or small acini was determined from these brightfield recordings by subtraction of the cell/acinus areas measured 10 s before and 30 s after the application of the agonists. In separate experiments, changes in $[Ca^{2+}]_i$ were monitored by digital imaging of fura-2 fluorescence ($R_{340/380}$ determined every 2.5 s). All experiments were carried out at 37 °C. All data are expressed as mean ± s.e.m. and were compared using an unpaired, two-tailed Student's *t* test.

Application of 10 μM ACh (6 min) stimulated a biphasic secretory response in acinar cells with the rate of exocytosis rising from a basal level of 0.27 ± 0.07 events/min/cell to a peak of 8.68 ± 1.17 events/min/cell within 30 s and then subsiding to 1.22 ± 0.24 events/min/cell after 5 min of treatment (n = 50). Application of 100 nM VIP stimulated a similar, albeit much smaller, response. The peak rate of exocytosis with VIP was only 0.83 ± 0.21 events/min/cell within 30 s (P <0.001 compared with ACh-treated cells), falling to 0.55 ± 0.21 events/min/cell after 5 min of treatment compared with a basal rate of 0.25 ± 0.08 events/min/cell (n = 29). Application of both ACh or VIP induced a rapid swelling of the cells which reached its maximum after ~2 s and persisted until the agonist was removed. The increase in cell/acinus area was 4.99 ± 0.35 % (n = 27) for 10 μM ACh and 4.88 ± 0.89 % (n = 11) for 100 nM VIP; there was no significant difference between the two means.

In fura-2 experiments, a biphasic increase in $R_{340/380}$ was observed during administration of 10 μM ACh. First, a transient rise from a basal value of 0.25 ± 0.002 to a peak of 0.91 ± 0.13 in the first 10 s, followed by a sustained plateau of 0.33 ± 0.002 (5-6 min; n = 26). However, 100 nM VIP produced a smaller and slower increase in $R_{340/380}$, with a peak of 0.34 ± 0.04 reached after 60 s, that was maintained while VIP was present in the dish (0.32 ± 0.002 after 5-6 min; P <0.01 compared with a basal value of 0.25 ± 0.001, n = 43).

In conclusion, when rat pancreatic acinar cells were stimulated with either ACh or VIP there was a close correlation between zymogen granule exocytosis and the increase in $[Ca^{2+}]_i$. However, both agonists led to comparable increases in cell volume despite a large difference in the Ca^{2+} signals.

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20P ENDOTHELIN STIMULATES A NIFEDIPINE-SENSITIVE STORE-FILLING Ca INFLUX BUT INHIBITS L-TYPE Ca CHANNELS IN MICROVASCULAR SMOOTH MUSCLE OF RAT RETINA *IN VITRO*

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Stimulation of endothelin Et_A receptors causes a transient elevation of cell $[Ca^{2+}]$ due to store release followed a sustained elevation in cell $[Ca^{2+}]$ due to Ca influx. In microvascular smooth muscle, this influx could be via voltage dependent L-type Ca^{2+} channels or through a voltage independent pathway that attempts to refill the Ca^{2+} stores. We report here that endothelin shifts Ca^{2+} influx from L-type Ca^{2+} channels to a pathway which does not depend on a change in membrane potential but is nevertheless inhibited by nifedipine.

Retinal microvessels were enzymatically dispersed from freshly killed rats (SD, either sex, 200-300 g), loaded with fura 2/AM and cell Ca^{2+} in the smooth muscle cells estimated by microfluorimetry. In separate experiments, perforated patch recordings, using electrodes filled with amphotericin, were made from single microvascular smooth muscle cells which remained attached to the vessel.

Endothelin-1 produced a rise in cell $[Ca^{2+}]$ that was sustained after endothelin had been washed out. Endothelin also completely blocked the voltage dependent L-type Ca^{2+} channels as measured in divalent free solution (Curtis & Scholfield, 1999a). In contrast to other effects of endothelin, the voltage dependent inward current recovered within 10 sec of washing out the endothelin.

Nifedipine (1 μM) in resting vessels produced a small fall in estimated cell $[Ca^{2+}]$ (by 16%; see also fig 1). Endothelin increased cell $[Ca^{2+}]$ from 106±10 which after a short transient phase, stabilised at a value of 265±19 nM. During this plateau phase of endothelin action, nifedipine reduced cell $[Ca^{2+}]$ from 265±19 to 123±12 nM (12 vessels, p<0.001). On washing out endothelin, cell $[Ca^{2+}]$ remained elevated (increased by 6%) and nifedipine also reduced cell $[Ca^{2+}]$ to the resing level (from 276±21 to 101±9 nM, P<0.001).

In another series of experiments, microvascular smooth muscle cells were held at –80 mV and treated with 10 mM caffeine for 10 sec to release store Ca^{2+}. The stores were allowed to refill for 120 sec in the presence of nifedipine (1 μM) and caffeine re-applied to assess the amount of refilling. The amount of Ca^{2+} released was monitored using the Ca^{2+}-activated Cl—conductance (Curtis & Scholfield, 1999b) and this was reduced by 31±6% (p<0.01, n=6) compared to that without nifedipine during the refilling period. Nifedipine added at the same time as caffeine had no effect.

Cyclopiazonic acid (CPA) blocks Ca^{2+} reuptake into Ca^{2+} stores. At 2 μM, CPA elevated cell Ca and this elevation was completely reversed by nifedipine (fig 1). CPA had no effect on the resting membrane potential of in 4 other cells (-45 mV).

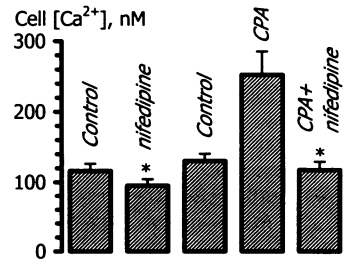

Figure 1. Resting cell $[Ca^{2+}]$ of 13 intact microvessels in normal, nifedipine (1 μM), CPA and nifedipine with CPA (bars are means and SEMs). Measurements were made at 3 min intervals and the asterisks show P<0.002.

These experiments suggest that the main Ca^{2+} store filling influx pathway in these cells is through one which is blocked by a low nifedipine concentration but is not mediated though classical voltage dependent L-type Ca^{2+} channels. This nifedipine sensitive store-filling pathway is also activated with endothelin, while at the same time, there is a shift away from voltage dependent Ca^{2+} influx.

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21P TRYPSIN STIMULATES THE NFκB SINGALLING PATHWAY IN NCTC 2544 TRANSFECTED WITH HUMAN PROTEASE-ACTIVATED RECEPTOR-2

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Protease-activated receptors (PARs) are a four-member family of seven transmembrane domain G-protein coupled receptors activated by a novel 'tethered ligand'. Human PAR-2, which is activated by trypsin, tryptase and the specific signal hexapeptide SLIGKV, has been reported to be present on epidermal keratinocytes, where it may play a role in regulating the proliferation, differentiation and inflammation in these cells (Derian *et al*, 1997; Wakita *et al*, 1997). In this study PAR-2 activation of the transcription factor NFκB, important in the regulation of many proinflammatory mediators (reviewed by Thanos & Maniatis, 1995), was investigated by stable expression of the receptor in the keratinocyte cell line NCTC 2544.

Activation of NFκB-DNA binding in nuclear extracts was measured by electrophoretic mobility shift assay (EMSA) and the translocation of NFκB to the nucleus was visualised by confocal microscopy. Inhibitory kappa B kinase (IKK) activity was assessed by *in vitro* kinase assay and the loss of IκBα from whole cell lysates was investigated by Western blotting. Each value is the mean ± s.e. of 3 separate experiments.

In NCTC 2544 cells transfected with PAR-2, 30nM trypsin was found to activate NFκB DNA -binding in nuclear extracts with a maximal increase in binding of 3.5 ± 0.3 fold at 60 min. This was accompanied by nuclear translocation of NFκB. Trypsin also stimulated a transient loss in the cellular expression of IκBα. The activities of the kinases that regulate the phosphorylation of IκB, IKKα and IKKβ, were also increased following trypsin stimulation. A 3.7 ± 0.2 and 5.4 ± 0.1 fold increase in the activity of IKKα and IKKβ was observed over 30 minutes. The protein kinase C activator phorbol myristate acetate (PMA) also stimulated NFκB-DNA binding, loss in the cellular expression of IκB isoforms and an increase in IKKα and β activity comparable to that observed for trypsin (fold stim 4.8 ± 0.7 for IKKα and 4.9 ± 0.1 for IKKβ). Chronic treatment with PMA (30nM, 24 h) abolished trypsin mediated NFκB translocation, IκBα loss, activation of IKKβ and reduced IKKα by 14.4 ±7%. Furthermore, the PKC inhibitor GF109203X (500nM) at a concentration that abolished PMA-stimulated NFκB translocation and IKK activity, only partially reduced trypsin-stimulated IKKα activity by 49.8 ± 8.2% and inhibited IKKβ activation by 76.4 ± 9.2%. Translocation of NFκB to the nucleus was not effected by GF109203X.

These findings indicate that both typical and atypical PKC are likely to be involved in the regulation of IKK and hence NFκB activity mediated by PAR-2 in NCTC2544 cells. Activation of this important inflammatory transcription factor by PAR-2 gives further support to previous findings indicating that PAR-2 may play a role in the regulation of inflammatory responses in the epidermis and other tissues.

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22P THE EFFECT OF H_2O_2 ON LIPOPOLYSACCHARIDE-STIMULATED NF-κB ACTIVATION IN RAT AORTIC SMOOTH MUSCLE CELLS

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Cellular exposure to oxidants such as the reactive oxygen intermediate hydrogen peroxide (H_2O_2) has been shown to lead to cell toxicity in the form of cell injury or apoptosis. In part, this is mediated via activation of homologues of the mitogen activated protein kinases, including the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 (Verhiej et al., 1996 & Goillot et al., 1997) and well as the transcription factor nuclear factor kappa B (NF-κB) (Meyer et al., 1993 & Schreck et al., 1991). NF-κB is regulated by inhibitory kappa B (IκB) proteins α, β, and ε which sequester NF-κB in the cytoplasm. IκB phosphorylation by the inhibitory kappa B kinase subunits IKKα and β initiate their ubiquitination and subsequent degradation freeing NF-κB to translocate to the nucleus and activate transcription of target genes (reviewed by Zandi et al., 1997). In this study we examined the effect of H_2O_2 on lipopolysaccharide (LPS)-stimulated NF-κB activation in rat aortic smooth muscle cells (RASMC).

NF-κB-DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) whilst IκBα β and ε protein expression was determined by Western blotting. IKKα and β kinase activities were measured by *in vitro* kinase assay following immunoprecipitation. All values represent the mean ± s.e.m. of at least 3 individual experiments.

In RASMC LPS (100μg/ml) induced NF-κB-DNA binding in a time-dependent manner reaching a maximum by 60 min and remaining sustained for up to 6h. LPS also stimulated a transient and rapid loss in IκBα maximal by 30 min and a slower more sustained loss in IκBβ and ε isoforms. Further experiments showed that LPS also activated IKKα as early as 15 min, giving a maximum response at 30 min and returning to basal values by 2 h. Activation of IKKβ also showed a similar time course. Pretreatment with 0.5 mM H_2O_2 reversed LPS-stimulated NF-κB-DNA-binding. 0.5mM H_2O_2 pretreatment also inhibited LPS stimulated loss of IκBα, β and ε (IκBα % control LPS = 5.8% ±, 2.87 LPS + 0.75 mM H_2O_2 = 73.4% ± 27.66). Furthermore, LPS-stimulated IKKα and β kinase activity was abrogated by pretreatment with 0.5mM H_2O_2 (IKK-α fold stim, LPS = 7.96 ±, 3.48 LPS + 0.5 mM H_2O_2 = 0 ± 0.13).

This study shows that H_2O_2 reverses LPS-stimulated activation of NF-κB signalling at the levels of NF-κB, IκB and IKK in RASMC indicating that this effect is mediated at the level of IKK or via an event upstream.

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23P REGULATION OF c-Jun-N-TERMINAL KINASE AND p38 MAP KINASE BY PROTEINASE ACTIVATED RECEPTOR-2 IN HUMAN SKIN EPITHELIAL CELL LINE NCT2544

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Proteinase activated receptor-2 (PAR-2) is a recently identified G-protein coupled receptor which is activated by trypsin or tryptase (Nystedt et al., 1994). Although its biological functions are not fully understood, potential roles in inflammatory responses, tissue repair and skin related disorders have been suggested (Santulli et al., 1995; Derian et al., 1997; Kong et al.,1997). The stress-activated protein kinases (SAPK), c-Jun N-terminal kinase (JNK) and p38 MAPK are thought to regulate proliferation and differentiation in keratinocytes. Therefore in this study we examined the mechanism by which PAR-2 activates JNK and p38 MAPK in human skin epithelial cells.

The human skin epithelial cell line NCTC2544 was stably transfected with a plasmid encoding human PAR-2 cDNA. The clone which responded to trypsin and PAR-2 activating peptide SLIGKV in inositol phosphate accumulation was isolated and utilized in further experiments. The JNK and p38 MAPK activities were measured by in vitro kinase assay using their specific recombinant substrates following immunocomplex precipitation. Each value represents the mean±SE of at least 3 independent experiments.

Initial results showed high inositol phosphate accumulation (72.8±7.2 fold of basal) in a PAR-2 transfected clone responded to trypsin (100nM), whereas no response was observed in blank-vector transfected cells. Trypsin (100nM) stimulated JNK activity peaked at 30 min at approximately 20 fold of basal (22.3±6.6). Similar activation of p38 MAPK was observed at a peak time of 15 min with approximately 25 fold of basal (24.9±12.4). The activation of these kinases were mimicked by the protein kinase C (PKC) activator 12-O-tetradecanoylphorbol 13-acetate (TPA) and the cytokine tumour necrosis factor-α (TNF-α). Chronic TPA pretreatment (30nM, 18hr) essentially abolished trypsin-stimulated JNK or p38 MAPK activation (96±2% and 93±5% inhibition respectively) and also responses to both TPA and TNFα. However, only partial inhibition of trypsin-stimulated JNK and p38 MAPK activity (12±15% and 36±9% respectively) was observed by pretreatment with 500nM of isotype-specific PKC inhibitor GF109203X, a concentration which abolished the responses to TPA. TNFα-stimulated JNK and p38 MAPK activity was not affected by GF109203X.

Our data shows that trypsin can activate both JNK and p38 MAPK in NCTC2544 cells through the novel G-protein coupled receptor PAR-2. PKC-downregulation and PKC-inhibitor treatment studies indicate both typical and atypical isoforms of PKC may be involved in PAR-2 mediated JNK and p38 MAPK activation in NCTC2544 cells.

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24P A POSSIBLE ROLE FOR PROTEIN KINASE C (PKC) ISOFORMS IN A_1 ADENOSINE RECEPTOR SIGNALLING TO THE NUCLEUS

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We have previously shown that A_1 adenosine receptor stimulated expression of a c-fos promoter-driven luciferase reporter gene involves pertussis sensitive Gi/o-proteins and MEK1, the upstream activator of ERK [Megson *et al*, 1998]. Here, involvement of PKC in A_1 adenosine receptor stimulation of the c-fos promoter was investigated and compared to that by the PKC-activator phorbol-12,13-dibutyrate (PDBu).

Two Chinese hamster ovary (CHO-K1) cell lines were used: i) stably expressing human adenosine-A_1 receptors (Am1CHO) and ii) Am1CHO cells secondarily transfected with a c-fos promoter-driven firefly luciferase reporter gene. For luciferase assays, cells were grown to confluency in 24 well plates and then serum starved in DMEM/F-12 (2 mM L-Glutamine) for 24 hrs. Inhibitors were added 30 min prior to the addition of the A_1 receptor agonist, N^6-cyclopentyladenosine (CPA 10^{-6} M) or PDBu (10^{-6} M). In some experiments, cells were treated with PDBu (10^{-6} M) for 24 hrs prior to agonist stimulation. After a 6 hr incubation period, cells were lysed and assayed using the Promega luciferase assay system. Western blotting of whole cell lysates (by 7.5% SDS-PAGE separation, transfer to nitrocellose and detection with PKC isoform-specific antibodies and ECL), was used to identify those isoforms downregulated by PDBu. Expression levels were quantified by densitometry. The data are expressed as mean ± SEM.

Table 1: Effect of 24hr PDBu treatment on PKC expression in Am1CHO (n=3). Results confirmed in Am1fosluc3 (n=1), not shown. np = not present

PKC isoform	α	β	δ	ε	ι	ζ	μ	θ
% inhibition	98±1	np	93±7	97±7	13±13	0	3±24	np

Table 2: Percentage inhibition of c-fos promoter driven luciferase expression by PKC inhibitors

PKC inhibitor		CPA-mediated response	PDBu-mediated response	n
24hr PDBu treatment		13.8 ± 7.6	100	3
Ro318220	10^{-5}M	100	100	3
Gö6976	10^{-5}M	52.5 ± 9.3	47.9 ± 6.0	5
	10^{-7}M	38.1 ± 3.4	30.1 ± 3.5	5
Gö6983	10^{-5}M	57.8 ± 11.1	100	4
	10^{-7}M	3.4 ± 7.2	30.7 ± 7.2	4
Bisindolylmaleimide I (GF109203X)	10^{-5}M	78.7 ± 7.4	100	3

In summary, the minimal effect of PKC down-regulation suggests the classical and novel PKC isoforms α, δ and ε are not involved in adenosine A_1 receptor signalling to the nucleus (Tables 1 & 2). However, partial inhibition of the A_1-mediated response by Gö6976 and Gö6983, and almost complete inhibition by Bisindolylmaleimde I and Ro318220 suggest a role for PKCμ and/or an atypical isoform.

KJH holds a Wellcome Prize Studentship.

Megson, A. C., Hill, K. J., and Hill, S. J., (1998) Naunyn-Schmiedeberg's Arch. Pharmacol., **358(1) S1**, 2921

25P ATTENUATION OF Ca^{2+} SIGNALLING BY INDUCTION OF TYPE II NITRIC OXIDE SYNTHASE IN RAT HEPATOCYTES

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Ca^{2+} and nitric oxide (NO) are both key signalling molecules implicated in a wide variety of cellular processes. We have previously demonstrated that NO donors and endothelial derived-NO (Patel *et al.*, 1999) can potentiate inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} signals in rat hepatocytes. In the present study we have examined the effects of induction of the type II isoform of NO synthase (NOS) on isolated hepatocyte Ca^{2+} signals.

Hepatocytes were isolated from male rat livers and were maintained in culture for 18 hours either in the absence or presence of a combination of lipopolysaccharide (10 μg/ml), tumor necrosis factor-α (500 U/ml), interleukin 1-β (15 U/ml) and interferon-γ (100 U/ml). Single cell cytosolic and luminal [Ca^{2+}] was measured by digital imaging microscopy of the Ca^{2+}–sensitive indicators fura-2 and fura-2 FF, respectively. For luminal Ca^{2+} measurements, cells were permeabilised with digitonin to gain access to intracellular Ca^{2+}stores. Immunoblotting and immunocytochemistry were performed with a monoclonal antibody raised to the murine macrophage type II NOS.

Exposure of hepatocytes to lipopolysaccharide in the presence of tumor necrosis factor-α, interleukin 1-β and interferon-γ induced expression of a 142 ± 5 KDa (n=3) protein that was recognised by an antibody raised to type II NOS of murine macrophages. This protein was expressed diffusely throughout the cytosol and concentrated in certain cytosolic regions. The percentage of cells that responded with an increase in cytosolic [Ca^{2+}] to both submaximal and supramaximal concentrations of the IP_3-forming agonist, vasopressin (VP) was markedly reduced in these induced cultures relative to control cultures (Table 1). The inhibitory effects of NOS induction could be reversed by carboxy PTIO (500 μM), a NO scavenger, and PPM18 (50 μM), an inhibitor of NOS expression (Table 1). 8-Bromo cyclic GMP (500 μM),but not 8-bromo cyclic AMP, mimicked the effects of induction (Table 1). Induction did not however affect release of Ca^{2+} from intracellular stores by IP_3 (n = 37 cells). A submaximal concentration of IP_3 (300 nM) released 20 ± 1 % and 20 ± 2 % of the ionomycin-sensitive Ca^{2+} store in control and induced cultures, respectively. Ca^{2+} release by a maximal IP_3 concentration (20 μM) was also similar in control (32 ± 1 %) and induced (35 ± 2 %) cultures.

These data suggest that expression of type II NOS in hepatocytes inhibits VP-dependent Ca^{2+} signals (possibly via cyclic GMP synthesis) at a locus upstream of the IP_3 receptor. Loss of signalling pathways in hepatocytes is likely to contribute to liver failure in pathological conditions such as septic shock.

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Culture incubation	Responsive cells (%) 2-5 nM VP	200 nM VP
Control	73 ± 2	85 ± 1
Induced	14 ± 3	41 ± 7
Induced + carboxy PTIO	68 ±9	87 ± 7
Induced + PPM18	54 ± 4	75 ± 5
8-Bromo cyclic GMP	23 ± 7	54 ± 9

Table 1: Responsiveness of hepatocyte cultures to VP. Data are means ± s.e.m. for 3-4 independent cultures.

26P EFFECTS OF MORPHINE ON THE RESPONSE TO HAEMORRHAGE AFTER PRIMARY THORACIC BLAST INJURY IN THE ANAESTHETISED RAT

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Primary blast injury to the thorax results in bradycardia, hypotension and apnoea (Guy *et al.* 1998). The bradycardia and apnoea are due to a vagal reflex (Ohnishi *et al.* 1997). Haemorrhage induces a biphasic chronotropic response: initial tachycardia followed by a vagally-mediated bradycardia (Little *et al.* 1989). The response to thoracic blast abolishes the first and augments the second (bradycardic) phase of the response to haemorrhage. By contrast, morphine attenuates the bradycardic response to simple haemorrhage in the absence of blast (Ohnishi *et al.* 1997) but does not significantly attenuate the response to blast (Kirkman *et al.* 1999). This study investigated the effects of morphine on the response to blood loss after thoracic blast.

Male Wistar rats (238-269 g body weight) were used in 2 groups. Surgical anaesthesia was induced and maintained with isoflourane (3.0-3.5% in O_2/N_2O, FIO_2=0.5). Following surgery anaesthesia was maintained with alphadolone/alphaxalone (19-21 $mg.kg^{-1}.h^{-1}$ iv). Heart period (HP) was measured from the electrocardiogram and mean arterial blood pressure (MBP) via the tail artery. Body temperature was maintained at 38.0°C. At the end of the study the animals were killed with an overdose of anaesthetic.

Both groups received a blast wave focused on the ventral thorax (Guy *et al.* 1998). 5 min later Group I (n=8) received 0.9% saline (1 $ml.kg^{-1}$ iv) while Group II (n=7) received morphine (0.5 $mg.kg^{-1}$ iv). 10 min after blast both groups received a haemorrhage of 40% total estimated blood volume (BV, 6.06 $mg.kg^{-1}$, Ohnishi *et al.* 1997) at 2% $BV.min^{-1}$. Blast (Group I) induced a significant bradycardia (HP increasing by 194±10 ms from 145±6 ms; mean±S.E.M. $P<0.05$, ANOVA) and hypotension (fall in MBP of 53±5 mmHg from 101±4 mmHg). The initial response in Group II was not significantly different from that in Group I. 10 min after blast HP in Group I was 168±8 ms while MBP was 87±7 mmHg. In Group I haemorrhage produced a significant bradycardia without an initial tachycardia: HP increased by 37±6 ms above pre-haemorrhage levels after the loss of 7 %BV and continued to rise by a maximum of 51±6 ms after the loss of 24 %BV while MBP fell throughout haemorrhage. By contrast in Group II haemorrhage induced a significant tachycardia but no bradycardia: HP was initially maintained at the pre-haemorrhage level of 193±12 ms until the loss of 17 %BV thereafter falling by 21±8 ms after the loss of 30 %BV. MBP remained significantly above that seen in Group I until the loss of 30 %BV.

These results indicate that after thoracic blast morphine abolishes the bradycardic response to haemorrhage, uncovers a tachycardic response, and maintains higher arterial blood pressure.

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27P ANTI-INFLAMMATORY EFFECTS OF HEAT-KILLED *MYCOBACTERIUM VACCAE* IN MURINE ANTIGEN-INDUCED AIRWAY INFLAMMATION

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Viable *Mycobacterium bovis*-Bacille-Calmette Guerin (BCG) inhibited ovalbumin (OA) induced airway eosinophilia in the mouse (Erb *et al.*, 1998). Furthermore, heat-killed *Mycobacterium vaccae* (*M. vaccae*) inhibited murine OA-induced IgE production (Wang & Rook, 1998). Neither of these non-specific immunotherapies have reported any anti-inflammatory effects in lung tissue. Therefore, the aim of this study was to investigate if heat-killed *M.vaccae* inhibited OA-induced inflammation in the murine lung.

Female C57B1/6J mice (18-20 g) were given *M.vaccae* (table 1) or vehicle (phosphate buffered saline) intraperitoneally (i.p.) at the time of i.p. antigen sensitisation on days 0 and 12 (10 µg OA with 20 mg aluminium hydroxide). Budesonide, 0.3 mg kg^{-1}, or vehicle were administered i.p. twice daily on days 21, 22 and 23 as a positive control. Mice were challenged with aerosolised OA on day 21 (10g l^{-1}, 60 mins) and again 4 h later. 72 hour after OA challenge, inflammatory cells were recovered from the airway lumen by bronchoalveolar lavage (BAL) and from the lung tissue by collagenase digestion. In a separate group of mice, lungs were insufflated. Differential cell counts and CD4+ T lymphocytes were measured in BAL and lung tissue digest (Underwood *et al.*, 1997).

Histopathology was assessed in paraffin embedded lung tissue using a scoring system, from 0 (no pathology) to 5 (severe pathology). BAL and lung tissue results are expressed as mean ± s.e.mean. Statistical significance was determined as $p<0.05$ using Kruskal-Wallis statistical test.

OA challenge produced a significant increase in lung tissue digest eosinophilia and CD4+ lymphocytes and lung tissue histopathology score. An OA-induced increase in BAL eosinophils and neutrophils was also observed. *M.vaccae* produced a dose-related reduction in eosinophils and CD4+ T-lymphocytes in lung tissue digest (table 1). A reduction in histology score was also observed with $1x\ 10^7$ and $1x10^8$ *M. vaccae*. In addition, BAL eosinophils and neutrophils were reduced after *M.vaccae* treatment (table 1). Budesonide showed a significant reduction in BAL neutrophils and eosinophils and lung tissue histopathology score.

In conclusion, this is the first study which demonstrates an anti-inflammatory effect of heat-killed *M.vaccae* on both BAL and lung tissue in OA challenged mice. These results provide further evidence that non-specific immunotherapy may be a novel therapy for the treatment of allergic asthma.

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Table 1. Effect of *M.vaccae* (cells per mouse, i.p.) against OA-induced airway inflammation in the mouse. $^{\dagger}p<0.05$ compared with the unchallenged, vehicle group: $^{*}p<0.05$ compared with the appropriate challenged, vehicle group.

Treatment Group	Group size	Unchallenged vehicle	Unchallenged M.vaccae 1 x 10^8	Challenged vehicle (1)	Challenged M.vaccae 1 x 10^6	Challenged M.vaccae 1 x 10^7	Challenged M.vaccae 1 x 10^8	Challenged vehicle (2)	Challenged budesonide (0.3 mg kg^{-1})
BAL ($x10^3$ cells ml^{-1})									
Eosinophils	10 - 12	0.3 ± 0.2	0 ± 0	69.3 ± 20.1†	68.2 ± 17.9	38.7 ± 10.9	4.4 ± 1.4*	109.8±37.5†	10.5 ± 2.3*
Neutrophils	10 - 12	0 ± 0	0 ± 0	8.9 ± 4.6	8.3 ± 3.4	9.1 ± 2.8	1.1 ± 0.3*	15.1 ± 7.2†	1.5 ± 0.9*
Lung Tissue ($x10^3$ cells mg^{-1})									
Eosinophils	10 - 12	0.9 ± 0.1	0.8 ± 0.1	3.1 ± 0.5†	2.8 ± 0.3	2.0 ± 0.2*	1.0 ± 0.1*	2.6 ± 0.4†	2.0 ± 0.2
CD4+ Lymphocytes	10 - 12	0.14 ± 0.02	0.16 ± 0.02	0.38 ± 0.06†	0.29 ± 0.03	0.29 ± 0.03	0.22 ± 0.03*	0.31 ± 0.03†	0.16 ± 0.02*

28P NO-STEROIDS: A CLASS OF NEW ANTI-ASTHMATIC AGENTS INDUCED BRONCHODILATION ON GUINEA-PIG TRACHEA *IN VITRO*

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Under physiological conditions endogenous nitric oxide (NO) suppresses airway plasma leakage. Nevertheless, when inducible NO synthase (iNOS) is overexpressed the increase of NO production enhances this pathophysiological effect. NO may be implicated in the maintenance of airway function and in the inflammatory process occuring in asthma. Studies have shown that the release of NO relaxes the bronchial smooth muscle, reduces inflammation and protects the inner walls of adjacent blood vessels. This is the reason why NO is also used therapeutically by inhalation in bronchitis (Thebaud B *et al.*, 1999).
In the present study we examine the possibility that a novel class of anti-inflammatory drugs, obtained by addition of a chemical moiety able to release NO to steroids, could modulate the methacholine-induced contraction of guinea-pig trachea in vitro.
We compared the efficacy of NCX-1004, NCX-1005, NCX-1010/1015, and NCX-1020 (Del Soldato, 1996) with the parent compounds hydrocortisone, dexamethasone, prednisolone, and budesonide, respectively. The experiments were performed with isometric techniques on precontracted guinea-pig trachea rings by methacholine as previously described (Farmer *et al.*, 1986). The compounds were tested alone on trachea rings with or without epithelium and in the presence or absence of 10^{-4}M L-NAME or $5x10^{-6}$M ODQ (Soluble guanylate cyclase inhibitor).

Table 1 shows that parent compounds poorly induced bronchodilation for the maximum used dose (10^{-4}M). On the contrary NO-derivatives induced a significative effect up to 45.9% (NCX-1010) of the maximal relaxation induced by 10^{-4}M papaverine. The lesion of epithelium or 10^{-4}M L-NAME did not affect the relaxation induced by NO-steroids, demonstrating that this effect is not epithelium dependant and does not involve the release of endogenous NO by epithelial cells. However, the presence in the organ bath of $5x10^{-6}$M ODQ decreased the relaxation induced by NO-derivative compound in a significative manner (85.9%, $P<0.01$) showing that the observed effect is due to the release of the NO-moiety acting in the tracheal smooth muscle.

Table 1: 10^{-4}M steroids and NO-steroids relaxation (%/10^{-4}M papaverine) in the guinea-pig trachea precontracted by $3x10^{-6}$M methacholine (* $P<0.05$, n=6).

Compounds	% Relaxation	Compounds	% Relaxation
Hydrocortisone	13.8 ± 1.9	Dexamethasone	11.0 ± 2.8
NCX-1004	30.4 ± 1.9*	NCX-1005	27.8 ± 1.9*
Prednisolone	8.1 ± 0.4	Budesonide	12.6 ± 2.5
NCX-1010	45.9 ± 6.1*	NCX-1020	27.6 ± 2.7*
NCX-1015	25.1 ± 4.4*		

Taken together these data suggest that NO-steroids could induce a concentration-dependant bronchodilation. NO released from NO-steroids may enhance the steroids' efficacy in treating asthma and other respiratory diseases. Thus these new agents warrant to be further investigated for their anti-inflammatory activity and safety.

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29P CHOLINESTERASE ACTIVITIES IN THORACIC TISSUES FROM NORMOTENSIVE (WKY) AND HYPERTENSIVE (SHR) RATS

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The cholinergic system is involved in the control of the respiratory functions and the thoracic blood circulation (Norel *et al.*, 1993, 1996; Walch *et al.*, 1997). Recent data have suggested that cholinergic mechanisms may be modified in SHR rats (Buccafusco, 1996). The aim of this study was to determine acetylcholinesterase (AChE: E.C.3.1.1.7) and butyrylcholinesterase (BChE: E.C.3.1.1.8) activities in the lung, diaphragm and thoracic aorta derived from WKY and SHR rats.

Male rats (20 weeks) were anaesthetised with pentobarbital sodium (60 mg/kg). Organs were removed and homogenised. Cholinesterase activities (U/g of tissue) were determined using Ellman's technique (1961) in presence of 1 mM acetylthiocholine. BW284c51 (1 μM) or iso-OMPA (10 μM) were used to selectively inhibit either AChE or BChE activities. Statistical analysis were performed using Student's t test, $P<0.05$ was considered statistically significant.

The results are presented in Table 1. In the lung, the ChE activity measured in SHR rats was significantly greater than that in WKY rats. In addition, in rat tissues, AChE is significantly predominant in the striated muscle and BChE is significantly the major activity in the smooth muscle. The rat thoracic aorta in which BChE activity is predominant may be an appropriate model for pharmacological and functional studies of BChE.

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Table 1. Cholinesterase activities measured in tissues derived from WKY and SHR rats

	AChE activity		BChE activity	
Preparation	WKY	SHR	WKY	SHR
Lung	0.19±0.02* (6)	0.29±0.02* § (6)	0.30±0.02 (6)	0.36±0.01 § (6)
Diaphragm	0.40±0.03* (4)	0.51±0.06* (4)	0.07±0.01 (4)	0.11±0.04 (3)
Thoracic aorta	0.10±0.01* (6)	0.05±0.01* § (5)	1.25±0.15 (6)	0.93±0.18 (5)

Results are means±s.e.mean and (n) indicates the number of rats used. * and § indicate $P<0.05$ vs corresponding BChE and WKY values, respectively.

30P MUSCARINIC RECEPTORS INVOLVED IN THE RELAXATION OF HUMAN PULMONARY VEINS

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Isolated human pulmonary veins with an intact endothelium relax when stimulated with acetylcholine (ACh). In contrast, neither contraction nor relaxation is observed in human pulmonary veins when the endothelium had been mechanically removed (Walch *et al.*, 1997). These data suggest the presence of muscarinic receptors only on the endothelium. Five homologous genes, encoding for the muscarinic receptors, have been described (m1-m5; Caulfield and Birdsall, 1998). The aim of this study was to characterise the muscarinic receptor subtype(s) involved in the ACh-induced relaxation of isolated human pulmonary veins.

Pulmonary venous preparations (3-4 mm internal diameter) were carefully removed from the macroscopically normal regions of human lung tissues obtained from patients who had undergone surgery for lung carcinoma. Isolated venous preparations with an intact endothelium were set up in an organ bath system containing Tyrode's solution and incubated (30 min) with or without a selective muscarinic antagonist. Subsequently, the preparations were contracted with noradrenaline (10 μM) and challenged with ACh (1 nM - 10 mM) applied in a cumulative fashion. ACh relaxed venous preparations with a pD_2 value of 5.82±0.09 (n=16). The apparent affinity values of the muscarinic antagonists calculated from the displacement of the concentration-response relation by one or two antagonist concentrations are shown in Table 1.

The profile of apparent pK_B values for the antagonists studied coincides with that previously reported for M_1 receptors but bears little resemblance to those for the M_2-M_5 receptors (Eglen *et al.*, 1996). These data suggest that M_1 muscarinic receptors on the endothelium of the human pulmonary veins are activated by ACh and this stimulation leads to vascular smooth muscle relaxation.

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Table 1. Apparent pK_B values for muscarinic receptor antagonists on the relaxation induced by ACh in human pulmonary veins based on the shift of the concentration-response curve at either one or two concentrations of antagonist.

	atropine (1 μM)	pirenzepine (0.5 μM, 1 μM)	methoctramine (5 μM, 50 μM)	himbacine (0.5 μM, 1 μM)	pFHHSiD (8 μM)	darifenacine (0.5 μM, 1 μM)
pK_B value	8.64±0.10 (5)	7.96±0.13 (7)	6.61±0.26 (5)	7.00±0.13 (3)	7.22±0.25 (5)	7.73±0.27 (4)

Values are the means ± s.e.mean derived from (n) different lung samples.

31P G-CSF PRODUCTION IS ELEVATED FROM ANTRAL BIOPSIES OF PATIENTS WITH GASTRITIS, BUT ONLY INCREASED BY INDOMETHACIN IN PATIENTS NEGATIVE FOR *HELICOBACTER PYLORI* INFECTION

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Gastritis in man is often associated with *Helicobacter pylori* infection (Gasbarrini *et al.*, 1997) or the chronic usage of non-steroidal anti-inflammatory (NSAIDs) (Fries *et al.*, 1991). In both cases there is a marked leukocyte infiltration, particularly of neutrophils (Wallace *et al.*, 1992). The colony stimulating factors, granulocyte (G-CSF) and granulocyte macrophage (GMCSF) stimulate maturation, activation and survival of neutrophils. Spontaneous GMCSF production is elevated in patients with gastritis, especially those positive for *H.pylori* infection, compared to controls. Indomethacin also increases GMCSF production from antral biopsies in culture (Breese *et al.*, 1999). In this study we have investigated the effect of the cyclo-oxygenase (COX) non-selective inhibitor, indomethacin, and the COX-2 selective inhibitor L745,377 (Chan *et al.*, 1995) on G-CSF production from gastric biopsies. We have also compared the spontaneous production of G-CSF, RANTES, IL-8 and eotaxin from patients with gastritis who were positive or negative for *H.pylori* infection. Antral biopsies, from routine endoscopy were equilibrated at (37°C, 5% CO_2) in DMEM supplemented with 2mM glutamine and antibiotics for 30min prior to incubation with indomethacin (10μM), L745,377 (10μM) or vehicle for 24h. Cytokine production was determined by specific sandwich ELISA.

Spontaneous G-CSF production by tissue from patients with gastritis who were positive for *H.pylori* infection (2.8 ± 0.6ng/mg protein, mean ± s.e.m., n=25) was significantly higher than that by tissue from gastritis patients negative for *H.pylori* (2.2 ± 0.7ng/mg, n=23, $p<0.05$ Mann-Whitney) and controls (1.1 ± 0.2ng/mg, n=16, $p<0.02$). Despite a trend towards greater G-CSF production there was no difference between biopsies of patients with gastritis, negative for *H.pylori* infection and controls. There was no difference between groups for RANTES, IL-8 or eotaxin. Moreover, G-CSF production was significantly reduced in the *H.pylori* negative gastritis group by indomethacin but not L745,377. The *H.pylori* positive group was not affected by NSAIDs (Fig 1).

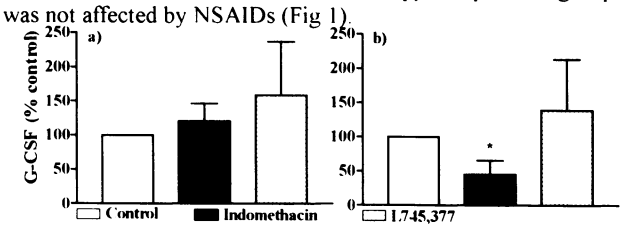

Figure 1: Percentage change in G-CSF production from antral biopsies by NSAIDs in patients with gastritis a) *H.pylori* positive and b) *H.pylori* negative *$p<0.01$ t test. Data represent mean and s.e.m.

Our data suggests that both GMCSF and G-CSF are involved in neutrophil activation in H.pylori induced gastritis but G-CSF is unlikely to be involved in NSAID induced gastritis.

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32P THE ACTIVATORS OF SOLUBLE GUANYLYL CYCLASE INHIBIT PGE_2 RELEASE UNDER CONDITIONS OF HIGH AND LOW ENDOGENOUS ARACHIDONIC ACID

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Nitric oxide synthase (NOS) and Cyclo-oxygenase (COX) are both important enzymes with homeostatic and pathogenic roles in the vasculature which are co-expressed constitutively (eNOS/COX-1) or co-induced (iNOS/COX-2) at the site of inflammation. Previously, we have demonstrated that in human vascular smooth muscle cells endogenous NOS activity is too low to influence COX-2. However, we showed that adding NO exogenously in the form of sodium nitroprusside (SNP) or the NO-independent guanylyl cyclase activator YC-1 inhibited PGE_2 release by cells stimulated to express COX-2 (Gitlin *et al.*, 1999). PGE_2 production by cells expressing COX-2 is greatly increased when phospholipase (PL) A_2 is also activated by agents such as bradykinin (BK)(Saunders *et al.*, 1999), a phenomenon which is likely to occur in vivo at the site of inflammation. Thus, we have furthered our studies by comparing the effects of SNP and YC-1 on PGE_2 produced by cells stimulated to express COX-2, using IL-1β, with those co-stimulated to activate PLA_2, with BK.

Human Vascular Smooth Muscle (HVSM) cells were cultured according to Bishop-Bailey *et al.*, 1998. After 6-10 weeks, the primary cultures were fully confluent, and cells passaged into 96 well culture plates. Two protocols were used in this study; in the first cells were co-treated with SNP or YC-1 and IL-1β (10ng/ml) to induce COX-2 (Bishop-Bailey *et al.*, 1998) for 24 hours. The medium was then removed and PGE_2 measured by radioimmunoassay. For the second protocol cells were treated with drugs and IL-1β for 24h and medium removed as above, fresh medium was then added containing the peptide BK (1μM) for 30 min, medium was then removed for analysis of PGE_2.

In the absence of IL-1β, SNP at 100μM for 24h stimulated PGE_2 release, an effect that was also observed when the same cells subsequently stimulated with BK. By contrast, SNP at 1mM or YC-1 at 10 or 100μM inhibited PGE_2 release by IL-1β stimulated HVSM cells before (figure 1A) and after stimulation with BK (figure 1B), no effect of these treatments were seen on cells cultured without cytokines.

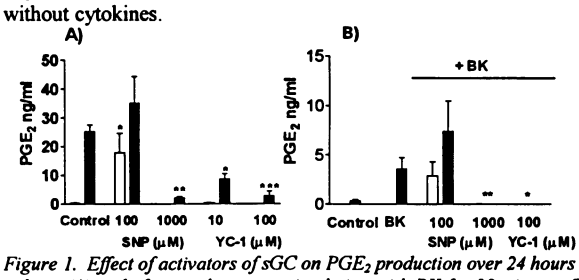

*Figure 1. Effect of activators of sGC on PGE_2 production over 24 hours in culture (A) and after a subsequent stimulation with BK for 30 minutes (B). Open bars represent data from control cells; filled bars represent cells treated with IL-1β. The data is mean ± s.e.m. for n=6-9 experiments. *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA followed by Dunn's multiple comparisons) represent differences between treated and controls.*

Here we have shown that SNP at 1mM and YC-1 at 10 and 100μM, inhibit PGE_2 production in cells expressing COX-2 and that this inhibition is still apparent when cells are subsequently stimulated with BK to activate PLA_2. Furthermore, we observed that when cells were treated with SNP at 100μM, in the absence of IL-1β, PGE_2 release was stimulated, suggesting that NO can positively as well as negatively regulate COX activity depending upon concentration and inflammatory state of the cell.

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33P VESICULAR INSERTION OF EPITHELIAL SODIUM CHANNELS INTO UROTHELIAL CELL MEMBRANES IS STIMULATED BY CYCLIC AMP

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It is clear that Na^+ absorbing epithelia are able rapidly to regulate the number of epithelial Na^+ channels (ENaC) in their apical membranes. This has been shown in A6 cells, Xenopus oocytes and salivary glands (Erlij *et al.*, 1991, Shimkets *et al.*, 1997, Dinudom *et al.*, 1998) and it involves transport of ENaC containing vesicles into and out of the plasma membrane. Our results reveal the same phenomenon in rabbit urinary bladder.

Urinary bladders were removed from Dutch rabbits of either sex (0.5-1.0 kg) and were mounted in Ussing chambers. The short-circuit current (SCC)was measured with a DVC 1000 voltage clamp. The SCC in bladder is equivalent to net Na^+ flow (Lewis & Diamond, 1976) Brefeldin A, forskolin and dibutyryl cAMP dissolved in ethanol were added to the mucosal surface of the bladder, both amiloride and cycloheximide were water soluble. Appropriate solvent controls were done as necessary. Normally a single experimental bladder was divided in two, one half acting as control tissue. Results are expressed as SCC inhibitable by amiloride at 10μM, a maximal concentration to block ENaC.

The SCC increases in the untreated bladder from 1.54 to 5.20 $\mu A.cm^{-2}$ a 4.58 fold increase over 6.25h. Addition of brefeldin (5 μM) completely blocked this rise in SCC. Cycloheximide (100 μM) an inhibitor of protein synthesis, had no effect on SCC until 4 h after the addition of the drug. The rate of increase in SCC was greater in the presence of forskolin (1 μM), which increases the intracellular cAMP (Figure 1). Rabbit urine diluted 1:10 with Krebs' solution reduced the SCC by 28±4% (n = 4) in a reproducible fashion, this effect was removed if the urine was previously boiled.

ENaC are inserted into the apical membrane of the rabbit bladder for at least 6 h following its removal from the animal. This involves insertion of ENaC containing membrane vesicles as it is completely blocked by brefeldin. Inhibition of protein synthesis has the same effect after a delay of 4h. ENaC activity is enhanced by forskolin which increases cAMP concentrations in the cells. We interpret these results to show a constitutive production and membrane insertion of ENaC which are normally degraded by urinary proteases. The importance of vesical Na^+ transport in mammals is that it is crucial to sensory transduction of pressure changes during filling and emptying of the bladder (Ferguson *et al.*, 1997).

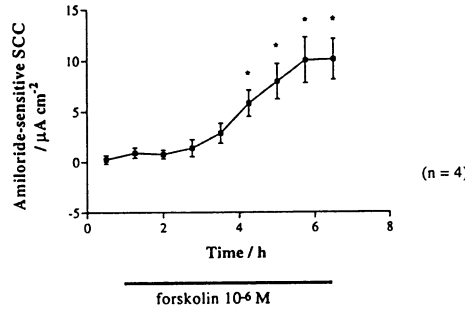

Figure 1. Increased amiloride-sensitive SCC in bladders treated with 1μM forskolin versus control preparations. (* $p<0.05$, Student's t-test).

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34P CALPAIN INHIBITOR 1 REDUCES THE RENAL DYSFUNCTION AND INJURY ASSOCATED WITH ISCHAEMIA-REPERFUSION OF THE KIDNEY OF THE RAT *IN VIVO*

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Activation of the neutral cysteine protease calpain is implicated in the pathophysiology of several disease states and the beneficial actions of its inhibition, e.g. by calpain inhibitor-I, have been demonstrated (Wang & Yuen, 1997). However, unlike other protease inhibitors such as chymostatin, calpain inhibitor-I also prevents the activation of nuclear factor-κB (NF-κB) (Lin *et al.*, 1995), which regulates the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Siebenlist *et al.*, 1994). The aims of this study were (i) to investigate the effects of calpain inhibitor-I in renal ischaemia-reperfusion injury in the anaesthetised rat and (ii) to determine one of the mechanisms by which calpain inhibitor-I may confer protection against ischaemia-reperfusion injury.

Thirty-eight male Wistar rats (200-250 g) were anaesthetised using sodium thiopentone (120 mg kg^{-1} i.p.) and tracheotomised. Following a midline laparotomy and isolation of the renal pedicles, the rats were randomly divided into five groups: (i) sham rats maintained under anaesthesia for the duration of the experiment, (ii) control rats subjected to bilateral clamping of the renal pedicles (45 min) followed by reperfusion (6 h), calpain inhibitor-I or chymostatin treated rats manipulated as described for the control group, but which received (iii) calpain inhibitor-I (10 mg kg^{-1} in 50 % (v/v) EtOH/saline, i.p.) or (iv) chymostatin (10 mg kg^{-1} in 50 % (v/v) EtOH/saline, i.p.) 30 min prior to clamping, (v) vehicle treated rats received 50 % (v/v) EtOH/saline only (1 ml kg^{-1} i.p.). On completion of experiments, renal function and injury were assessed by measurement of the plasma levels of urea and creatinine and urinary concentrations of glutathione-S-transferase (GST) and N-acetyl-β-D-glucosaminidase (NAG). In addition, iNOS and COX-2 protein expression in renal tissue was assessed using immunohistochemical analysis.

	N	Urea (mmol L^{-1})	Creatinine (μmol L^{-1})	GST (μg L^{-1})	NAG (i.u. L^{-1})
sham	8	8 ± 1	35 ± 2	28 ± 4	15 ± 2
control	8	28 ± 1*	213 ± 7*	92 ± 5*	54 ± 9*
calpain inhibitor-I	8	20 ± 2+*	127 ± 14+*	25 ± 5+	25 ± 3+*
chymo-statin	8	26 ± 2*	178 ± 5*	106 ± 9*	47 ± 5*
vehicle	6	27 ± 1*	205 ± 6*	105 ± 7*	44 ± 2*

Table 1: Effects of calpain inhibitor-I and chymostatin on biochemical markers of glomerular function and tubular injury subsequent to renal ischaemia-reperfusion. Data are expressed as mean ± s.e. mean, *$P<0.05$ vs. sham group, +$P<0.05$ vs. control group, analysed using one-way ANOVA followed by a Dunnett's post significance test.

Bilateral renal clamping (45 min) followed by reperfusion (6 h) of rat kidneys produced significant increases in the plasma levels of urea and creatinine and the urinary concentrations of GST and NAG (Table 1). Administration of calpain inhibitor-I 30 min prior to ischaemia-reperfusion significantly reduced renal dysfunction and injury (Table 1). Chymostatin or vehicle (50% v/v EtOH), did not have a significant effect on renal biochemical parameters on comparison with the control group. In addition, calpain inhibitor-I, but not chymostatin, substantially reduced iNOS and COX-2 expression in renal tissues (data not shown).

These data demonstrate that calpain inhibitor-I significantly reduces the renal dysfunction and injury associated with ischaemia-reperfusion in the anaesthetised rat. We propose that one mechanism of protection involves the inhibition of the activation of NF-κB and of NF-κB-dependent proteins including iNOS and COX-2.

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35P INVESTIGATION OF THE ROLE OF α_{1A}- AND α_{1D}- ADRENOCEPTORS IN THE CONTROL OF THE "MICTURITION REFLEX" IN THE ANAESTHETIZED MALE RAT

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Evidence indicates that α_1-adrenoceptors are involved in the storage and release of urine (see de Groat *et al.*, 1999). However, the role of different α_1-adrenoceptor subtypes in these processes has yet to be resolved. Hence, the present experiments were carried out to determine the effects of the selective α_{1A}-adrenoceptor antagonist RS100329 (Willams *et al.*, 1999) and BMY7378, a selective α_{1D}-adrenoceptor antagonist (Goetz *et al.*, 1995), on the micturition reflex.

Experiments were performed on spontaneously-breathing male Sprague Dawley rats (255-385g) anaesthetized with urethane (1.2 g kg^{-1} i.v.). "Micturition reflexes" were evoked by distension of the urinary bladder with saline infusion (0.05 ml min^{-1}). Intraluminal bladder and urethral perfusion pressures were recorded as described by Kakizaki *et al.* (1997). The pressure threshold, amplitude and duration of the bladder contractions and associated urethral responses were measured. Changes (%) after test drug (i.v.) were compared with vehicle (0.04 M) lactic acid controls using unpaired Student's *t*-test; a level of $P<0.05$ was taken as significant.

Distension–induced bladder contractions were accompanied by contractions of the urethra. RS100329 (0.1 & 0.3 mg kg^{-1}; n = 4) significantly decreased the baseline urethral pressure (0.1 mg kg^{-1}, -23 ± 5%, mean ± s.e.mean) and, at the higher dose, significantly reduced background activity (45 ± 19%). Reflex urethral contractions were significantly attenuated by 0.3 mg kg^{-1} of RS100329 (-66 ± 8%). Further, RS100329 tended to cause bursts of oscillations in urethral pressure, as previously reported for doxazosin (Snowball *et al.*, 1999). However, neither dose of RS100329 affected the bladder. BMY7378 (0.1-1.0 mg kg^{-1}; n=3-4) significantly reduced baseline urethral pressure (0.1 mg kg^{-1}, -13 ± 5%) and, at the highest dose, blocked background activity. Reflex urethral contractions were significantly attenuated by 0.3 and 1.0 mg kg^{-1} (0.3 mg kg^{-1}, -53 ± 2%). In addition, BMY7378 reduced the pressure threshold for the micturition reflex.

The above data show that RS100329 and BMY7378 have a similar potency in attenuating reflex urethral contractions. As these drugs have similar affinities for the α_{1D} subtype and RS 100329 has approximately 1,000x higher affinity for the α_{1A} subtype compared to BMY7378, it would seem that this effect is, at least in part, mediated by the α_{1D} subtype. The bursts of oscillations in urethral pressure may be attributed to blockade of α_{1A}-adrenoceptors. However, the decrease in the pressure threshold is similar to that observed with 8-OH-DPAT (Snowball *et al.*, 1999) and thus may be related to BMY7378 partial agonist action at 5-HT_{1A} receptors (Yocca *et al.*, 1987).

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36P UPREGULATION OF THE ORL1 RECEPTOR IN MICE LACKING THE ORPHANIN FQ PEPTIDE GENE

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The cloning of the ORL1 receptor and characterisation of its endogenous ligand orphanin FQ (OFQ) has led to a number of studies to address the physiological role of this system (see Henderson & McKnight, 1994). Mice deficient in OFQ have now been generated by deletion of exon 3 of the OFQ gene (Chen *et al.*, 1999). These mice showed increased pain sensitivity at both the spinal and supraspinal level suggesting an antinociceptive role for this peptide (Chen *et al.*, 1999). Increased morphine dependence but not tolerance was also reported indicating specificity in its functional anti-opioid effects (Kest *et al.*, 1999). Quantitative autoradiographic mapping of the ORL1 receptor in the brains of mice deficient in the OFQ gene has now been performed to provide an indication of the endogenous tone within the system.

Brain sections (20µm) from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mice for the OFQ gene were cut for total and non-specific binding using a mapping interval of 300µm. Slides were pre-incubated in buffer (50mM Tris HCl 0.9% NaCl pH 7.4) for 30 minutes. [^{3}H] *leucyl*-nociceptin (0.4nM) binding was carried out in an incubation buffer (50mM Tris HCl, 3mM $MgCl_2$, 0.2mM EGTA, $1.26x10^3$ u/l bacitracin and 0.1% bovine serum albumin pH 7.4). for three hours and non-specific binding was determined using 1µM unlabelled nociceptin. Slides were rinsed in ice cold buffer (50mM Tris HCl, 3mM $MgCl_2$ and 1% BSA pH 6.8) for 15 minutes. Films were dried and apposed to tritium sensitive hyperfilm (Amersham) for three weeks and analysed using video-based computerised densitometry on an MCID image analyser (Imaging Research Canada).

A 15% and 30% upregulation of the ORL1 receptor expression was identified in the brains of +/- and -/- mice respectively ($P<0.05$ ANOVA) (Table 1). The upregulation of the ORL1 receptor in the absence of the OFQ peptide gene suggests the existence of endogenous tone within the system. This may be particularly important in the regulation of pain transmission as this increase is most profound in regions associated with nociceptive processing.

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Table 1: Specific binding (fmol/mg tissue; mean ± s.e.m. n=3) of [^{3}H] *leucyl* nociceptin in brain regions of OFQ knockout mice.

Region	+/+	+/-	-/-	% Change +/-	% Change -/-
Amygdala	77.1 ± 5.2	87.6 ± 11.7	107.2 ±15.2	13.6	39.0
Periaquaductal Gray	39.0 ± 8.6	60.3 ± 8.6	82.1 ± 3.0	54.5	110.5
Motor Cortex	62.6 ± 10.4	72.6 ± 10.4	86.0 ± 6.3	15.9	37.3
Rostral Somatosensory Cortex	56.4 ± 6.4	56.3 ± 6.4	72.5 ± 6.6	-0.2	28.6
Caudal Somatosensory Cortex	62.9 ± 4.7	65.3 ± 4.7	78.7 ± 5.2	3.8	25.0

37P CHARACTERISATION OF [^{3}H]QUISQUALATE BINDING TO RAT RECOMBINANT METABOTROPIC GLUTAMATE 1a AND 5a RECEPTORS AND TO RAT BRAIN SECTIONS

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Group I metabotropic glutamate receptors, which couple to phosphoinositide hydrolysis, are activated by the weak group I selective agonists S-DHPG and S-3HPG and the potent non-selective agonist quisqualate. The distribution of the sites of synthesis and protein expression of mGlu1 and 5 receptors has been studied by *in situ* hybridization histochemistry and immunohistochemistry. However, the lack of potent and highly selective group I receptor radioligands has hindered radioautographic investigations of the unequivoical distribution and abundance of these receptors in brain sections. For group II receptors, this has recently been resolved by the radiolabelling of two highly selective agonists, namely DCG-IV and LY354740 [Mutel et al., 1998; Schaffhauser et al., 1998].

The preparation of [^{3}H]quisqualate has now allowed its binding affinity for recombinant rat mGlu1a and b as well as 5a receptors to be characterized and, after its affinity for ionotropic glutamate receptors was eliminated, the evaluation of its binding characteristics to group I receptors in rat brain sections by quantitative receptor radioautography.

Binding experiments were performed with pre-washed membranes of HEK293 cells, transiently expressing recombinant rat metabotropic glutamate 1a and 5a receptors, and [^{3}H]quisqualate (s.a. 35 Ci/mmol; TRK 1070) in a 20mM HEPES-NaOH buffer with 2mM $MgCl_2$, 2mM $CaCl_2$ at pH 7.4. Saturation isotherms were monophasic and gave K_D values of 27 ±4 and 81 ±22 nM and Bmax values of 6 ±1.2 and 5 ±1.6 pmoles/mg protein for mGlu1a and mGlu5a receptors, respectively. Several compounds inhibited the binding concentration-dependently with Ki values from 11 nM for quisqualate to 900 µM for $CdCl_2$ on mGlu1 and 30 nM for quisqualate to 400 µM for CHPG on mGlu5 receptors. 2-Methyl-4-CPG was 16-fold more potent on mGlu1 than mGlu5 whereas $CdCl_2$ was 34 times more potent on mGlu5 than mGlu1. Interestingly, CHPG and AIDA did not show any preferential inhibitory action. Finally, GTPγS did not inhibit the binding on either receptor and several compounds with unexpected activity on these receptors were identified.

The distribution and abundance of binding sites in pre-washed rat brain sections was studied radioautographically using 10 nM [^{3}H]quisqualate in 50mM Tris-HCl buffer with 2mM $MgCl_2$ and 2mM $CaCl_2$ at pH 7.4. The rank order of high density binding sites (0.5-2.2 pmol/mg prot) was cerebellar molecular layer, dentate gyrus, cerebral cortex, striatum and thalamus. Binding to ionotropic glutamate receptors was inhibited by 30 µM kainate leaving mGlu1 and 5 receptors unaffected (= 55-75% of total binding, depending upon the brain region) whereas binding to the latter was inhibited by 100 µM s-DHPG.

We conclude that [^{3}H]quisqualate is a new tool with which to analyse, under defined binding conditions, group I metabotropic glutamate receptors and to map their distribution in the CNS.

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38P NOVEL APPROACH FOR THE QUANTITATIVE DESCRIPTION OF 5-HT$_{1A}$ RECEPTOR AGONISM *IN VIVO*: DIFFERENTIATION BETWEEN FULL, PARTIAL AND SILENT AGONISTS

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It is well established that agonists for the 5-HT$_{1A}$ receptor have anxiolytic and antidepressant properties. Furthermore, it has been suggested that different therapeutical indications may require different profiles of agonist intrinsic activity (see De Vry, 1995). However, despite the abundance of literature on the function and characteristics of 5-HT$_{1A}$ receptors, no studies have been reported using an integrated pharmacokinetic-pharmacodynamic (PK-PD) approach. As a consequence, intrinsic activity and potency have not been quantitatively studied *in vivo*. Therefore, we have developed an *in-vivo* model for PK-PD analysis of 5-HT$_{1A}$ receptor agonists, using the hypothermic response in rats. In this study, we have used this model to estimate intrinsic activity and potency of R-8-OH-DPAT, S-8-OH-DPAT, buspirone, its active metabolite 1-(2-pyrimidinyl)-piperazine (1-PP) and N-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-N-2-pyridinylcyclohexanecarboxamide (WAY100635).
Male Wistar rats (296 ± 2 g mean ± s.e.mean, n=63) were equipped with a telemetric temperature probe and catheters for i.v. drug administration and arterial blood sampling, enabling simultaneous measurement of pharmacokinetics and pharmacodynamics. Body temperature was measured continuously for 8 hours, giving detailed time-effect profiles following administration of R-8-OH-DPT (1 mg/kg in 5 min and 3 mg/kg in 5, 15 and 30 min), S-8-OH-DPAT (5 and 15 mg/kg in 15 min), buspirone (a gift from Bristol Myers Squibb, 5 and 15 mg/kg in 15 min), 1-PP (10 mg/kg in 15 min), WAY 100635 (3 mg/kg in 15 min) or vehicle (saline in 5, 15 and 30 min). Blood concentrations of the drugs were determined using HPLC. The pharmacokinetics were described using standard two- and three-compartment models. The formation of 1-PP in buspirone-treated rats could be described with the use of a primary-metabolite, two-compartment model (Rowland & Tucker, 1986). The hypothermic effect was described using a model based on the indirect pharmacodynamic response model (Dayneka, et al. 1993) with a set-point function. Parameter estimates (mean ± s.e.mean) for half life, clearance and volume of distribution were 150 ± 19 min, 23 ± 3 ml/min and 2.1 ± 0.35 l, respectively for R-8-OH-DPAT (n=27); 334 ± 36 min, 16 ± 1 ml/min and 3.4 ± 0.35 l, respectively for S-8-OH-DPAT (n=12); 30 ± 1 min, 22 ± 2 ml/min and 0.69 ± 0.065 l, respectively for buspirone (n=12), 80 ± 7 min, 9 ± 1 ml/min and 0.97 ± 0.17 l, respectively for 1-PP (n=6) and 23 ± 3 min, 42 ± 7 ml/min and 0.72 ± 0.11 l, respectively for WAY100635 (n=6). The fraction buspirone metabolised to 1-PP was 7 ± 2 % (n=12). A significant decrease in temperature was observed after administration of R-8-OH-DPAT (4 ± 0.3 °C) S-8-OH-DPAT (3.2 ± 0.2 °C), buspirone (2.8 ± 0.3 °C), 1-PP (1.6 ± 0.2 °C) but not for WAY 100635 (0.0 ± 0.2 °C). The time courses of effects could be described with the indirect physiological response model with set-point control. In estimating pharmacodynamic parameters for buspirone its active metabolite 1-PP was taken into account using an interaction model. From the PK-PD analysis, *in vivo* concentration-effect curves could be estimated that clearly demonstrate the differences in agonist potency and intrinsic activity (Figure 1).
In conclusion, our integrated PK-PD approach provides a novel framework for quantitative study of 5-HT$_{1A}$ receptor agonism and enables estimation of intrinsic activity and potency *in vivo*.

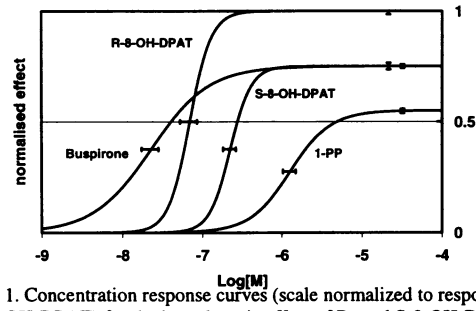

Fig. 1. Concentration response curves (scale normalized to response to R-8-OH-DPAT) for the hypothermic effect of R- and S-8-OH-DPAT, buspirone and 1-PP.

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39P MUTATION OF AN ISOLEUCINE RESIDUE IN M2 ALTERS THE Ca^{2+} PERMEABILITY OF 5-HT_{3A} RECEPTORS

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Changes in intracellular [Ca^{2+}] play critical roles in both the acute and long-term responses of neurones to extracellular stimuli. Voltage-gated Ca^{2+} channels provide the major route of Ca^{2+} entry, but Ca^{2+}-permeable neurotransmitter-gated ion channels are also important. One such protein is the 5-HT_3 receptor, and we have used site directed mutagenesis of the pore lining region (M2) to examine the role of isoleucine (I) 294 in Ca^{2+} permeation in the recombinant homomeric 5-HT_{3A} receptor.

Mutations were performed using the Kunkel method on the full length 5-$HT_{3A(b)}$ subunit DNA inserted into the expression vector pRc/CMV to exchange I^{294} for alanine (A). Mutant DNA was then stably transfected into HEK 293 cells, and the expressed 5-HT_3 receptors were examined using radioligand binding, Ca^{2+} imaging and whole-cell voltage clamp as previously described (Hargreaves *et al.*, 1996).

Radioligand binding using [^{3}H]granisetron, a 5-HT_3 receptor antagonist, revealed saturable specific binding for both wild type and mutant I^{294}A receptors, with no signifcant difference in affinity (K_d = 0.31 ± 0.09 nM and 0.32 ± 0.19 nM respectively, n=3). Examination of receptor function using whole-cell voltage clamp revealed that both wild type and mutant DNA produced receptors with a rapidly desensitizing, inward-rectifying 5-HT-induced current.

Whole-cell Ca^{2+} imaging using Fura-2 loaded cells revealed 5-HT induced, concentration dependent increases in intracellular [Ca^{2+}], with an EC_{50} of 2.9 ± 0.4 μM (n=3) in wild type receptors, however, no increase in intracellular [Ca^{2+}] was observed for cells expressing I^{294} mutant receptors at concentrations of 5-HT up to 300 μM.

We conclude that changing $I^{294}$to A in the pore lining region of the 5-HT_{3A} receptor subunit results in a functional receptor but ablates Ca^{2+} permeability in a similar manner to mutation of the homologous leucine residue in the α7 nicotinic acetycholine receptor (Bertrand *et al.*, 1993).

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40P DESENSITISATION PROFILES OF ENDOGENOUS M_3-MUSCARINIC RECEPTOR AND RECOMBINANT α_{1B}-ADRENOCEPTOR IN SH-SY5Y HUMAN NEUROBLASTOMA ASSESSED BY A DIRECT MEASURE OF G-PROTEIN COUPLING

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The α_{1B}-adrenoceptor (α_{1B}-AR) and the M_3-muscarinic (M_3-AChR) receptor are known to activate similar second messenger cascades but the interactions between these two receptor systems have not yet been studied extensively at the level of the G-protein. The present study addressed this by employing a novel [^{35}S]-GTPγS-G_α-immunoprecipitation assay to investigate the interaction of the two receptor systems at the level of $G_{q/11}$ using an α_{1B}-AR-transfected human neuroblastoma which endogenously expresses muscarinic receptors, predominantly of the M_3-subtype.

Saturation binding assays were performed by incubation of cell membranes with [^{3}H]prazosin or [^{3}H]NMS for 1 hour at 37°C. Phentolamine (10μM) or atropine (10μM) defined the NSB. For assessment of G-protein activation, cell membranes were incubated with [^{35}S]-GTPγS (8nM), GDP (1μM) and agonist for 2 min at 30°C. The G_α-[^{35}S]-GTPγS complex was then solubilised prior to immunoprecipitation with anti-sera and protein A-sepharose (Akam *et al.*, 1998). In experiments where prestimulation of intact cells was performed prior to membrane preparation, cell suspensions were incubated with the agonists noradrenaline (NA) or methacholine (MCh) for 0-300 seconds at 37°C. The agonist incubation was terminated by 30-fold dilution with ice-cold buffer and immediate centrifugation (4 min, 900g, 4°C).

A stable clone of the SH-SY5Y cell line, chosen for its similar maximal $G_{q/11}$ activation in response to either NA or MCh, expressed the human α_{1B}-AR with a B_{max} of 3.9±0.3pmols/mg protein (n=4); the M_3-AChR density of this clone was 272±30.8 fmols/mg protein (n=4). NA and MCh were efficacious at stimulating $G_{q/11}$ with an EC_{50} of 94±16 nM and 40±6μM, respectively (n=3). The maximal G-protein activation elicited by NA and MCh was 687±42% basal and 443±55 % basal, respectively (n=3). Stimulation of cells with agonist produced no significant effect on either receptors' density over the timescale studied (0-300s) ($p>0.05$, Student's t test, n=3). Pre-treatment of intact cells with agonist prior to measurement of G-protein activation by a successive incubation with agonist revealed that the α_{1B}-AR-mediated stimulation of $G_{q/11}$ activation was reduced by pretreatment with NA (30μM, 30s) (maximum response55.5±5.8 % of control, n=3) but not MCh (100μM, 0-300s). In contrast, the M_3-AChR-mediated stimulation of $G_{q/11}$ activation was reduced by either NA pretreatment (30μM, 30s) (47.2±5.7 % of control, n=3) or MCh (100μM, 30s) (44.7±2.1% of control, n=4). The desensitisation effect of MCh prestimulation upon a subsequent MCh-induced activation of $G_{q/11}$ was concentration dependent (prestimulation with MCh (30μM) produced desensitisation with $t_{½}$=100.6±31.5s, MCh (1mM) $t_{½}$=20.9±6.2s, n=3) but the maximal desensitisation effect was not significantly different for both concentrations (MCh (30μM), 39.2±4.2 % control and MCh (1mM), 41.8±4.4 % control, $p>0.05$, Student's t test, n=3). Incubation of cells with Ro-31-8220 (10μM) for 10mins prior to treatment with NA (30μM, for 30s) did not affect the desensitisation of the $G_{q/11}$ activation produced by a subsequent stimulation with NA (30μM) or MCh (1mM) (n=2).

The present findings indicate that noradrenaline pretreatment could reduce MCh-stimulation of $G_{q/11}$ but that the reverse was not true. Both the M_3-AChR and the α_{1B}-AR can be homologously desensitised with rapid timescales. The receptor density of both the α_{1B}-AR and the M_3-AChR were unchanged by agonist stimulation, indicating that a direct uncoupling of receptor from G-protein may be a mechanism for this desensitisation. The PKC inhibitor, Ro-31-8220 was ineffective at blocking the desensitisation produced by NA stimulation suggesting that PKC is unlikely to play a role in this effect.

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41P INFLUENCE OF GENETIC POLYMORPHISMS IN THE β_2-ADRENOCEPTOR ON DESENSITIZATION IN HUMAN LUNG MAST CELLS

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Recent studies indicate that the beta$_2$-adrenoceptor (β_2-AR) is polymorphic. Single base substitutions leading to amino acid changes at positions 16 (arg to gly) and 27 (gln to glu) have been shown to influence receptor desensitization (Green *et al.*, 1995). The aim of the present study was to establish whether genetic polymorphisms in the β_2-AR influence the degree of functional desensitization in human lung mast cells (HLMC).

Long-term (24 h) exposure of HLMC to isoprenaline (ISO, 10^{-6} M) led to a reduction in the subsequent ability of ISO (10^{-10}-10^{-5} M) to inhibit the IgE-mediated release of histamine from these cells ($P<0.0001$). This was reflected by a reduction in the maximal response (E_{max}) attained (from 57±2 to 29±2%, $P<0.0001$) and a decrease in the pD_2 value (from 8.9±0.1 to 8.1±0.1, $P<0.0001$) for ISO following the desensitizing treatment (n=72). However, there was considerable variability in the extent of desensitization with some HLMC preparations resistant and others highly susceptible to desensitization. In order to establish whether genotypic differences in the β_2-AR influenced the wide variability in desensitization, genomic DNA was extracted from stored lung tissue specimens. Following polymerase chain reaction (PCR), the PCR products containing the polymorphisms of interest were sequenced using a DNA sequencer in order to establish amino acid substitutions at positions 16 (arg/gly) and 27 (gln/glu). The influence of genotype on E_{max} and pD_2 values for ISO following control (24 h buffer) and desensitized (24 h, 10^{-6} M ISO) treatments is given in the table.

	Control		**Desensitized**	
genotype	E_{max} (%)	pD_2	E_{max} (%)	pD_2
gln27 (n=25)	54±2	8.9±0.1	23±2	7.9±0.2
glu27 (n=17)	59±3	8.9±0.1	40±1	8.5±0.1
gln/glu27 (n=30)	57±2	8.7±0.1	26±3	7.8±0.2
arg16 (n=18)	54±3	8.8±0.1	21±2	7.8±0.2
gly16 (n=26)	59±3	8.9±0.1	32±1	8.3±0.1
arg/gly16 (n=28)	58±2	8.8±0.1	30±3	7.9±0.2

Analysis of position 27 indicates that HLMC preparations expressing mutant (glu27) β_2-ARs were resistant to desensitization when compared with preparations that expressed wild-type (gln27) β_2-ARs and heterozygotes. This difference in the susceptibility to desensitization was statistically significant ($P=0.04$). Analysis of position 16 indicates that HLMC preparations expressing mutant (gly16) β_2-ARs tended to be more resistant to desensitization when compared with preparations that expressed wild-type (arg16) β_2-ARs and heterozygotes, but this tendency was not statistically significant ($P=0.2$).

In conclusion, these data suggest that genetic polymorphisms in the β_2-AR can influence the degree of functional desensitization in HLMC.

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42P THE EFFECT OF GUANINE NUCLEOTIDES AND RECEPTOR DENSITY ON AGONIST BINDING TO HUMAN RECOMBINANT ADENOSINE A_1 RECEPTORS

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Members of the G-protein coupled receptor superfamily typically display high and low affinity agonist binding components representing G-protein coupled and uncoupled receptor states. In membrane binding studies high, but not low, affinity agonist binding is inhibited by guanine nucleotides. This behaviour can be described qualitatively by the ternary complex model of agonism (TCM, De Lean *et al.*, 1980). The adenosine A_1 receptor provides a suitable test system for investigating the quantitative predictions of the TCM and related models because of the large differences in agonist affinities between states (Cohen *et al.*, 1996). We have examined the effects of guanine nucleotides and differences in receptor density on agonist binding to the A_1 receptor and compared our results with the predictions of the TCM.

Membranes from CHO cells stably expressing high (9pmoles/mg protein; 3µg/well) and low (0.4pmoles/mg protein; 30µg/well) levels of the A_1 receptor were incubated in a buffer of 20mM HEPES, 100mM NaCl, 10mM $MgCl_2$, 30µg/ml saponin and 0.3U/ml adenosine deaminase, pH 7.4, with radiolabelled antagonist ([^{3}H]DPCPX; 0.5nM) and increasing concentrations of the agonist N^6-cyclohexyladenosine (CHA; 3pM - 100µM) and guanine nucleotide for 1 hr at 25°C. Bound radioligand was separated by rapid filtration using a 96 well harvester and measured using solid scintillation spectrometry. TCM simulations were performed using a numerical approach. Fitting both experimental and simulated data to a non-interconverting two site model provided an accurate description of the inhibition curves.

[^{3}H]DPCPX binding was inhibited by CHA in a biphasic manner with pIC_{50} values of 8.56 ± 0.05 and 5.72 ± 0.07 (low expression, n = 5) and 8.58 ± 0.12 and 5.63 ± 0.03 (high expression, n = 5). The proportion of high affinity binding sites ($fraction_H$) was 0.83 ± 0.02 and 0.48 ± 0.01 in low and high expression cell lines respectively. The proportion of high affinity binding sites observed in the high expression cell line was 11-fold greater than the proportion predicted by the TCM.

Increasing concentrations of GDP (Table 1) or GTP (data not shown) reduced both the p IC_{50} and proportion of high affinity CHA binding sites in both cell lines, consistent with the qualitative predictions of the TCM. However, these results are in contrast to the quantitative simulations of the TCM which predict only very small changes in $fraction_H$ as pIC_{50H} decreases with added guanine nucleotide (data not shown).

Table1: The effect of GDP on CHA binding to A_1 receptors.

	low expression			high expression		
[GDP]	$fraction_H$	pIC_{50H}	pIC_{50L}	$fraction_H$	pIC_{50H}	pIC_{50L}
0	0.83	8.56	5.72	0.48	8.58	5.63
1µM	0.74	7.52	5.79	0.36	7.94	5.63
3µM	0.57	7.39	5.79	0.31	7.72	5.62
10µM	0.41	6.93	5.73	0.28	7.31	5.60
100µM			5.68*			5.64*

n=4-5; s.e.mean: 0.01-0.05 ($fraction_H$), 0.04-0.14 (pIC_{50H}); 0.03-0.12 (pIC_{50L}). * only one binding component observed; H = high affinity, L = low affinity.

These results indicate that differences in receptor density and the effects of guanine nucleotides are qualitatively, but not quantitatively, similar to the predictions of the ternary complex model.

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43P INCREASES IN INWARD CURRENTS AND AGONIST POTENCY AT P2X$_7$ RECEPTORS FOLLOWING REPEATED AGONIST APPLICATION

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The biophysical properties of the P2X$_7$ receptor are complex and exhibit species differences. Thus, upon repeated agonist application inward currents through mouse P2X$_7$ receptors increase (Chessell et al., 1997) whereas this phenomenon of current growth has not been reported for the rat or human orthologues (Surprenant et al., 1996; Rassendren et al., 1997). In the present study we have further examined the reasons for the species differences in current growth.

Inward currents were recorded from HEK293 cells expressing recombinant P2X$_7$ receptors. Whole cell recordings were made at 22°C in cells voltage clamped at –90mV and studied in an extracellular solution containing no added divalent cations except 0.5mM $CaCl_2$ (Chessell et al., 1997). To measure current growth ATP or 2'&3'-O-benzoylbenzoyl-ATP (BzATP) was applied repeatedly to the cells for 2s every 30s. To determine potency, increasing concentrations of agonist were applied for 0.5s (rat P2X$_7$) or 2s (mouse and human P2X$_7$) every 2min. In some studies agonist potency was also determined in cells which had been exposed to 1mM ATP for 2s every 30s until there was no further increase in inward current. Data are the mean±s.e.mean of at least 5 experiments

Repeated application of 100μM BzATP resulted in significant current growth at the mouse P2X$_7$ receptor (response to 4th application of BzATP 143±17% of 1st response; p<0.05 ANOVA and Tukey's test) but not at the rat P2X$_7$ receptor. However, the potency of BzATP was much higher at the rat P2X$_7$ receptor (pEC$_{50}$ 5.17±0.09) than at the mouse P2X$_7$ receptor (pEC$_{50}$ < 4) such that 100μM was a supra-maximal concentration at the rat P2X$_7$ receptor but threshold at the mouse P2X$_7$ receptor. When sub-maximal concentrations of BzATP were applied to the rat P2X$_7$ receptor, current growth occurred. Thus, using 1, 3 and 10μM BzATP, significant increases in inward current occurred after 14, 5 and 4 applications of BzATP, respectively. Current growth was also observed using a submaximal concentration of ATP (300μM). In addition, current growth was observed when applying sub-maximal concentrations of 30 or 100μM BzATP or 300μM ATP to the human P2X$_7$ receptor.

The potency of BzATP for rat P2X$_7$ receptors was greater (P<0.05; Student's t-test) in cells repeatedly exposed to 1mM ATP (pEC$_{50}$=5.39±0.04) than in naïve cells (pEC$_{50}$=5.17±0.09). Similar increases in agonist potency occur at the human P2X$_7$ receptor (Chessell et al., 1999). The pEC$_{50}$ of BzATP at mouse P2X$_7$ receptors could not be determined since a maximum response was not obtained. However, when studies were performed in a buffer with 140mM Na-glutamate replacing NaCl, agonist potency increased and in this buffer the BzATP pEC$_{50}$ for mouse P2X$_7$ receptors was greater (P<0.05; Student's t-test) in cells repeatedly exposed to ATP (4.58±0.09) than in naïve cells (4.08±0.08).

These results suggest that current growth can be observed at rat, human and mouse P2X$_7$ receptors provided that sub-maximal concentrations of agonist are applied. The failure to observe this phenomenon in previous studies was probably due to the use of supra-maximal agonist concentrations. Previously, current growth was thought to be due to channel dilation and an increase in channel conductance (Chessell et al., 1997). However, the finding that agonist potency is increased after repeated agonist application in this, and previous studies (Chessell et al., 1999), suggests that changes in agonist affinity may also contribute to current growth.

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44P CAN THE P2X$_7$ RECEPTOR EXIST IN MORE THAN ONE AFFINITY STATE FOR AGONISTS?

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The P2X$_7$ receptor is a ligand-gated cation channel activated by extracellular ATP (Surprenant et al., 1996). The ionic-selectivity of the channel changes upon repeated agonist application suggesting that the channel can dilate and become permeable to large cations (MW <900 Da; Surprenant et al., 1996). Furthermore, agonist potency is thought to increase upon channel dilation (Chessell et al., 1999). In this study we show that agonist potency for stimulating ethidium (MW=314 Da) influx through the P2X$_7$ receptor can vary.

Studies were performed on HEK293 cells expressing recombinant P2X$_7$ receptors and grown in 96 well plates. Prior to study, growth media was replaced with Dulbecco's modified Eagles medium (DMEM) or sucrose buffer comprising (mM) :- 10 Hepes, 10 glucose, 5 KCl, 0.5 $CaCl_2$, 280 sucrose (pH 7.4). In some studies the ATP metabolising enzyme, apyrase (1unit ml^{-1}), was added to the DMEM (DMEM/apyrase) or 1unit ml^{-1} apyrase and 10mM $MgCl_2$ were added to the sucrose buffer (SAM buffer). After various times at 37°C, cells were washed 3 times with 22°C sucrose buffer. In some studies additional treatments were performed and the cells washed again. After 10min at 22°C, ethidium accumulation was initiated by addition of a mixture of ethidium (100μM) and agonist. Reactions were terminated after 2 min at 22°C by ethidium removal. Ethidum accumulation was measured in a plate reader (Ex 530nm ;Em 620nm). Data are the mean±s.e.mean of 3-5 experiments.

The pEC$_{50}$ for 2'&3'-O-benzoylbenzoyl-ATP (BzATP) at the human P2X$_7$ receptor was 5.5±0.05 when determined in cells pre-incubated in DMEM for 1hr at 37°C prior to study but 5.1±0.08 in cells pre-incubated in DMEM/apyrase. When cells were pre-incubated in sucrose buffer for 1hr the BzATP pEC$_{50}$ was 6.0±0.09 whereas in cells pre-incubated in SAM buffer for 1 hr the pEC$_{50}$ of BzATP was 5.2±0.06. This suggested that release of endogenous ATP may have affected the subsequent potency of BzATP. Indeed, in sucrose buffer ATP possessed relatively high affinity for human P2X$_7$ receptor (pEC$_{50}$ 4.8±0.11). Furthermore, in cells incubated for 1hr in DMEM/apyrase, subsequent exposure to 30μM BzATP for 1min followed by washing, resulted in an increased potency for BzATP (pEC$_{50}$ 6.3±0.13). Similar results were obtained with 100μM ATP (pEC$_{50}$ values 5.2±0.07 and 6.3±0.08 in control and ATP treated cells, respectively). The ATP and BzATP-induced changes in potency persisted for at least 1hr. When cells were incubated at 37°C in sucrose buffer for 1hr, subsequent addition of SAM buffer for 10mins followed by washing decreased the pEC$_{50}$ of BzATP to 5.1±0.07 compared to a pEC$_{50}$ of 6.2±0.13 in control cells. At the mouse P2X$_7$ receptor pEC$_{50}$ values for BzATP were 4.6±0.07, 4.3±0.06 and 3.9±0.06 in cells pre-incubated at 37°C for 1hr in sucrose, DMEM or DMEM/apyrase, respectively. If cells expressing the mouse P2X$_7$ receptor were pre-incubated in DMEM/apyrase, exposed to 128μM BzATP for 5 min and washed, the pEC$_{50}$ for BzATP was 5.3±0.08.

This study has shown that agonist potency for the P2X$_7$ receptor can vary and that two extremes of potency exist. When cells are pre-incubated under conditions which reduce the potential for P2X$_7$ receptor activation by endogenous ATP a low potency is obtained. In contrast, if cells are exposed to exogenous agonist a higher potency is obtained. These data confirm reports that agonist affinity for the P2X$_7$ receptor can be modified by receptor activation (Chessell et al., 1999). Since potency estimates in this study were determined by measuring ethidium influx, it is unlikely that the potency differences reflect agonist actions at the "channel" and "large-pore" forms of the receptor as proposed previously (Chessell et al., 1999) but may reflect the ability of P2X$_7$ receptors to exist in more than one affinity state.

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45P THE ADENINE NUCLEOTIDE $P2Y_1$ RECEPTOR (HIGHLY ABUNDANT IN BRAIN) COUPLES TO BOTH N-TYPE Ca^{2+} AND M-TYPE K^+ CHANNELS IN NEURONS

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The $P2Y_1$ receptor is a G protein-linked receptor for adenine nucleotides known to stimulate the phosphoinositide signaling pathway (Simon et al., 1995). This receptor is highly abundant in the brain (Webb et al., 1998). We have investigated the coupling of the molecularly-defined rat brain $P2Y_1$ receptor to neuronal N-type Ca^{2+} channels and to M-type K^+ channels, by heterologous expression in rat superior cervical sympathetic (SCG) neurons.

Single SCG neurons were isolated from 15-19 day old rats (killed by CO_2 asphyxiation). 4-6 hr after plating, the neurons were injected into the cytoplasm with $P2Y_1$ cRNA (1.25 μg/μl) or into the nucleus with cDNA (0.15 μg/μl) plus cRNA/cDNA for jellyfish Green Fluorescent Protein (GFP). Membrane currents were recorded using whole-cell or perforated patch-clamp configuration (data obtained in both configurations were indistinguishable) from GFP-labelled cells after incubation at 37°C for 14-24 h (see details in Filippov et al., 1998).

In $P2Y_1$ cRNA injected neurons, adenosine diphosphate (ADP) inhibited the Ca^{2+} current ($I_{Ca(N)}$) by 64.0 ± 1.7 % (mean ± s.e.m., n=16), with an IC_{50} of 8.2 ± 0.9 nM. In the presence of the $P2Y_1$ antagonist, A3'P5'P (10 μM), the IC_{50} for ADP increased to 126.6 ± 20.9 nM (n=5). Inhibition was voltage-dependent, a strong depolarizing prepulse decreasing inhibition from 60.0 ± 6.1 % to 28.6 ± 6.8 % (n=5). *Pertussis* toxin (PTX) pretreatment (0.5 μg/ml, overnight) reduced $I_{Ca(N)}$ inhibition to 33.3 ± 8.5 % but did not prevent its voltage-dependence. Similar results were obtained after $P2Y_1$ cDNA injections. 2-Methylthio-ADP inhibited $I_{Ca(N)}$ with IC_{50} of 0.57 ± 0.05 nM (n=3) and 2-methylthio-ATP with IC_{50} of 2.55 ± 0.28 nM (n=4).

ADP also inhibited the M-current ($I_{K(M)}$) by 59.2 ± 3.39 % (n=10), with an IC_{50} of 6.9 ± 0.8 nM. This inhibition was not affected by PTX. In accordance with earlier data (Filippov et al., 1998), neither current was inhibited by more than 8-12% in control neurons not injected with $P2Y_1$ cRNA.

Thus, the $P2Y_1$ receptor couples with equal and very high effectiveness to at least two G-proteins to inhibit both neuronal $Ca^{2+}{}_N$ and $K^+{}_M$ currents: coupling to $Ca^{2+}{}_N$ channels involves both PTX-sensitive and PTX-insensitive G proteins, whereas coupling to $K^+{}_M$ channels is mediated only by a PTX-insensitive G protein. Similar effects in the brain could affect both synaptic transmission and neuron excitability.

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46P P2X RECEPTORS ON GUINEA-PIG PELVIC GANGLION NEURONS EXHIBIT NOVEL SENSITIVITY TO ANTAGONISTS SURAMIN, CIBACRON BLUE AND PPADS

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$P2X_{(1-7)}$ receptors are ligand gated ion channels activated by extracellular ATP (Burnstock, 1997). In our previous studies, we have shown that the P2X receptors on rat pelvic ganglion neurons are of the $P2X_2$ subtype, similar to those on rat superior cervical ganglion (SCG) and coeliac ganglion (Zhong *et al.*, 1998, 1999a). However, two distinct P2X receptors coexist on guinea pig SCG neurons. While both are slowly-desensitising, one is sensitive to αβ-methylene ATP (αβmeATP) and the other is not (Zhong *et al.*, 1999b). In this study, we examined the P2X receptors on guinea pig pelvic ganglion neurons, to determine whether they are similar to those on rat pelvic ganglion, or those on guinea pig SCG.

Single neurons of the pelvic ganglia from male guinea pigs (150-250g) were enzymatically isolated and maintained in tissue culture for up to 24 hrs. Whole cell voltage-clamp recording was carried out at a holding membrane potential of -60mV. All results are expressed as mean ± s.e.mean.

Rapid application of αβmeATP and ATP 100μM on to isolated pelvic ganglion neurons (>300 cells) of guinea pig induced three types of inward currents. About 10% neurons showed predominantly fast-desensitising or clearly biphasic responses. The remaining 90% neurons showed slowly-desensitising responses, which were composed of αβmeATP-sensitive and –insensitive components. We have concentrated on the pharmacological characterisation of these slowly-desensitising responses. To isolate the αβmeATP-sensitive slowly-desensitising receptors, we used αβmeATP as the selective agonist. However, there is no selective agonist available for the αβmeATP-insensitive receptors. Thus, we selected neurons (approximately 5% of total) where the ratio of maximal response to αβmeATP and ATP (αβmeATP/ATP ratio)<0.1, and used ATP as the agonist. The response to αβmeATP (100μM) was inhibited by PPADS (10μM) to 19±3% (n=9) of control after 4-min preincubation. A 2-min preincubation with suramin (100μM) or cibacron blue (50μM) inhibited the αβmeATP response to 5±1% (n=6) and 35±1% (n=4) of control, respectively. In contrast, on the αβmeATP-insensitive receptors, while the response to ATP (100μM) was inhibited by PPADS (10μM) to 18±3% (n=4) of control after 4-min preincubation, it was not sensitive to the antagonism by suramin (100μM) and cibacron blue (50μM). The response to ATP (100μM) was 122±12% (n=3) and 122±8% (n=4) of control, respectively after 2-min preincubation with these antagonists (neither was significantly different from control, $p>0.05$, Student's t-test).

To conclude, there are at least three different P2X receptors on guinea pig pelvic ganglion neurons. The proportion and combination of each type vary greatly from cell to cell. The novel selectivity to antagonists demonstrated by these receptors suggests that either novel P2X receptors are present in these neurons, or the guinea pig P2X receptors have novel pharmacology compared with their rat homologues.

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47P N-(3-OXODODECANOYL)-L-HOMOSERINE LACTONE CAUSES BRADYCARDIA IN CONSCIOUS RATS

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N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), a quorum-sensing signal molecule produced by *Pseudomonas aeruginosa* (Winson *et al.*,1995), has been shown to affect cytokine production by eukaryotic cells (Telford *et al.*,1998) and to inhibit contraction in porcine arterial smooth muscle (Lawrence *et al.*,1999). Here, we assessed the haemodynamic effects of OdDHL in conscious, male, Long Evans rats (350-450g, n=9). Animals were chronically instrumented with pulsed Doppler probes and intravascular catheters to allow assessment of changes in heart rate (HR), mean arterial blood pressure (MAP), and renal (R), mesenteric (M), and hindquarters (H), vascular conductance (VC). All surgery was carried out under sodium methohexitone anaesthesia (40-60 $mgkg^{-1}$ i.p., supplemented as required)(Gardiner *et al.*,1995). Animals were given i.v.(0.1 ml) doses of OdDHL (up to 10 $mgkg^{-1}$) in vehicle (50:50 acetonitrile/sterile dextrose (5%)). Table 1 summarises some of the results. OdDHL caused a marked bradycardia, which was maximal around 3 min after administration. There was a slight pressor effect, but this was not different from that seen with vehicle, although the latter had no significant influence on HR. The bradycardic effect of OdDHL was accompanied by modest reductions in MVC and HVC; such changes were not seen with vehicle (Table 1). In a separate experimental group of animals (n=3), the bradycardic effect of OdDHL ($10mgkg^{-1}$) was abolished (-3±5 beats min^{-1} at 3 min) in the presence of atenolol and atropine ($1mgkg^{-1}$,' $1mgkg^{-1}h^{-1}$ for both).

Table 1. Baseline cardiovascular variables and values recorded 3 min after administration of OdDHL ($10mgkg^{-1}$) or vehicle in the same conscious Long Evans rats (n=9) on different days. Values are mean ± s.e.mean; *P<0.05 versus baseline (Friedman's test).

		Baseline	3 min
HR	OdDHL	345±7	262±10*
(beats min^{-1})	Vehicle	336±6	345±7
MAP	OdDHL	100±2	106±2*
(mmHg)	Vehicle	103±2	107±1*
RVC	OdDHL	67±6	68±6
([kHz $mmHg^{-1}$]10^3)	Vehicle	66±6	69±6
MVC	OdDHL	68±9	56±7*
([kHz $mmHg^{-1}$]10^3)	Vehicle	69±8	68±7
HVC	OdDHL	43±3	38±2*
([kHz $mmHg^{-1}$]10^3)	Vehicle	41±2	41±2

Collectively, these data indicate that OdDHL may interact with mechanisms selectively modulating sinoatrial node discharge rate. However, in contrast to OdDHL, specific bradycardic agents may also cause hypotension in conscious rats (Gardiner et *al.*, 1995). Although OdDHL inhibits P2Y-receptor-mediated effects (Saleh *et al.*, 1999), this is not a likely explanation of the present findings (see Ralevic & Burnstock, 1998, for review).

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48P REGULATION OF I_{KR} BY FORSKOLIN REDUCES THE BLOCKING EFFECT OF E4031 IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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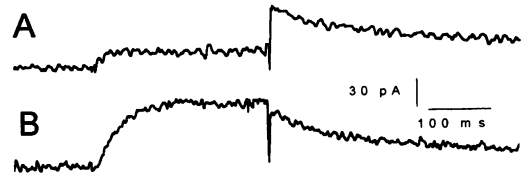
The delayed rectifier potassium current in the heart plays a fundamental role during repolarization and is thought to consist of a rapidly activating current, I_{Kr}, and a more slowly activating current, I_{Ks} (Sanguinetti and Jurkiewicz, 1990). I_{Ks} is known to be modulated by neurotransmitters and hormones and we have recently found that I_{Kr} is also regulated in a similar manner in guinea-pig isolated ventricular myocytes (Heath and Terrar, 1999). The possibility that this regulation influences the action of E4031 was investigated.

Single cells were isolated from the hearts of guinea-pigs (male 0.5 – 0.7 Kg) and cells were continuously superfused with balanced salt solution at 36 °C. I_{Ks} was blocked using 350 μM thiopentone in all solutions and I_{Kr} was studied using switched voltage clamp with sharp electrodes. I_{Kr} was activated by 0.3 s step depolarization from a holding potential of –40 mV to a test potential of +20 mV and studied as deactivating tail currents or as the E4031-sensitive current. Data are mean ± s.e.mean and Student's t test was used for statistical analysis with P<0.05 taken to indicate significance.

In the presence of forskolin, I_{Kr} is enhanced through a decrease in C-type inactivation. This regulation is inhibited by buffering of cytoslic Ca and by block of I_{Ca}. (Heath and Terrar, 1999). I_{Ca}. We investigated the possibility that the regulation of I_{Kr} may influence block by the methanesulfonanilide compound E4031. Experiments were carried out in the continuous presence of 1 μM forskolin. To study the effect of E4031 when C-type inactivation was not reduced, 2 μM nifedpine was present in the extracellular solution to block I_{Ca} and electrodes contained 50 mM BAPTA. These conditions inhibited the regulation of I_{Kr} and the E4031-sensitive current showed the typical rectification during the depolarization (Fig. A, averaged



from three cells). The mean tail current amplitude was 79 ± 11 and after exposure to 1 μM E4031, I_{Kr} tails were reduced to 14 ± 2 pA (n=5), a decrease of 81 ± 3 % (n=5 p<0.01). When electrodes contained no BAPTA and there was no nifedipine present, forskolin reduced the inactivation of I_{Kr} (Fig. B) increasing I_{Kr}. Under these conditions, tail current amplitude was 111 ± 13 pA (n=9) and this was reduced to 54 ± 12 pA (n=6; P<0.01) after exposure to 1 μM E4031. This was a reduction of only 54 ± 4%, which was significantly less (p<0.01) than the 81 ± 3 % decrease caused by E4031 when inactivation was not influenced by forskolin. These data are consistent with a reduced sensitivity of I_{Kr} to block by E4031 when the channel is regulated by forskolin and this may contribute to the reduced effect of this compound to prolong APD under conditions of sympathetic stimulation in the heart.

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49P EFFECT OF CYTOCHALASIN D ON VASCULAR REACTIVITY AND CELLULAR OUTGROWTH IN THE RABBIT CAROTID ARTERY

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Actin filaments form an important component of the cytoskeleton and are critical for the regulation of various cell functions including muscular contraction and migration (Tobacman, 1996; Schedlich *et al.*, 1997). Cytochalasin D (cyt D) is a fungal metabolite which inhibits microfilament function and polymerisation by blocking actin monomer addition at the rapidly growing end of actin filaments (Cooper, 1987). The aim of this study was to investigate the effect of cyt D on the phenylephrine induced contraction in the isolated rabbit carotid artery and on cellular outgrowth *in vitro*.

Segments (2 mm width) from carotid arteries of male New Zealand White rabbits (2.2-3.5 kg) were mounted in isolated organ baths (5 ml) for isometric force measurements at 6 g loading tension. Cumulative dose response curves of phenylephrine were monitored after a 30 min incubation with 0 (n=7), 0.01 (n=6), 0.1 (n=7) or 1 (n=7) μM cyt D and were expressed as percentage of the contraction evoked by 50 mM potassium chloride before addition of cyt D. To determine the effect of cyt D on cellular outgrowth, rabbit carotid artery explants (2 mm width) were individually incubated in 2 ml Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum to stimulate cellular outgrowth. Segments were separately treated with either 0 (n=13), 0.01 (n=13), 0.1 (n=13) or 1 (n=14) μM cyt D. Cells migrated from the explant after 10 days of incubation in a humidified 5% CO_2 incubator at 37°C, were harvested by trypsinisation, stained with propidium iodide, counted by flow cytometric analysis and expressed as percentage of the amount of cells migrated from the explant without cyt D. Data are presented as mean ± s.e. mean. Comparison between the groups were made by one-way analysis of variance followed by Dunnett's multiple comparison *post-hoc* test. Statistical significance was assumed at $p<0.05$.

The maximal normalised phenylephrine induced contraction decreased from 134±3% in the control group to 113±8% in the presence of 0.1 μM ($p<0.05$) and to 27±4% in the presence of 1 μM cyt D ($p<0.01$). There was no significant difference between the control and the 0.01 μM cyt D groups (134±3% versus 133±2%, respectively). In contrast to the maximum normalised force development, the sensitivity to phenylephrine was not influenced by the exposure to cyt D. Incubation with 0.1 μM cyt D decreased the cellular outgrowth to 31±4% ($p<0.01$) as compared to control. Cellular outgrowth could not be detected by flow cytometry after incubation with 1 μM cyt D. In contrast, no significant difference in cell number was observed after incubation with 0.01 μM cyt D (94±6%).

In conclusion, these results suggest that the actin integrity is compromised by cyt D and that this integrity is somehow essential for optimum vascular contractility and cell migration.

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50P INVOLVEMENT OF AN ENDOTHELIN-1-DEPENDENT ANTI-APOPTOTIC EFFECT IN THE PROLIFERATIVE RESPONSE OF CULTURED ENDOTHELIAL CELLS TO α_2-ADRENERGIC RECEPTOR STIMULATION

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Activation of endothelial α_2-adrenergic (AR) receptors has been shown to regulate endothelin-1 (ET-1) production. Furthermore, ET-1 is known to induce endothelial cell (EC) proliferation. Therefore, we have investigated if activation of α_2-AR triggered EC proliferation and involved ET-1 production as an intermediate.

Primary cultures of porcine aortic EC (n=20, passages 1 and 2) were used. The release of ET-1 by confluent EC in serum-free medium (DMEM) was measured by standard radioimmunoassay. To measure proliferation, EC were seeded at a concentration of 2,000 cells/well containing DMEM supplemented with 10% fetal bovine serum (FBS). At day 0, drugs were added (or not), and the medium was replaced at days 2 and 4. Starting at day 1, cells were counted every 24 hours up to day 6. Chromatine cleavage, an index of apoptosis, was measured at day 4 after 24 hrs [^{3}H]-thymidine incorporation. Results were analysed by ANOVA/Scheffé's *F* test and expressed as mean±SEM.

Norepinephrine stimulated EC proliferation: at day 6, EC number (x 1,000) was 47±2 ($P<0.05$, n=6) compared to 31±1 in serum-DMEM alone. This proliferative effect was blocked ($P<0.05$) by yohimbine (10 μmol/L), a selective α_2-AR antagonist (27±1) but unaffected by propanolol ($P<0.05$), a β-AR antagonist (45±2). Oxymetazoline (OXY, 10 μmol/L), an α_2-AR agonist, stimulated EC proliferation: at day 6, EC number (x 1,000) was 82±5 ($P<0.05$, n=12) compared to 49±2 in serum-DMEM alone. The proliferative effect of OXY was reduced by yohimbine (10 μmol/L; 60±3, $P<0.05$), and prevented by BQ788 (1 μmol/L), a selective ET_B receptor antagonist (52±4, $P<0.05$), but not by ET_A receptor inhibition (BQ 123, 1 μmol/L; 80±4). ET-1 (1 nmol/L) stimulated EC proliferation from 48±2 to 77±3 ($P<0.05$). This proliferative effect of ET-1 was reduced by BQ788 (65±4, $P<0.05$) but unaffected by BQ123 (79±3).
OXY (10 μmol/L) stimulated ET-1 release (0.12±0.01 pg/μg protein, $P<0.05$) compared to basal (0.08±0.01).
OXY-induced ET-1-dependent EC proliferation involved activation of the p38 mitogen-activated protein (MAP) kinase pathway. Selective inhibition of this pathway by SB 203580 (10 μM, n=12) slowed EC proliferation in the presence of OXY (55±3, $P<0.05$) below the control growth response obtained in the presence of serum-DMEM alone (66±3) or SB 203580 alone (67±3). In contrast, inhibition of the MAP kinase kinase (MEK) pathway by PD 98059 (10 μM) did not prevent the proliferative effect of OXY on EC (76±3) compared to 82±5 in the presence of OXY alone.
Finally, chromatine cleavage (n=12) measured in control conditions, *i.e.* in the presence of serum-DMEM alone (4.3±0.3%) was decreased by OXY alone (2.5±0.4%, $P<0.05$) whereas ET-1 alone (1 nmol/L) had no significant effect (3.8±0.3%). However, when OXY or ET-1 were combined with SB 203580, chromatine cleavage strongly increased to 11.6±2.9% and 15.2±2.9%, respectively ($P<0.05$).

In summary, activation of α_2-AR triggers EC proliferation: this effect is mediated 1) by an increase in ET-1 production and 2) an activation of the p38 MAP kinase pathway. This results in a reduction of apoptosis.

In conclusion, α_2-AR-mediated EC proliferation may be the consequence of an anti-apoptotic effect rather than a proliferative effect.

51P ACTIONS OF *O*-RAFFINOSE CROSS-LINKED HAEMOGLOBIN ON ISOLATED SEGMENTS OF RAT THORACIC AORTA

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Attempts to produce safe and reliable substitutes for human haemoglobin for transfusion purposes have, been complicated by undesirable side-effects, such as the inappropriate vasoconstriction seen with diaspirin cross-linked haemoglobin, through the binding of nitric oxide (NO) (Freas *et al.*, 1995; Callingham *et al.*, 1999). A further modified haemoglobin with a potential use as a blood substitute is *o*-raffinose cross linked and polymerised human haemoglobin (ORCLHb; Hemolink™, Hemosol Inc, Etobicoke, Canada), which has been suggested to be less prone to cause rises in blood pressure (Freedman *et al.*, 1997). It was decided to see whether or not ORCLHb was capable of contracting isolated segments of rat aorta and antagonising the actions of endothelium-dependent and -independent vasodilators.

Thoracic aortae, from male Wistar rats, were cut into 3 mm rings, mounted in individual organ baths containing aerated (95% O_2/5% CO_2) Krebs-Henseleit solution at 37°C and attached to isometric strain gauges for measurement of tension. Eicosanoid production was prevented by the addition of indomethacin (10^{-5} M). All rings were tested for the presence of endothelium by addition of carbachol (10^{-5} M) to rings pre-contracted with phenylephrine (10^{-6} M). Statistical evaluation was by the alternate Welch *t*-test.

ORCLHb (10^{-5} M) inhibited the cumulative, endothelium-independent relaxation induced by sodium nitroprusside (SNP), in rings of aorta pre-contracted with phenylephrine (10^{-5} M). The EC_{50} value (mean ± s.e. mean) for SNP increased from 2.1 ± 0.1 x 10^{-8} M to 1.17 ± 0.05 x 10^{-7} M ($P < 0.001$, n = 4 with 4 rings used from each animal). Under the same conditions, ORCLHb (10^{-5} M) reversed the endothelium-dependent relaxation induced by carbachol (10^{-5} M) to the original level of pre-contracted tone or even higher, as did human oxyhaemoglobin (10^{-5} M) and L-NAME (10^{-4} M). ORCLHb added to rings, not pre-contracted, produced a concentration-dependent increase in tension with an EC_{50} value of 5.0 ± 0.7 x 10^{-6} M (n = 8 with 2 or 3 rings used from each animal). The contractions caused by ORCLHb were completely antagonised by the α_1-adrenoceptor selective agonist, prazosin (10^{-6} M), but the α_2-adrenoceptor selective agonist, yohimbine (10^{-7} M), had no effect. Incubation with L-NAME (10^{-4} M) potentiated the actions of ORCLHb and of haemoglobin by factors of 3.9 and 3.1, respectively.

Following ultra-centrifugation, at 270,000 *g* for 14 h, of ORCLHb, the supernatant produced no changes in isometric tension of rings of rat aorta, when applied in volumes corresponding to those used to dissolve the ORCLHb. When the pellet was re-suspended in saline solution, a concentration-dependent increase in tension was obtained similar to that seen with ORCLHb solutions that had not been centrifuged.

In these experiments, it appears that ORCLHb antagonises the actions of SNP to relax rings pre-contracted with phenylephrine, probably by binding NO. However, the ability of ORCLHb to contract rings in the absence of phenylephrine is antagonised by prazosin, suggesting that it could also act through α_1-adrenoceptors.

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52P HUMAN PROSTATE-ARTERY SELECTIVITY OF TAMSULOSIN, DOXAZOSIN AND ALFUZOSIN

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Alpha$_1$-adrenoceptor antagonists are used in the treatment of benign prostatic hyperplasia, but their use is often associated with cardiovascular side-effects (Chapple & Chess-Williams, 1999). Tamsulosin is the only clinically used α_1-antagonist that discriminates between α_1-adrenoceptor subtypes and this receptor-selectivity may contribute towards the "uro-selectivity" observed in clincial trials. The aim of this study was to examine the tissue-selectivity of tamsulosin, doxazosin and alfuzosin for the human prostate compared with human mesenteric arteries *in vitro*.

Prostatic strips from patients undergoing transurethral resection of the prostate and mesenteric arteries (endothelium denunded) from anterior bowel resections were set up under 1g tension in gassed Krebs-bicarbonate solution at 37°C. After equilibration, cumulative concentration-response curves to noradrenaline (prostate) or phenylephrine (arteries) were obtained in the absence and presence (60min incuabation) of tamsulosin (0.1-10nM for prostate, 10-100nM for artery), doxazosin (0.1-1.0nM for prostate, 0.3-1.0 for artery) or alfuzosin (0.1-1.0 for prostate, 1-3nM for artery). Affinity values (mean pK_B±sem) were calculated from the shifts of agonist curves as described previously (Noble et al., 1997). Experiments were conducted in the presence of corticosterone (10μM), cocaine (10μM) and propranolol (1μM).

Noradrenaline and phenylephrine produced concentration-dependent contractions of isolated human prostate and mesenteric arteries respectively.

Tamsulosin exhibited a 10-fold selectivity for the prostate over the artery; alfuzosin displayed some selectivity (2.5-fold) for the prostate and doxazosin was the only antagonist to exhibit a selectivity (3.7-fold) for the vascular tissue.

Table1. Prostate-artery selectivity of antagonists in clinical use

Antagonist	n	Prostate pK_B	n	Mesenteric arteries pK_B	Selectivity for prostate
Doxazosin	15	7.47 ±0.09	13	8.02 ±0.16	0.28
Alfuzosin	14	7.62 ±0.09	12	7.22 ±0.08	2.51
Tamsulosin	21	9.90 ±0.40	16	8.90 ±0.15	10.00

Selectivity is calculated as the ratio of the molar affinities (K_B). Affinity values are apparent values for doxazosin and alfuzosin on the prostate and also tamsulosin on the artery (Schild slope = 0.46±0.15).

The results support findings in the clinic, where tamsulosin has minimal effects on blood pressure and alfuzosin is well tolerated, while doxazosin exerts significant blood pressure lowering effects in hypertensive patients. The relatively low affinity of tamsulosin at the artery receptor supports the concept that the α_{1B}-adrenoceptor predominantly mediates contraction in the human vascular system.

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53P EVIDENCE THAT THE ANTI-ANGINAL AGENT, RANOLAZINE, BEHAVES AS A WEAK β-ADRENOCEPTOR ANTAGONIST

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Ranolazine has demonstrated anti-anginal effects in clinical trials and myocardial anti-ischaemic activity in several *in vitro* and *in vivo* animal models, without producing significant haemodynamic effects. To date, the anti-ischaemic mechanism of action of ranolazine has not been clearly identified and a beneficial effect on myocardial cell metabolism has been suggested (Hara *et al.*, 1999). However, a preliminary study has shown that ranolazine has weak affinity for β_1-adrenoceptors (Ferrandon *et al.*, 1992). Therefore, the objective of this work was to evaluate whether ranolazine exerted functional β-adrenergic receptor antagonist activity.

Radioligand binding was performed in rat hearts for β_1- or guinea-pig lungs for β_2-adrenoceptor affinity. Interaction of ranolazine with β_1 and β_2-adrenoceptors was studied in functional *in vitro* and *in vivo* procedures. *In vitro*, experiments were carried out in rat isolated left atria which were electrically driven (4 Hz), in which a cumulative concentration response curve to isoprenaline (0.01 nM to 1 μM) was constructed. *In vivo* haemodynamic studies were conducted in pithed rats. Cumulative incremental doses of isoprenaline (0.63 ng/kg to 0.63 mg/kg, i.v.) induced depressor and chronotropic responses. Animals respectively received vehicle (NaCl 0.9 %, i.v., n=12), atenolol (0.04 to 2.5 mg/kg i.v., n=6 rats per dose), ICI 118551 (0.01 to 0.63 mg/kg i.v., n=6 or 7 rats per dose) or (+/-) propranolol (0.01 to 0.63 mg/kg i.v., n=6) or ranolazine (2.5 to 80 mg/kg i.v., n=6 or 7 rats per dose) 10 min. prior to isoprenaline.

Radioligand binding affinity was observed for ranolazine at β_1 and β_2-adrenoceptors (IC_{50} values 5.0 (4.0-6.3) and 1.6 (1.0-2.5) μM, respectively). In rat isolated atria, ranolazine (0.32 to 10 μM) competitively antagonised isoprenaline-induced positive inotropic responses, with an apparent pA_2 value of 5.9. In pithed rats, isoprenaline decreased diastolic arterial pressure (max. decrease: 46.2±1.5 %; $p<0.001$) and increased heart rate (max. increase: 54.1±2.9 %; $p<0.001$). Ranolazine dose-dependently displaced isoprenaline-induced decreases in diastolic arterial pressure (dose ratio 12.2 with 80 mg/kg of ranolazine), suggesting competitive antagonism. Similarly, ranolazine dose-dependently shifted the isoprenaline-induced increases in heart rate (dose ratio 20.3 with ranolazine at 80 mg/kg), also suggesting competitive antagonism.

Collectively, these results demonstrate that ranolazine behaves as a weak β_1- and β_2-adrenoceptor antagonist.

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54P CAROTID VASCULAR EFFECTS MEDIATED BY α_1-ADRENOCEPTORS IN ANAESTHETISED PIGS: POSSIBLE IMPLICATIONS FOR MIGRAINE THERAPY

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It has recently been shown that both α_1- and α_2-adrenoceptors are operative in mediating vasoconstriction in the porcine carotid vasculature (Willems *et al.*, 1999). The present study set out to elucidate which α_1-adrenoceptor subtypes mediate vasoconstriction in this vascular bed, with special emphasis on arteriovenous anastomoses (AVAs). For this purpose, we gave 10 min intracarotid infusions (0.1 ml min^{-1}) of phenylephrine (1-10 μg kg^{-1} min^{-1}) in pentobarbital anaesthetised, bilaterally vagosympathectomised female pigs (12-14 kg; n=35), treated with either vehicle (distilled water; n=8) or the following subtype-selective antagonists: 5-methylurapidil (α_{1A}; 300 or 1000 μg kg^{-1}, n=6 each), L-765,314 (α_{1B}; 300 or 1000 μg kg^{-1}, n=6 and 3, respectively) or BMY 7378 (α_{1D}; 300 μg kg^{-1}, n=6). Total carotid blood flow was measured with an electromagnetic flow meter, whereas its AVA and capillary fractions were calculated using the radioactive microsphere method (see Saxena, 1995). All data are expressed as mean±s.e.mean. Baseline values (n=35) of heart rate (beats min^{-1}), mean arterial blood pressure (mmHg) and total carotid, AVA and capillary conductances (10^{-2} ml min^{-1} $mmHg^{-1}$) were 100±2, 93±2, 123±6, 102±6 and 30±2, respectively. Whereas the treatments caused only minor systemic and carotid haemodynamic changes, phenylephrine produced a slight tachycardia (maximum change: 28±5%). Phenylephrine dose-dependently decreased total carotid conductance (maximum decrease: 74±4%), which was confined to the AVA fraction (maximum decrease: 92±3%; see Fig. 1); the capillary fraction was not modified. Whereas L-765,314 (1000 μg kg^{-1}) abolished the phenylephrine-induced vasoconstriction of carotid AVAs, the same dose of 5-methylurapidil attenuated this response only partially; BMY 7378 was ineffective (Fig. 1). These results, coupled to the binding affinities of the above antagonists at α_{1B}-adrenoceptors, suggest that mainly α_{1B}-adrenoceptors mediate vasoconstriction of dilated carotid AVAs in anaesthetised pigs. Since this *in vivo* model may predict anti-migraine activity (see Saxena, 1995), an agonist at α_{1B}-adrenoceptors could provide a new avenue for the development of migraine abortive drugs.

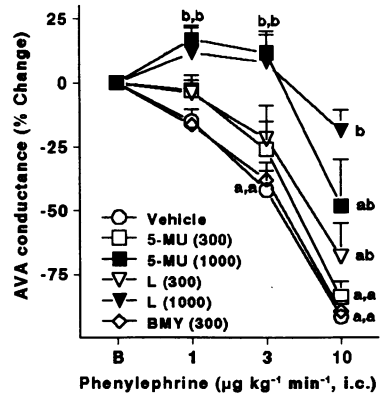

Fig. 1
Percent changes in AVA conductance induced by 10 min intracarotid infusions of phenylephrine in anaesthetised pigs, treated either with vehicle (control) or the subtype-selective antagonists.
a, P<0.05 vs. baseline; b, P<0.05 vs. control.

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55P INFLUENCE OFAT$_1$-RECEPTOR BLOCKADE WITH LOSARTAN, IRBESARTAN AND TELMISARTAN ON THE FACILITATION OF SYMPATHETIC NEUROTRANSMISSION BY ANGIOTENSIN II IN THE PITHED RAT MODEL

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Numerous studies have shown that Angiotensin II enhances sympathetic nervous transmission (Saxena et al. 1992).
The pithed rat is a high-renin model, in which endogenously formed angiotensin II facilitates neurally mediated increments in vascular resistance (Kaufman et al. 1985). In the pithed rat, the AT_1-receptor antagonist losartan was shown, in a single dose study, to attenuate hemodynamic responses to spinal cord stimulation (Wong et al. 1992). The objective of the present study was to quantify the inhibitory effect of the AT_1-receptor blockers losartan, irbesartan and telmisartan on sympathetic neurotransmission and to compare the potency of these agents. In the male, normotensive pithed rat model (weight 255-320g), we studied the effect of AT_1-blockade by losartan (1, 3, 10 and 30 mg/kg), irbesartan (3, 10, 30 and 60 mg/kg) and telmisartan (0.3, 1, 3 and 10 mg/kg) on the sequelae of stimulation of thoraco-lumbar sympathetic outflow (n=6 in each group). Rats were treated with propranolol 1 mg/kg i.v., tubocurarine 2.5 mg/kg i.v. and atropine 2 mg/kg i.p. prior to stimulation. Bilateral adrenalectomy and vagotomy were performed. Diastolic and systolic blood pressure were monitored using a carotid arterial catheter. Linear regression was performed and analysis of covariance was used to determine differences between regression lines. Sympathetic nervous system stimulation (2 Hz, 15 sec.) caused an increase in DBP of 87.0 ± 2.4 mmHg in the control group (mean ± SEM). The increase in DBP could be dose-dependently reduced by AT_1-receptor blockade. A maximal reduction in DBP-rise was observed after administration of losartan 10, irbesartan 30 and telmisartan 3 mg/kg (ΔDBP's of 52.8 ± 7.8 , 45.2 ± 5.7 and 57.0 ± 5.9 mmHg, respectively (control vs. all three agents: $p<0.01$). The doses which reduced ΔDBP by 20 mmHg (ED20, half of the maximal inhibition, expressed as log mol/kg) were telmisartan −5.5, losartan −5.1 and irbesartan −4.8 , respectively ($p<0.05$ between regression lines). Surprisingly, the highest doses caused less than maximal reduction in the rise of DBP. (ΔDBP's after administration of losartan 30, irbesartan 60 and telmisartan 10 mg/kg were 68.1 ± 5.1 , 59.6 ± 3.7 and 73.0 ± 6.8 mmHg, respectively). We conclude that the effects of stimulation of the thoraco-lumbar sympathetic outflow on DBP are counteracted by AT_1-receptor blockade. The order of potency of the drugs proved telmisartan>losartan>irbesartan. It is noteworthy that the order of potency, concerning the inhibition of angiotensin II-induced contraction of vascular smooth muscle, was reported to be telmisartan>irbesartan>losartan (Wienen et al. 1993, Cazaubon et al. 1993). This discrepancy might contribute to the individual pharmacological profiles of AT_1-blockers.

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56P THE RESPONSES OF THE DIGITAL ARTERY OF THE FALLOW DEER, *DAMA DAMA*, TO 5-HT IN THE WINTER OF 1998-1999

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The vasoactive responses of the digital artery of the fallow deer, *Dama dama*, to a variety of pharmacologically active substances, including the vasoconstrictor, 5-hydroxytryptamine (5-HT), differ when the animals are exposed to different climatic temperatures. It has been proposed that these differences provide an energy efficient means of maintaining deep body temperature during cold weather (Callingham *et al.*, 1998; Milton *et al.*, 1999). It was also found that the digital artery of the deer does not contain muscarinic acetylcholine receptors, and thus resembles the horse (Callingham *et al.*, 1998). Bailey & Elliott (1998a) have suggested that 5-HT could play a role in the pathogenesis of laminitis, in which an abnormally high tonic constriction of the distal blood vessels of the forelimb is involved. It was decided to investigate the manner in which 5-HT caused contractions of deer digital artery rings, in an attempt to see if there were any similarity with its actions in the corresponding horse artery.

The experiments described here were carried out during the mild winter of 1998-1999, when responses to nitro-vasodilators were unchanged from those seen in the summer months (Milton *et al.*, 1999). Digital arteries were dissected from the forelimbs of fallow deer, immediately after the animals had been killed for venison under European Union red meat regulations. The arteries were kept in ice cold modified Krebs-Henseleit saline solution for transport to the laboratory. After removal of surplus connective tissue, 2-3 mm wide rings were cut, mounted, in saline solution at 37° C, aerated with 95% O_2, 5% CO_2, on hooks attached to strain gauges. An initial tension of approximately 3 g was applied and changes in tension recorded.

In all experiments the presence of a functional endothelium was determined by contracting the arterial rings with L-phenylephrine (10^{-6} M) and then applying the endothelium dependent relaxant, histamine (10^{-5} M). In some experiments the endothelium was removed by rubbing the luminal wall with a stainless steel wire. Cumulative concentration response curves to 5-HT were obtained both in the presence and absence of the endothelium, and showed no significant differences between the two EC_{50} values: (mean ± s.e. mean) $7.3 \pm 0.50 \times 10^{-8}$ M, n = 7 and $1.30 \pm 0.11 \times 10^{-7}$ M, n = 3, with and without endothelium, respectively. The 5-HT_{2A} receptor antagonist, ketanserin (10^{-7} M), significantly increased the EC_{50} value of 5-HT to $2.0 \pm 0.25 \times 10^{-8}$ M, $n = 4$, $P < 0.001$. Increases in EC_{50} values were also seen with 10^{-9} and 10^{-9} M ketanserin. The affinity constant for ketanserin was $3.14 \pm 0.01 \times 10^8$ M^{-1}. Rauwolscine (10^{-7} M), an antagonist at 5-HT_{2B} receptors, also reduced the responses to 5-HT with an EC_{50} value of 5.5×10^7 M^{-1} The 5-$HT_{1B/1D}$ antagonist, pindolol (10^{-7} M), had no effect on the EC_{50} value of 5-HT. However, this concentration appeared to have a non-competitive effect in that it reduced by 50%, the maximum contraction achieved with 5-HT.

These results appear to show that 5-HT is an agonist in the digital artery of the fallow deer, acting mainly through 5-HT_2 receptors. This is in contrast to the equine digital artery where 5-$HT_{1B/1D}$ receptors are the main subtype responsible for vasoconstriction (Bailey & Elliott 1998b).

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57P ANTIPLATELET AND ORAL ANTITHROMBOTIC ACTIVITY OF SL 65.0472

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SL 65.0472 (7-fluoro-2-oxo-4-[2-[4-(thieno[3,2-*c*]pyridin-4-yl) piperazin-1-yl]ethyl]-1,2-dihydroquinoline-1-acetamide) is a potent antagonist of vasoconstriction mediated by 5-HT_{1B} and 5-HT_{2A} receptors in vivo (O'Connor *et al.*, this meeting). This study examined its effects on 5HT-induced human platelet activation in vitro, and in rat and rabbit platelet-rich thrombosis models.

Citrated rat, rabbit or human platelet-rich plasma (PRP) was incubated with SL 65.0472 for 1 min (37°C) before adding EDTA (5mM) followed by 1μM 5-HT. Shape change was monitored by turbidimetry. Aggregation was induced in citrated human PRP by 5 μM 5-HT + threshold concentrations of ADP (0.5-1μM) or collagen (0.3 μg/ml) and was measured by turbidimetry. Thrombosis was induced in an A-V shunt containing a scraped nylon monofilament connecting a carotid artery to a jugular vein for 15 min in pentobarbital-anaesthetised CD rats (Berry *et al.*, 1994), or a shunt containing a silk thread between a carotid artery and jugular vein in pentobarbital anaesthetised rabbits (Herbert *et al.*, 1993). The thrombus wet weights were determined. Femoral arterial thrombus formation was induced in anaesthetised rabbits (chlorpromazine + ketamine) by application of an electric current (3mA, 5 min) to the adventitial surface. The thrombotic end-point was the delay to occlusion (i.e. zero flow) measured with a doppler flow probe (Berry *et al.*, 1996).

SL 65.0472 inhibited 5-HT-induced platelet shape change (IC_{50} values = 34.9, 68.9 and 226 nM in rat, rabbit and human PRP respectively, calculated from log-concentration response curves of data pooled from 3 experiments), and inhibited human platelet aggregation induced by 5-HT in the presence of ADP and collagen with mean (±s.e.m.) IC_{50}s of 49±13 and 48±6 nM respectively (n = 6 determinations). In the rat A-V shunt model, SL65.0472 given 2h p.o. prior to shunt assembly led to a dose-dependent diminution in thrombus weight from 164±7.7 mg (mean±s.e.m.) to 84±11 mg after 1 mg/kg ($p<0.01$, Dunnett's test, n=8/group). No further decrease was observed after 3 mg/kg. In the rabbit A-V shunt model, thrombus weights were reduced from 36±5.4 mg to 21.1±3.5 mg ($p<0.05$, Dunnett's test) after 10 mg/kg p.o. 1h before shunt assembly, with lower doses being inactive. SL 65.0472 dose-dependently (1-20 mg/kg p.o.2h prior to electrical lesion) increased the delay to femoral arterial occlusion from 17.5±1.5 min to 61.6±11 min after 20 mg/kg p.o. (n = 10/group, $p < 0.05$ Kruskall-Wallis test).

Thus the anti-5HT_{2A} component of SL 65.0472 is reflected by its ability to inhibit 5-HT-induced platelet activation, and to inhibit platelet rich thrombus formation.

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O'Connor S.E., Grosset, A. Duval, N. *et al.* (2000) This meeting

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58P 5-HT_{1B} AND 5-HT_{2A} RECEPTOR ANTAGONIST PROPERTIES OF SL 65.0472 *IN VIVO*

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Two receptor subtypes have been implicated in 5-HT-induced vasoconstriction, 5-HT_{2A} receptors and a subtype previously classified as 5-$HT_{1\text{-like}}$ but now considered likely to be the 5-HT_{1B}-receptor (Sgard *et al.*, 1996). 5-HT_{2A} receptors also mediate the prothrombotic effects of 5-HT. This study describes SL65.0472 (7-fluoro-2-oxo-4-[2-[4-(thieno[3,2-*c*] pyridin-4-yl) piperazin-1-yl] ethyl]-1, 2-dihydroquinoline-1-acetamide), a novel 5-HT receptor antagonist, in models of vasoconstriction mediated by 5-HT_{1B} and 5-HT_{2A} receptors and in a dog coronary thrombosis model.

Sumatriptan-induced saphenous venoconstriction in the anaesthetised dog (Drieu La Rochelle & O'Connor, 1995) was used as a model of a 5-HT_{1B} mediated response. Saphenous vein diameter (SVD) was measured by sonomicrometry. An i.v. dose of sumatriptan (29 ± 8.2 μg kg^{-1}, n = 10) was chosen for each animal to give ~30 % reduction in SVD and was repeated after i.v. administration of vehicle (5 % glucose) or SL65.0472 (3, 10 and 30 μg kg^{-1} i.v.). SL65.0472 produced dose-related inhibition of sumatriptan-induced reductions in SVD with an ID_{50} of 10.8 ± 1.6 μg kg^{-1} (n = 5) and a maximum inhibition of 90.2 ± 4.3 % after 30 μg kg^{-1} ($P < 0.01$ v vehicle group). Data are mean ± s.e.m.. 5-HT_{2A}-mediated vasoconstriction was studied in rats as increases in mean arterial blood pressure (MAP) produced by 5-HT 30 μg kg^{-1} i.v. in male Sprague-Dawley rats (200-300 g) anaesthetised with sodium pentobarbitone 60 mg kg^{-1} i.p., pithed via the orbit and artificially ventilated. MAP was measured in the carotid artery. 5-HT-induced increases in MAP were measured 75 min after SL65.0472 (3-100 μg kg^{-1} p.o., n=6-9) or 5 min after SL65.0472 (0.3-10 μg kg^{-1} i.v., n = 6). SL65.0472 inhibited 5-HT pressor responses with ID_{50} values of 1.38 μg kg^{-1} i.v. (95 % c.l. 1.15-1.64) and 31.1 μg kg^{-1} p.o. (95 % c.l. 22.6-42.6).

In an anaesthetised dog coronary thrombosis model (Duval *et al.* 1996) endothelial lesions of the circumflex coronary artery with a critical stenosis created regular cyclic variations in blood flow (CFV). CFV frequency and minimum coronary flow (MCBF) were monitored for 120 min after drug or vehicle administration (n = 5/group). Vehicle had no effect on CFV frequency (6.8 ± 0.9 h^{-1} pre-treatment, 7.2 ± 1.0 h^{-1} post-treatment) or on MCBF (3.3 ± 2.2 ml min^{-1} pre-treatment, 3.9 ± 1.7 ml min^{-1} post-treatment). SL65.0472 30 μg kg^{-1} i.v. decreased CFV frequency (7.2 ± 1.0 h^{-1} pre-treatment, 0.6 ± 0.6 h^{-1} post-treatment, $P < 0.05$) and increased MCBF (1.2 ± 0.8 ml min^{-1} pre-treatment, 31.8 ± 8.4 ml min^{-1} post-treatment, $P < 0.05$). Data are mean ± s.e. mean.

Therefore SL65.0472 is a potent antagonist of 5-HT_{1B} and 5-HT_{2A} mediated vasoconstriction *in vivo* and inhibits coronary artery thrombosis (presumably via its 5-HT_{2A} antagonist properties).

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59P NITRIC OXIDE REGULATES NMDA-EVOKED DOPAMINE RELEASE IN THE RAPHE NUCLEI AND FRONTAL CORTEX OF THE FREELY MOVING RAT

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The free radical Nitric oxide (NO) acts as an intercellular messenger in the brain and a number of roles have been proposed. The generation of NO by the neuronal nitric oxide synthase (nNOS) has been linked to the activation of glutamatergic-N-methyl-D-aspartate receptors (Garthwaite & Boulton, 1995). A clear link has been established between NMDA receptor activation and the release of dopamine (DA) in several brain regions (Whitton, 1997) and NO has been implicated in this process, in striatal slices (Hanbauer *et al.*, 1992). In the present study we have investigated the regulation of NMDA-evoked DA release by NO in the raphe nuclei (RN) and frontal cortex (FC) of freely moving rats, using in vivo microdialysis.

Male Wistar rats (270-330g) were anaesthetised using halothane and concentric dialysis probes implanted into either the RN or FC, as previously described (Pallotta et al., 1998). The following day rats were dialysed with artificial cerebrospinal fluid (composition in mM: KCL 2.5; NaCL 125; $MgCL_2$ 1.18, $CaCL_2$ 1.26). After a one hour equilibration period, four 30 minute basal samples were collected prior to drug infusion. The following drugs were infused alone or in combination with NMDA (25-100μM), via the dialysis probe: the NMDA antagonist 2-amino-5-phosphonopentanoic acid (AP5: 100μM), the nNOS inhibitor 7-Nitroindazole (7NI: 1mM) or the NO donor S-nitroso-N-acetylpenicillamine (SNAP: 500μM-5mM). DA levels were measured using HPLC with electrochemical detection as previously described (Whitton & Fowler, 1991). All data are represented as percentage of basal levels ± s.e.mean, n=5. Statistics were performed using one way ANOVA followed by Dunnetts test.

In both the FC and RN, NMDA caused a significant ($p<0.05$) fall in extracellular DA (with 100μM NMDA levels fell to 30±2% in the RN and 25±5% in the FC), which was blocked by AP5. Infusion of AP5 alone increased extracellular DA in both brain regions. 7NI abolished the effects of NMDA infusion and in fact increased DA levels to 215±17% in the RN and 200±28% in the FC. SNAP caused a concentration-dependent decrease in dialysate DA of up to 30±5% in the RN. In the FC, low SNAP concentrations (500μM-1mM) also caused a decrease, but after infusion of 5mM SNAP, levels of DA rose to 150±12% above basal.

The present findings support the modulation of DA release by NMDA and suggest that NO may be involved in regulating this process in the RN and FC of the rat. In both regions the increase in dialysate DA following AP5 alone suggests a tonic role of NMDA receptors in modulating DA release. Moreover the 7NI data suggests that this may depend on tonic activity of nNOS. The biphasic effect of SNAP on DA release in the FC is similar to that seen in other studies on monoamine release (eg. Kaehler *et al.*, 1999), suggesting a complex role of NO in regulating monoamine levels.

JCES holds an MRC studentship.

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60P DIURNAL VARIATION IN 5-HT OVERFLOW IN THE SCN OF THE RAT IN RESPONSE TO NMDA RECEPTOR STIMULATION

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The terminals of the serotonergic raphe-hypothalamic tract and the glutamatergic retino-hypothalamic tract converge in the ventro-lateral region of the suprachiasmatic nucleus (SCN), the site of the mammalian biological clock (Moga and Moore, 1997). We have investigated the effect on 5-hydroxytryptamine (5-HT) overflow from the SCN of the excitary amino acid receptor agonist NMDA, administered at two time points, or zeitgeber times (ZT;ZTO = lights on) in conscious rats by *in vivo* microdialysis. Male Wistar rats (240-280g), were housed under a 12:12 h light:dark cycle. Concentric microdialysis probes were implanted adjacent to the SCN. Probes were continuously perfused (1.2 μl/min) with aCSF containing 1μM citalopram. Drugs were infused via the probe over a 60 min period at ZT 6 and 18. 5-HT levels in three 15 min pre-intervention control samples (mean ± sem 36.3±8.89 and 28.1±5.34 fmol for ZT6 and ZT18 respectively) and eight subsequent dialysate samples were analysed by HPLC-ECD. Statistical analysis was carried out by one way ANOVA with repeated measures with Student Newman Keuls post hoc test. NMDA receptor stimulation at ZT 6 failed to affect (100 μM) or increased (1 mM) (62±26.9%; $p<0.05$) 5-HT overflow, an effect that was blocked by the non-competitive NMDA antagonist MK-801 (10 μM). At ZT 18, NMDA (100 μM) induced a decrease (48.0±21.5%; $p<0.05$) whereas administration of NMDA (1 mM) resulted in an increase 180±38.8%; $p<0.05$) in dialysate 5-HT concentration, effects that were blocked by the competitive antagonist AP5 (100 μM) but not by MK-801. Concurrent infusion of idazoxan (1 μM) with NMDA (100 μM) significantly elevated SCN 5-HT release at ZT6 and ZT18, suggesting that part of the inhibitory effect on 5-HT release of NMDA receptor stimulation may be mediated by noradrenaline release. These data show that the effect of NDMA receptor stimulation on 5-HT overflow in the SCN is both concentration and zeitgeber time-dependant. The significance of the observed diurnal variation in terms of regulating inputs to the circadian clock over 24 hours remains to be elucidated.

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61P THE 5-HT_{1B} AGONIST, CP93129, INHIBITS GABA RELEASE FROM SLICES OF RAT GLOBUS PALLIDUS AND REVERSES AKINESIA FOLLOWING INTRAPALLIDAL ADMINISTRATION IN THE RESERPINE-TREATED RAT

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5-HT_{1B} receptors function as heteroreceptors, reducing the presynaptic release of neurotransmitters such as dopamine. Since 5-HT_{1B} receptors are densely expressed on GABAergic neurone terminals in the globus pallidus (GP), their activation may also reduce GABA release. Excess GABA release in the GP, from overactive striatopallidal projections, contributes to the akinetic symptoms of Parkinson's disease (PD). Therefore, this study examined whether (i) 5-HT_{1B} receptor activation reduces GABA release in the GP and (ii) activation of 5-HT_{1B} receptors in the GP proffer symptomatic relief in a rodent model of PD.

All experiments were performed on male Sprague Dawley rats (250-350g). A 2-stimulation, depolarisation-evoked (25mM KCl) release paradigm was used for in vitro slice superfusion studies as previously described (Mayfield & Zahniser, 1993). Briefly, 25mM KCl-evoked [^{3}H]GABA release was measured before (S1), and after (S2) the addition of the 5-HT_{1B} agonist CP93129 (Macor *et al.*, 1990). The S2/S1 ratio was used to quantify [^{3}H]GABA release in the presence of CP93129 or vehicle (Kreb's buffer). Specificity of action of CP93129 was confirmed in some slices by addition of the 5-HT_{1B} antagonist isamoltane (10μM). Significant differences in release were assessed using a one-way ANOVA ($p<0.05$).

Rats were cannulated in the GP, as described previously (Maneuf *et al.*, 1993). 5 days later, rats were injected with reserpine (5 mg kg^{-1}; s.c.). 18 h later, when a stable akinesia was obtained, rats received unilateral injections of CP93129 (110, 220 or 330 nmole in 0.5μl PBS) or vehicle (PBS alone) into the GP and their locomotor activity was monitored for 5 h. After a minimum 2 h rest period, animals received a second dose of CP93129 and were monitored for a further 5 h. Net contraversive rotations (indicative of a unilateral reversal of akinesia) were counted in 10 min time bins over each 5 h period. Significant differences in total rotations / 5 h were assessed using a one-way ANOVA ($p<0.05$). Receptor specificity of CP93129 was confirmed in a separate experiment by injection of 10 nmole isamoltane / vehicle between consecutive doses of CP93129 (220 nmole). Net rotations / 5 h were compared pre and post isamoltane / vehicle using a paired t-test ($p<0.05$).

In slices of GP, CP93129 (0.6 - 16.2μM) produced a significant decrease in 25 mM KCl-evoked [^{3}H]GABA release (maximum 52.5 ± 4.5 % inhibition with 16.2μM). Isamoltane (10μM) fully antagonised the effects of CP93129 (5.4μM). Intrapallidal injection of CP93129 in the reserpine-treated rat produced a significant increase in net contraversive rotations / 5 h (Table 1). Isamoltane (10 nmole), but not vehicle, inhibited CP93129 (220 nmoles)-induced rotations by 84 ± 6% (n=6).

[CP93129] nmole	Vehicle	110	220	330
Rotations / 5h	1 ± 0.5	64 ± 17*	170 ± 29*	285 ± 60*

Table 1: Effect of CP93129 on locomotor activity in the reserpine treated rat. Data are mean ± s.e.m (n=8-11 animals per group). * = significantly different to preceding dose or vehicle ($p<0.05$).

The reduction of GABA release seen following application of CP93129 to slices of GP indicates that 5-HT_{1B} receptors function as heteroreceptors in this region. Furthermore, in vivo activation of 5-HT_{1B} receptors in the GP can alleviate the akinesia produced in the reserpine rat model of PD. These data support the idea that 5-HT_{1B} receptor agonists may provide a novel therapeutic approach to PD.

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62P CHARACTERISATION OF 5-HT EFFLUX FROM THE RAT SPINAL CORD DORSAL HORN *IN VIVO*

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5-HT neurotransmission is proposed to play an important role in the processing of sensory information at the level of the spinal cord (Sawynok & Reid, 1996). Using microdialysis sampling *in vivo*, we have studied 5-HT efflux in the dorsal horn of the rat lumbar spinal cord. An initial characterisation of dialysed 5-HT is presented. The effect on 5-HT efflux of local depolarisation induced by increased extracellular K^+ concentration inhibition of re-uptake by paroxetine, blockade of action potential generation by tetrodotoxin (TTX) and antagonism of Ca^{2+} entry into the nerve terminal by increased extracellular Mg^{2+} concentration are reported.

Methodology was adapted from Gerin & Privat (1996). Briefly, male Wistar rats (250-350 g; Charles Rivers) were anaesthetised under halothane (4% induction, 1.5% maintenance in O_2) and mounted on a stereotaxic frame. Vertebra Th_{13} was exposed and immobilised with the use of a vertebral clamp such that the dorsal surface of the underlying spinal cord lay on the horizontal plane. A burr hole was then created in the dorsal aspect of the vertebra through which the microdialysis probe was introduced at an angle of 16° from horizontal. This placed a 1.5 mm length of dialysis membrane (Hemophan o.d. 218 μm; Gambro U.K. Ltd.) unilaterally into the dorsal horn of the L3/L4 lumbar region of the spinal cord. The position of the probe was fixed by application of dental cement around the probe and anchorage screws located in the Th_{13} and L_1 vertebrae and anaesthesia reduced to 0.75-1% halothane in O_2/N_2O (1:1). The probe was perfused at a rate of 2 μl.min^{-1} with an artificial extracellular fluid solution (aECF) for 120 min post implantation before 15 min samples of dialysate were collected. After 45 min to estimate basal efflux, separate groups received one of the following treatments: i) 5 min perfusion with high K^+ aECF (60 mM); ii) 75 min perfusion with aECF including paroxetine (1 μM); iii) 75 min perfusion with aECF including TTX (1 μM); iv) 75 min perfusion with a modified aECF containing 12 mM Mg^{2+}, zero Ca^{2+}. Following the treatment period perfusion was switched back to aECF alone for a further 60 min, or for a further 45 min in the case of high K^+ perfusion. A control group was perfused with normal aECF throughout (0-180 min). Samples were assayed for 5-HT content by HPLC allied to electrochemical detection. Data are presented as mean percentage of basal efflux (mean concentration in the initial three samples (0-45 min)).

Basal 5-HT efflux was 9.6 ± 0.7 fmol.20 μl^{-1} (mean ± s.e. mean, n=24). Perfusion with high K^+ aECF induced a marked increase in 5-HT efflux to 314 ± 65% of basal efflux ($P<0.01$, ANOVA with repeated measures, n=5). Basal 5-HT efflux was also significantly increased by paroxetine to a maximum of 231 ± 32% of the level in the absence of paroxetine ($P<0.01$, two-way ANOVA compared to control group, n=5). Perfusion with TTX or high Mg^{2+} aECF significantly reduced efflux to a minimum of 8 ± 5 and 45 ± 9% of basal efflux respectively ($P<0.01$, two-way ANOVA compared to control group, n=5 and n=4 respectively).

These results indicate that physiologically meaningful changes in extracellular 5-HT can be monitored *in vivo* in the rat spinal cord. Moreover, the calcium-dependency and TTX-sensitivity of 5-HT efflux indicate that the majority of the basal extracellular level of 5-HT was derived from neuronal activity in this study.

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63P THE EFFECTS OF 5-$HT_{1B/1D/1F}$ RECEPTOR LIGANDS ON THE ACTIVITY OF NUCLEUS TRACTUS SOLITARIUS (NTS) NEURONES IN ANAESTHETIZED RATS: AN *IN VITRO* IONOPHORETIC STUDY

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It has been suggested that activation of 5-HT_{1D} receptors inhibits and activation of 5-HT_{1B} receptors potentiates the vagal afferent evoked excitatory input to NTS neurones (Wang *et al.*, 1998). The present study was carried out to further investigate the effects of ligands selective for 5-HT_{1B}, 5-HT_{1D} and/or 5-HT_{1F} receptor subtypes, sumatriptan, CP-93,129 and GR55562, on the ongoing activity of NTS neurones *in vivo*.

Male rats (280-390 g) were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p., supplemented with 20 mg kg^{-1}, i.v. as required) and artificially ventilated. "Piggy-back" glass microelectrodes, comprising a single recording electrode and a 5-barrelled electrode, were used for recording ongoing NTS neuronal activity (see Wang et al., 1998). NTS neurones were identified by orthodromic excitation from stimulation of the cervical vagus (1 ms, 10-200 μA, 0.2-1 Hz) and by their location 300-700 μm below the brain surface and above the dorsal vagal nucleus (see Wang *et al.*, 1998). All drugs were applied ionophoretically. Drugs were classed as evoking excitation or inhibition if, during the ejection period, activity was increased or decreased by at least 20% of baseline.

51 NTS neurones were recorded with either ongoing activity or which were induced to fire by the excitatory amino acid DL-homocysteic acid. Application of sumatriptan (20 mM, pH4, 10-120 nA), a 5-$HT_{1B/1D/1F}$ receptor agonist (see Hoyer et al., 1994), current-dependently inhibited (-69 ± 7%, mean ± s.e.mean, of baseline firing rate) the activity of the majority (26 of 29) of NTS neurones tested. In contrast, application of CP-93,129 (10 mM, pH4, 20-120 nA), a 5-HT_{1B} receptor agonist (see Hoyer et al., 1994) current-dependently excited (82 ± 15% above baseline firing rate) 14 of 17 NTS neurones tested. In the same neurone, sumatriptan and CP-93,129 had opposite effects in 4 of 6 neurones tested. Application of GR55562 (10 mM, pH4, 20-80 nA), a 5-$HT_{1B/1D}$ receptor antagonist (Connor *et al.*, 1995, MacLean *et al.*, 1996), failed to attenuate the sumatriptan induced inhibition. In fact, GR55562 alone also caused inhibition in the majority (15 of 19) of NTS neurones tested in a similar manner to sumatriptan. When GR55562 and sumatriptan were applied together, the inhibition was found to be additive in 5 of 8 neurones tested.

These results suggest that it is impossible to characterise the inhibitory effect of sumatriptan as being due to activation of 5-$HT_{1B/1D}$ receptors on NTS neurones using GR55562. The mechanism of the inhibitory effect of both GR55562 and sumatriptan remains to be determined. However, it is unlikely that this is due to local anaesthetic effects as spike amplitudes were unaffected.

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64P REVERSAL OF A SCOPOLAMINE-INDUCED DEFICIT IN OBJECT DISCRIMINATION BY A SELECTIVE 5-HT_6 RECEPTOR ANTAGONIST, Ro-046790, IN RATS

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Evidence suggests that 5-HT_6 receptors may regulate cholinergic mechanisms involved in cognition (Bentley *et al*, 1997; Rogers *et al*, 1999). This study determined the effect of 4-amino-N-(2,6-bis-methyl-amino-pyrimidin-4-yl)-benzene sulphonamide (Ro-046790), a selective 5HT_6 receptor antagonist (Sleight *et al*, 1998) in a novel object discrimination test involving working memory and attention.

On two occasions, at 7 day intervals, 6 groups (n=9-12) of male Lister-hooded rats (250-400g), each received one of the following drug combinations in a random order; pre-treatment with saline or scopolamine hydrobromide (0.1, 0.5, 1 mg kg^{-1} i.p.) 20 min prior to saline (1 ml kg^{-1}) or Ro-046790 (10 mg kg^{-1} i.p.). Twenty min later rats were tested using an object discrimination task adapted from Ennanceur and Delacour (1988). Each rat was habituated to the test arena for 1 h, 24 h prior to the test, which comprised habituation (3 min no object) followed by two consecutive 3 min trials (T1 and T2) with two identical objects (a1 and a2) in T1 and a novel (b) and familiar object (a) in T2, separated by 1 min in the home cage. Exploration was defined as the time spent touching, sniffing, licking or chewing the object. Data are expressed as time (median and interquartile range (I.Q.R.)) spent exploring the two objects in T1 and the novel and familiar object in T2. Within group analysis was performed using a Wilcoxon-paired rank test and between group analysis was performed using a Mann Whitney "U" test.

All groups spent equal time exploring the two objects in T1. Compared with saline/saline (58, 32s; median, interquartile range) the total time spent exploring both objects during T2 was only significantly reduced ($p < 0.05$) by the highest dose of scopolamine and Ro-046790 did not affect the total T2 exploration time (66, 18; of the novel object in T2. Ro-046790 did not alter the object discrimination in T2 when given alone but completely reversed the scopolamine (0.5 and 1 mg kg^{-1})-induced attenuation, and even caused these rats to spend more time exploring the novel object than saline/saline controls.

Treatment	Time (s) at b (median, I.Q.R)	Time (s) at a (median, I.Q.R)
Saline/saline n = 44	30 (24)	12 (11)**
0.1 scopolamine/saline n = 12	26 (16)	6 (14)
0.5 scopolamine/saline n = 12	9 (19) ††	7 (4)
1 scopolamine/saline n =11	14 (10) ††	10 (11)
Saline/Ro-046790 n = 9	38 (19)	21 (9)*
0.5 scopolamine/Ro-046790 n = 12	36 (23) ^{ᵛᵛ}	10 (15)**
1 scopolamine/Ro-046790 n = 12	41 (35)^{††ᵛᵛᵛ}	17 (18)**

Table 1; Comparison between the time spent exploring the novel (b1) and familiar object (a1) in T2; ** $p < 0.01$ and * $p < 0.05$ from the novel object in the same treatment group, †† $p < 0.01$ and † $p < 0.05$ from novel object in saline/saline controls and ^{ᵛᵛᵛ} $p < 0.001$ and $^{ᵛᵛ}p<0.01$ from the novel object in the appropriate scopolamine/saline control

These data demonstrate that a 5-HT_6 receptor antagonist can reverse a scopolamine-induced novel object discrimination deficit and imply that 5HT_6 receptors may be involved in regulating cholinergic mechanisms involved in working memory or attention.

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65P [^{3}H]LY354740 BINDING TO mGlu2/3 RECEPTORS IN THE PERFORANT PATH: EFFECTS OF IONS AND LESIONS

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Metabotropic glutamate receptors, regulating the release of glutamate from terminals of the perforant path projecting to the hippocampus, are thought to be mGlu2 autoreceptors. The binding properties of the selective group II agonists [^{3}H]LY354740 and [^{3}H]DCG-IV to rat brain homogenates and tissue sections have been recently reported (Schaffhauser et al., 1998; Mutel et al., 1998). Evidence was presented for their preferential, but not exclusive, affinity for mGlu2 over mGlu3 receptors.

We first examined the influence of different cations on the binding selectivity of [^{3}H]LY354740 for the one versus the other receptor in membranes of cells transfected with the rat mGlu2 and mGlu3 receptors, as well as in membranes of cells transfected with different chimaera and mutants of these two receptors. The differential properties of cations (Mg^{++}, Ca^{++}, Cd^{++} and Zn^{++}) were then used to study in detail the binding of [^{3}H]LY354740 to the perforant path and entorhinal cortex in rat brain. We also investigated the influence, on the binding, of unilateral perforant path lesions. The lesions were made using a knife cut approach (see Kirby & Higgins,1998) and the animals were sacrificed 6 days post-lesion.

[^{3}H]-LY354740 (in 50mM Tris-HCl with 2mM $MgCl_2$, pH 7.4) has a slightly higher affinity for mGlu2 (K_D = 20 ±5 nM) than mGlu3 (K_D = 53 ±8 nM) receptors; this rank order of affinity was preserved in chimaera containing the respective mGlu2 (CH1) and mGlu3 (CH2) N-terminal domains. In rat brain cortex homogenates, the radioligand bound to two sites with K_D values of 5 ±0.7 and 60 ±27 nM. The binding to mGlu2 (but not mGlu3) receptors was inhibited by $ZnCl_2$ (2, 3 or 10mM). Interestingly, 10mM $ZnCl_2$ increased the specific binding to a chimaera containing the N-terminal domain of mGlu3.

The cation-dependency of [^{3}H]-LY354740 binding to parasagittal sections of rat brain was evaluated by quantitative radioautography as previously described (Schaffhauser et al., 1998). In the majority of brain regions, the presence of either 2mM $MgCl_2$ or $CaCl_2$ was essential for high levels of specific binding since, in their absence, little or no binding occurred. Whereas $ZnCl_2$ (in the presence of either Mg^{++} or Ca^{++} ions) markedly decreased the binding to these brain regions it increased the, otherwise very low, binding in several thalamic nuclei. Unilateral perforant path lesions reduced the binding to the lacunosum moleculare, subiculum and dentate gyrus molecular layer by ~70% of the non-lesioned contralateral side..

We conclude that [^{3}H]-LY354740 binds to different brain regions in a cation-dependent manner In the perforant path, it appears to bind presynaptically to mGlu2 autoreceptors regulating the excitatory input to the hippocampal formation.

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66P HISTAMINE INHIBITS DEPOLARISATION-INDUCED DOPAMINE-DEPENDENT RELASE OF GABA IN RAT STRIATUM VIA AN ACTION ON H_3-RECEPTORS

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We have reported that histamine H_3-receptor agonists selectively inhibit that portion of depolarisation-induced [^{3}H]-GABA release from terminals in the substantia nigra pars reticulata which is dependent on concurrent dopamine D_1-receptor activation (Garcia *et al.*, 1997). The striatonigral projection neurones have collaterals which terminate in areas of the striatum. We have set out to determine whether depolarisation-induced D_1-receptor-dependent [^{3}H]-GABA release in slices of rat striatum is also inhibited by histamine H_3-agonists.

Striatal slices, microdissected from vibratome-cut slices (300 µm) from Wistar rat brain, were loaded with 80 nM [^{3}H]-GABA and then transferred to a superfusion apparatus (20 parallel chambers) as described for nigral slices (Garcia *et al.*, 1997). The slices were superfused at 0.5 ml.min^{-1} with Krebs-Henseleit medium containing 10 µM nipecotic acid, 10 µM aminooxyacetic acid and, in all but the initial series of experiments, 10 µM sulpiride. Four or five fractions were collected at 4 min intervals before changing to a solution containing 15 mM K^+. Drugs under test were present for 12 min before changing to the depolarising solution, after which a further 6 fractions were collected. The tritium content of each fraction was expressed as a fraction of the tritium content of the slices at the beginning of that interval (Garcia *et al.*, 1997). All experiments were repeated at least 3 times.

The depolarisation-induced release of [^{3}H]-GABA was potentiated in the presence of the D_2-receptor antagonist sulpiride (10 µM), but further addition of the D_1-receptor antagonist SCH 23390 (1 µM) reduced the release to control levels in the absence of sulpiride. Sulpiride (10 µM) was present in all subsequent experiments. Depolarisation-induced [^{3}H]-GABA release, usually in the range 2 to 6-fold of basal, was strongly Ca^{2+}-dependent (84 ± 6% reduction when Ca^{2+} was omitted) and was markedly reduced by 100 µM histamine (78 ± 3% inhibition) and by 1 µM immepip, a selective H_3-receptor agonist (81 ± 5% inhibition). For both agonists the inhibition was reversed by 1 µM thioperamide to a level not significantly different from control. The IC_{50} values for histamine and immepip were 1.3 ± 0.2 µM and 15.5 ± 2.3 nM, respectively. The inhibitory action of 1 µM immepip was also reversed by the selective H_3-receptor antagonist clobenpropit (EC_{50} 7.3 ± 2.1 nM; K_d 0.11 ± 0.04 nM). In slices from reserpinised animals, in which striatal dopamine is reduced to very low levels (Garcia *et al.*, 1997), increasing K^+ to 15 mM produced little or no increase in [^{3}H]-GABA release, but in the presence of the D_1-agonist R(+)-SK&F 36933 (1 µM) release was increased 1.7 - 6.2-fold by the depolarising stimulus. This stimulated release was reduced to near basal levels by 1 µM immepip (90 ± 5% inhibition).

These data provide evidence that dopamine D_1-receptor-dependent release of [^{3}H]-GABA in rat striatum is inhibited by activation of histamine H_3-receptors. Some of this inhibition may be at the level of dopamine release (Schlicker *et al.*, 1993), but the high level of inhibition of [^{3}H]-GABA release in reserpinised animals in the presence of SK&F 36933 indicates that the major effect is probably mediated by H_3-receptors on the terminals of recurrent collaterals of GABA projection neurones.

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67P $GABA_B$ RECEPTOR-MEDIATED INHIBITION OF [^{3}H]GABA RELEASE FROM CEREBROCORTICAL SLICES IS NOT ALTERED IN GENETIC ABSENCE EPILEPSY RATS FROM STRASBOURG

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Genetic Absence Epilepsy Rats from Strasbourg (GAERS) are a validated animal model of absence epilepsy (Vergnes *et al*, 1982). Absence seizures are generated by the cerebral cortex and thalamus, and $GABA_B$ receptors have been implicated in the causation of these seizures (Marescaux *et al*, 1992). We previously reported an alteration in $GABA_B$ receptor modulation of [^{3}H]GABA release in GAERS thalamic slices *in vitro* (Sutch and Bowery, 1999). To determine whether the alteration was specific to the thalamus, we have extended these investigations of GAERS and non-epileptic control (NEC) rats to measure [^{3}H]GABA release from cerebrocortical slices.

[^{3}H]GABA release was measured using cross-chopped cerebrocortical slices (250μm x 250μm) prepared from 13-week-old female GAERS and NEC. Slices were suspended in artificial CSF (aCSF) containing 100μM β-alanine and 50μM aminooxyacetic acid and incubated with 45nM [^{3}H]GABA for 20min. After 3 washes, slices were perfused at 0.4ml.min^{-1} with aCSF additionally containing 10μM NNC-711 (Suzdak et al., 1992). After 36min perfusion, perfusate fractions were collected every 4 minutes. Release was stimulated at 48 (S1) and 88 (S2) minutes by the application of biphasic current (40mA, 4Hz) for 3.5min. The $GABA_B$ agonist (-)Baclofen (Bac) and antagonist CGP52432 (Lanza et al., 1993), were included 30min before S2. Release levels were calculated as percent fractional release (FR). Basal FR levels were subtracted from each stimulated FR. The S2/S1 ratio was then calculated. Statistical analysis comparing drug-treated S2/S1 to control S2/S1 was by one-way ANOVA with post-hoc Dunnett's test. Values are given as mean±s.e.mean.

There was no difference between NEC and GAERS in S1 levels of [^{3}H]GABA release. (-)Baclofen (1-10μM) inhibited cortical release from GAERS and control rats. This inhibition was blocked by the $GABA_B$ antagonist CGP52432.

Table 1 [^{3}H]GABA release from cortical slices (n=4-10).

	Control rats	GAERS
control S2/S1	1.05±0.08	0.93±0.05
3 μM (-)Bac	0.68±0.06 **	0.76±0.04 **
10 μM CGP52432	1.50±0.26	1.43±0.05
(-)Bac + CGP52432	1.38±0.18	1.36±0.07

** P<0.01 compared to respective control S2/S1 values

These data suggest that the modulation of [^{3}H]GABA release by $GABA_B$ receptors is no different in cerebrocortical slices from GAERS and non-epileptic control rats. This is in contrast to the findings with thalamic slices, where 12 min preincubation with (-)Baclofen (up to 10 μM) did not reduce release from NEC slices but significantly inhibited release from GAERS thalamic slices by 45±11% (Sutch and Bowery, 1999).

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68P THE ANTICONVULSANT, LAMOTRIGINE, RECIPROCALLY MODULATES GLUTAMATE AND GABA RELEASE IN THE RAT ENTORHINAL CORTEX

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We have recently demonstrated that the anticonvulsant, phenytoin, decreases the release of glutamate, but increases release of the inhibitory transmitter, GABA, in the entorhinal cortex (EC; Cunningham and Jones 1998; Cunningham *et al.*, 1999). We have now examined a newer drug with a similar clinical usage, lamotrigine (LTG). Like phenytoin, LTG exerts a voltage and frequency dependent blockade of Na^+-channels and it has been suggested that this accounts for it's ability to inhibit veratridine-evoked glutamate release *in vitro* and *in vivo* (see Walker and Sander, 1999). We have used whole cell patch-clamp recordings to examine the effect of LTG on the release of glutamate and GABA in the EC.

Slices of EC from adult rats were prepared and maintained *in vitro* by conventional means. Recordings were made from neurones in layer V. For recording spontaneous excitatory postsynaptic currents (EPSCs) electrodes were filled with a solution containing (in mM): Cs methanesulphonate (130), HEPES (5), EGTA (0.5), $MgCl_2$ (1), NaCl (1), $CaCl_2$ (0.34) and QX-314 (5). Inhibitory postsynaptic currents (IPSCs) were recorded with electrodes filled with a solution containing (in mM) CsCl (135), HEPES (10) $MgCl_2$ (2), QX-314 (5) $CaCl_2$ (0.5) and EGTA (5). Membrane potential was clamped at -60mV.

The effect of LTG on glutamate release was tested in 3 neurones in the presence of tetrodotoxin (TTX, 1μM) to record miniature EPSCs (mEPSCs) i.e. activity independent events. In pooled data from these neurones (minimum of 100 events per neurone), mEPSCs had mean (±S.E.M) amplitude of 7.2±0.15 pA and an inter-event interval of 970±50 ms. LTG (10μM) had no effect on amplitude (7.1±0.16 pA), but significantly (Kolmogorov-Smirnov test; P<0.0001) increased the inter-event interval to 1615±122ms, reflecting a decrease in frequency.

Spontaneous $GABA_A$-receptor mediated IPSCs were recorded in 6 neurones (with AMPA and NMDA receptors blocked with appropriate antagonists). In pooled data IPSCs had an amplitude of 12.0±0.2 pA, and an inter-event interval of 243.2±11.6 ms. LTG (50μM) significantly (P<0.0001) reduced the inter-event interval to 187±8 ms with a small, increase in the peak amplitude (13.6±0.25pA; p>0.001). Miniature IPSCs were recorded in three further neurones in the presence of TTX. These had a mean amplitude of 18.7±1.0 pA and a inter-event interval of 249±13 ms. LTG again caused a significant (P<0.0001) reduction in inter-event interval, (19.8±1.0 pA, 176±7 ms). These results suggest that lamotrigine has a presynaptic action on inhibitory terminals to increase GABA release.

Thus, LTG, like phenytoin, can increase GABA release whilst reducing glutamate release. That both effects persist in TTX shows that they are independent of actions on Na^+ channels. The similarity between the two drugs suggests common sites of action in presynaptic terminals, and we are currently attempting to define the mechanisms involved.

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69P AMILORIDE DRUGS BOTH BLOCK AND ENHANCE THE ACTION OF H$^+$ IONS ON RAT DORSAL ROOT GANGLION NEURONES

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Some amiloride-sensitive Na^+ channels are directly gated by endogenous ligands. These include the peptide-gated channels of molluscan neurones (Green et al 1994; Lingueglia et at 1995; Jeziorski et al 2000) and the acid sensing ion channels (ASICs) of mammalian neurones (Waldmann and Lazdunski 1998; Chen et al 1998). It was recently shown that the FMRFamide-response of the snail *Helisoma trivolvis* is enhanced by amiloride; related drugs benzamil and EIPA were more effective. The overall effect of each drug depended on its relative potency as an antagonist and enhancing agent (Jeziorski et al 2000).

Here we report that responses to H^+ ions of isolated sensory neurones from rat dorsal root ganglia (DRG) are also enhanced as well as blocked by amiloride drugs. DRG were removed from 1 to 3 day neonate rats, anaesthetized and sacrificed by cervical dislocation. Neurones were dispersed on collagen plates after dissociation with trypsin. Whole cell, and outside-out patch, recordings were made 1 to 17 days later with patch pipettes filled with (in mM): KCl 140, $MgCl_2$ 1, KEGTA 5, HEPES 10, adjusted to pH 7.4 with KOH. The external solution contained (mM): NaCl 140, KCl 1, $MgCl_2$ 1, $CaCl_2$ 1, MES 10, adjusted to pH 5 and 6, or 7.3 (HEPES 10), with NaOH. Amiloride (Sigma) and benzamil (Sigma) stock solutions were prepared in distilled de-ionized water. EIPA (5-(N-ethyl-N-isopropyl)-amiloride; Sigma), was prepared as stock 20 mM solution in DMSO.

Many small and larger sized neurones responded to local application of acid solutions. The inward current responses varied in different neurones, as has already been described by Bevan and Yeats (1991). Some were activated at pH 6; others required pH 5 for activation. Response were often biphasic, but some neurones only showed a fast response. Similarly, there was a variation in the effects of the amiloride drugs. In many cases, the fast current was reduced, with little effect on the sustained current. In others there was a clear enhancement of the slower component. This was most notable on unitary currents with benzamil.

Of the acid sensing channels cloned, only ASIC1 and ASIC3 occur in DRG. Waldmann et al (1997) noted that amiloride has a complex effect on heterologously expressed ASIC3 (DRASIC); it antagonized the fast current, but the sustained current was higher. ASIC1 is blocked by amiloride (see Chen et al 1998). Thus the enhancing effect of benzamil may result from an action on contained ASIC3, but the neuronal response is more sensitive to H^+ ions than heterologously expressed ASIC3.

We thank the Wellcome Trust for financial support.

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70P PHARMACOLOGICAL CHARACTERIZATION OF THE HUMAN BOMBESIN RECEPTOR SUBTYPE 3 (BRS-3) RECEPTOR ENDOGENOUSLY EXPRESSED IN NC1-N417 CELLS

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It has recently been reported that a small cell lung carcinoma cell-line, NCI-N417, endogenously expresses the human BRS-3 receptor (Ryan *et al.*, 1998). However, only a limited number of ligands were used to characterise this receptor and the pharmacology identified (Ryan *et al.*, 1998) differed to that previously reported for the recombinant receptor (Wu *et al.*, 1996). Therefore, the present study was undertaken to further characterise this receptor in NCI-N417 cells using a wider range of ligands.

NCI-N417 cells were seeded into Costar 96 well black walled plates (20,000 cells per well), cultured overnight in RPMI 1640 medium containing 10% foetal calf serum and 1% L-glutamine, and then loaded with the cytoplasmic calcium indicator Fluo-3AM (4μM) in the presence of 2.5mM probenecid at 37°C for 60 min. The cells were then washed 4x with, and finally resuspended in, Tyrode's medium containing 2.5mM probenecid, before being incubated with buffer alone or antagonist for 30min. Fluorescence was monitored using a FLIPR (λ_{EX}= 488nm, λ_{EM}= 540nm) before and after the addition of various agonists (10pM-10μM).

A range of synthetic and natural bombesin ligands were tested, and all acted as agonists, with varying degrees of potency and efficacy (Table 1), except the cyclic peptide [H-D-2-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-2-Nal-NH2] which acted as an antagonist with an apparent pK_B of 7.92±0.03 (n=8).

The agonist profile obtained in the present study was consistent with NCI-N417 cells expressing the human BRS-3 receptor (Wu *et al.*, 1996; Ryan *et al.*, 1998). Moreover, the pharmacology identified in the present study was more consistent with that previously reported for the recombinant receptor (Wu *et al.*, 1996) than was the pharmacology reported from the original study of NCI-N417 cells (Ryan *et al.*, 1998),

Table 1. Agonist pharmacology at the endogenous human BRS-3 receptor in NCI-N417 cells.

Ligand	pEC_{50}	Efficacy %
Bombesin (BN)	<5	23 at 10μM
Neuromedin B (NMB)	5.65±0.07	100
Neuromedin C	<5	20 at 10μM
Ranatensin	5.59±0.12	100
[D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]BN(6-14)	7.88±0.04	100
PG-L	5.54±0.05	100
Gastrin releasing peptide	<5	23 at 10μM
[D-Tyr^6, βAla^{11}, Phe^{13}, Nle^{14}]BN(6-14)	8.02±0.03	100
AcNMB(3-10)	5.73±0.03	100
[D-Phe^1, Nle^9]litorin	5.96±0.05	100
[D-Phe^6]BN(6-13) propylamide	5.86±0.09	100
[D-Phe^6, Phe^{13}]BN(6-13)propylamide	6.83±0.03	100

Data are mean±S.E.M., n=5-12.

most notably in showing that [D-Phe^6]BN(6-13)propylamide and [D-Phe6, Phe13]BN(6-13)propylamide were full agonists. Furthermore, a peptide antagonist with reasonable affinity was characterised, providing a useful additional tool compound for the study of this receptor. In conclusion, these data clearly demonstrate the utility of NCI-N417 cells as a model to study the pharmacology of the human BRS-3 receptor in a native environment.

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71P LACK OF TOLERANCE TO REDUCED WEIGHT GAIN RESULTING FROM CHRONIC (14 DAY) ADMINISTRATION OF 5-HT_{2C} RECEPTOR AGONISTS TO RATS

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5-HT_{2C} receptor activation reduces feeding in rodents, and is thought to mediate the action of the clinically effective anorectic, fenfluramine (Vickers et al., 1999). However, while fenfluramine is chronically effective in man, tolerance rapidly develops to the hypophagic effects of both fenfluramine and 5-HT_{2C} receptor agonists after repeat injection of rats (Aulakh et al, 1995; Rowland and Carlton, 1986). The effect of chronic delivery of serotonergic anorectics by osmotic mini-pump, on rat body weight and feeding was therefore studied.

In study 1, male hooded Lister rats (180-200 g) were implanted with subcutaneous osmotic mini-pumps (under isoflurane anaesthesia), that delivered either PEG-300 vehicle, 6 mg/kg/day d-fenfluramine (d-FEN, n=10), 12 mg/kg/day m-chlorophenylpiperazine (mCPP, n=8) or 36 mg/kg/day Ro 60-0175 (n=6), a selective 5-HT_{2C} receptor agonist (Martin et al, 1998) for 14 days. All drug treatments reduced weight gain compared with vehicle controls ($p<0.001$; ANOVA), such that, by day 14, d-FEN-, mCPP- and Ro 60-0175-treated rats weighed 5 %, 8 %, and 10 % less than controls, respectively. All treatments reduced food intake; although only up to day 10 ($p<0.01$, ANOVA's across treatment group at each day). Water intake was unaffected by any treatment. In study 2, male hooded Lister rats (250-290 g) were implanted with mini-pumps that delivered either saline (14 days), 6 mg/kg/day d-FEN (7 or 14 days), or mCPP (7 or 14 days). Rats treated with d-FEN or mCPP for 14 days gained less weight than vehicle-treated rats ($p<0.001$). In contrast, rats treated with d-FEN or mCPP for only 7 days showed a similar reduction in weight gain from day 1-7, but on withdrawal from day 8-14 gained more weight than either 14 day vehicle- or 14 day drug-treated animals (Table 1). Both d-FEN and mCPP reduced feeding during treatment (most marked: days 1-7). Interestingly, animals given d-FEN for only 7 days ate more than vehicle controls during days 8-14 when no longer receiving drug. This hyperphagic response upon withdrawal was not seen in rats that received 7 day mCPP, however. As in study 1, effects on water intake were small and inconsistent and rats did not exhibit sedation, serotonin syndrome or motoric disturbance (daily observations).

Table 1: Weight gain data for rats treated for 7 or 14 days (Study 2)

Treatment (n=9)	Duration	Weight gain (g) to day 7	Weight gain (g) to day 14
Vehicle	14 days	71.4 ± 3.6	111.9 ± 3.8
D-FEN	14 days	40.3 ± 2.5 #	74.2 ± 2.9 #
	7 days	37.3 ± 2.5 #	88.6 ± 3.7 *
mCPP	14 days	43.0 ± 2.5 #	79.6 ± 2.3 #
	7 days	43.3 ± 2.3 #	90.7 ± 3.7 *

# $p<0.01$ vs Vehicle by ANOVA and Newman-Keuls test; * $p< 0.01$ vs relevant 14 day treatment, unpaired t-tests (planned comparison).

Thus, osmotic mini-pump delivery of the 5-HT releasing agent, d-FEN, or the 5-HT_{2C} receptor agonists, mCPP or Ro 60-0175 reduced body weight gain, an effect maintained for 14 days. The observation that weight gain in rats withdrawn from drug is greater than when drug is maintained on days 8-14 suggests that tolerance did not develop to the drug. The lack of rebound hyperphagia in rats withdrawn from mCPP in study 2 suggests that hypophagia may only partially account for the maintainance of reduced body weight.

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72P INFUSION OF NICOTINE CAN INCREASE THE OPERANT SELF-ADMINISTRATION OF ETHANOL

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There is considerable evidence that dependence on alcohol and on nicotine are associated; a very high proportion (over 90%) of alcoholics smoke. The present study investigated the effects of chronic nicotine infusions on operant self-administration of alcohol, over a range of nicotine doses which give blood concentrations corresponding to those achieved in humans during smoking.

Male Lister rats, 450 - 550g at start, n = 9-14 per treatment group, were used. They were trained to press levers to obtain alcohol, using the "sucrose fading" method of Grant and Sampson (1985), in which the animals learn to press a lever to obtain sucrose, then the solution is gradually changed to sucrose plus alcohol, then alcohol alone. The operant schedule used was a variable interval (15s) with 30 min access per day, 5 days per week. The rats were trained to respond for 5% ethanol, then osmotic minipumps were implanted, which provided 28 days of either 0.25, 2.5 or 7 mg/kg/24h nicotine; controls were implanted with sham pumps. After two days recovery time, the rats were tested for two weeks for operant self-administration of 5% ethanol.

The results were analysed by two way analysis of variance.

Infusion of 2.5 mg/kg nicotine increased the responding for self-administration of 5% ethanol ($P < 0.05$). During the first week after implantation of the minipumps the number of responses on the alcohol-delivery lever by the rats receiving 2.5 mg/kg/24h nicotine was significantly higher than that of control animals (Table 1; $P < 0.05$, $F(1,20) = 4.7$, compared with corresponding control values over all 5 days of that week). The number of rewards obtained and amount of alcohol consumed were also significantly increased. By the third week of infusion, however, the differences were no longer significant. The 0.25 and 7 mg/kg doses of nicotine had no significant effects on the responding for alcohol.

The results show that nicotine can increase responding for alcohol. The effect could be due to an increase in the pharmacological effects of ethanol, such as those responsible for the reinforcing actions. Humans who smoke titrate their intake of nicotine within narrow limits; the dose response relationship seen in these results may illustrate a comparable situation.

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Table 1. Values are numbers of responses on alcohol delivery lever in 30 min, mean ± s.e.m. Bs = baseline values; Mp = values after implantation of osmotic minipumps; Mon, Tues etc = test days during the week; Nic 2.5 = nicotine infusion at 2.5 mg/kg/24h

Treatment	Bl Mon	Bs Tues	Bs 1 Wed	Bs Th	Bs Fri	Mp Mon	Mp Tues	Mp Wed	Mp Th	Mp Fri
Controls	88± 25	61± 13	76 ± 14	60 ± 12	58 ± 14	56 ± 13	54 ± 13	54 ± 12	53 ± 8	53 ± 12
Nic 2.5	60 ± 10	64 ± 14	58 ± 6	55 ± 5	64 ± 9	95 ± 12	89 ± 17	86 ± 7	83 ± 13	85 ± 12

73P THE ANANDAMIDE TRANSPORT INHIBITOR AM404 IS AN AGONIST AT THE RAT VANILLOID RECEPTOR (VR1)

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It has recently been reported that olvanil is a potent inhibitor of anandamide transport in human astracytoma cells, whilst other vanilloid ligands such as capsaicin and capsazepine were without effect (Beltramo & Piomelli, 1998). This was attributed to the close structural similarity between olvanil and the anandamide transport inhibitor *N*-(4-hydroxyphenyl)-arachidonylamide (AM404) (Szallasi & Blumberg, 1999).

However, olvanil also acts as an agonist at the vanilloid receptor (VR1), which is a ligand gated ion channel expressed in nociceptors (Szallasi & Blumberg, 1999). To date, no studies have been published investigating the possible interaction between AM404 and VR1 receptors. The present study was therefore undertaken to address this.

HEK293 cells, stably expressing rat VR1 receptors, were seeded into Costar 96 well black walled plates (25,000 cells per well) and cultured overnight in minimum essential medium (MEM), containing 10% foetal calf serum, 2mM L-glutamine, and 400μg ml^{-1} G418. Cells were loaded with the cytoplasmic calcium indicator Fluo-3AM (4μM) at 25°C for 120 min. The cells were then washed 4x with, and finally resuspended in, Tyrode's medium, before being incubated with buffer alone or antagonist for 30min. Fluorescence was monitored using a FLIPR (λ_{EX}= 488nm, λ_{EM}= 540nm) before and after the addition of various agonists (10pM-10μM).

Capsaicin, olvanil and AM404 all increased intracellular calcium ($[Ca^{2+}]_i$) with a range of potencies (Table 1). All were full agonists compared to capsaicin and displayed similar kinetics. Application of a range of concentrations of the VR1 antagonist capsazepine (10pM-10μM) inhibited the respose to each agonist in a concentration dependant manner (Table 1). Capsazepine did not display agonist dependent antagonism.

Table 1. Agonist potencies and antagonist affinities at rat VR1 receptors

Agonist	pEC_{50}	capsazepine (pK_B)
capsaicin	8.25 ±0.11	7.21 ±0.06
olvanil	8.37 ±0.06	7.18 ±0.03
AM404	6.96 ±0.04	7.35 ±0.06

Data are mean ± S.E.M., n=4.

In conclusion, the present study has demonstrated that AM404 is a full agonist at the rat VR1 receptor and thus data obtained from *in vivo* studies using this compound, must be interpreted with a degree of caution.

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74P THE RESPONSES OF CULTURED SENSORY NEURONS AND CHO CELLS EXPRESSING THE RAT VANILLOID RECEPTOR (VR1) TO HEAT AND CAPSAICIN: STUDIES WITH INTRACELLULAR CALCIUM IMAGING

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Whole-cell and single channel recordings have demonstrated that the cloned VR(vanilloid)1 receptor is a non-selective cation channel activated by heat in the noxious range, as well as capsaicin (Caterina *et al.*, 1997), and this receptor may account for the response of sensory neurons to these two stimuli (Cesare & McNaughton, 1996; Nagy & Rang, 1999a). Here we have examined the effect of chemical agonists, antagonists and heat stimulation on intracellular calcium levels ($[Ca^{2+}]_i$) in chinese hamster ovary (CHO) cells transfected with the rat VR1 receptor and in rat dorsal root ganglion (DRG) neurons.

Cells grown on glass coverslips were loaded with fura-2AM, and ratiometric fluorescence measurements of individual cells taken with a cooled CCD camera. The cells were continuously perfused via a pipette positioned close to the recording site. The temperature of the perfusate was controlled with a regulated Peltier device, and monitored at the tip of the pipette by a miniature thermocouple. The heat stimulus consisted of a linear increase (1°/sec) from 25°C to 50°C. $[Ca^{2+}]_i$ is expressed as the ratio of fura-2 fluorescence at 340 and 380 nm excitation, which increases in a linear fashion with Ca^{2+} concentration. Data are expressed as mean ± s.e. mean; *n* being the number of cells tested.

VR1-transfected CHO cells and a subset (34%) of DRG neurons responded to capsaicin and heat with rapid and substantial rises in $[Ca^{2+}]_i$. Heat stimulated $[Ca^{2+}]_i$ rises were non-linear and occured at an estimated threshold temperature of 42.6 ± 0.3 °C (n=117) in transfected cells and 42 ± 0.6 °C (n=18) in DRG neurons. 'Non-responsive' DRG cells typically showed a small rise in $[Ca^{2+}]_i$ with no clear threshold when the temperature was increased, similar to that seen in non-transfected CHO cells. The average maximum ratio increase over basal in response to heat stimulation was 3.98 ± 0.19 (n=115) in VR1 transfected cells, compared with 0.24 ± 0.05 in non-transfected cells. Omission of extracellular Ca^{2+}, reduced the heat stimulated $[Ca^{2+}]_i$ rise in responding DRGs by 74 ± 4% (n=24) and 78 ± 2 % (n=54) in VR1-transfected CHO cells. The small non-specific response in DRGs was unaffected by omission of Ca^{2+}.

A strong correlation (Spearman-Rank test) between the amplitudes of responses to heat and capsaicin was found in VR1-transfected CHO cells (R=0.86, P<0.0001; n=115) but not in responding DRG neurons (R=0.25, P=0.36, n=15). A similar lack of correlation was found for electrophysiological responses in DRG neurons (Nagy & Rang, 1999a), where there is evidence that different channel populations account for responses to the two stimuli (Nagy & Rang, 1999b).

The effect of two capsaicin antagonists, capsazepine and ruthenium red was measured on the responses of VR1-transfected CHO cells. Capsazepine (10 μM), a competitive capsaicin antagonist, abolished the response to capsaicin (10 or 100 nM), whereas the response to heat was reduced by 41±3% (n=86). Ruthenium red (10 μM) reduced the heat response by 78 ± 1 (n=88) to a level not significantly different from that in non-transfected CHO cells.

These results demonstrate that noxious heat, as well as capsaicin, stimulates influx of external Ca^{2+} in a responsive subset of cultured DRG neurons and through recombinant rat VR1 receptors expressed in CHO cells. The effects of heat on non-transfected CHO cells and non-responsive DRG neurons is similar, producing only small increases in $[Ca^{2+}]_i$. The finding that capsezepine is fairly ineffective at blocking heat responses in VR1-transfected CHO cells is in agreement with electrophysiological data on DRG neurons (Nagy & Rang, 1999b). Additionally, the fact that the present data from DRGs is consistent with electrophysiological findings (Cesare & McNaughton 1996; Nagy & Rang 1999a,b) shows that calcium imaging provides an alternative approach to studying the physiology and pharmacology of heat transduction.

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75P THE EFFECT OF THE HIGHLY SELECTIVE ADENOSINE A1 AGONIST GR79236 IN MODELS OF NOCICEPTIVE, ACUTE AND CHRONIC INFLAMMATORY PAIN

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Adenosine A1 receptor agonists have been shown to inhibit mechanical and thermal nociceptive thresholds (Sawynok et al., 1986), and inhibit c fibre evoked response, windup, post discharge and both phases of formalin induced firing of dorsal horn neurones in rats (Reeve et al., 1995). Clinical studies have also shown adenosine to be analgesic against mustard oil (Segerdahl et al., 1995) and in neuropathic pain (Sollevi et al., 1995). We have investigated the effect of the selective A1 agonist GR79236 (Gurden et al., 1993), N-[(1S, trans)-2-hydroxycyclopentyl] adenosine in models of nociceptive, acute (carrageenan and Freund's Complete Adjuvant-FCA) and chronic inflammatory pain (FCA). Male Random Hooded rats (180-220g) were fasted overnight. GR79236 was administered 30mins before either 100µl of 2% carrageenan or 100µl of 1mg/ml FCA into the left hind paw. In a model of chronic inflammatory hyperalgesia GR79236 was administered 2 days after FCA. The effect of GR79236 on carrageenan and FCA induced decrease in weight bearing on the inflamed left hind paw (dual channel weight averager. Clayton et al., 1997) was determined 3 and 6 hours respectively, after the inflammatory insult, or in the model of chronic inflammatory hyperalgesia 6 hours after GR79236. The effect of GR79236 on carrageenan induced decrease in thermal paw withdrawal latency (Hargreaves et al., 1988) was also determined 3 hours post carrageenan. The effect of GR79236 on carrageenan and FCA induced paw oedema was assessed using a plethysmometer. Anti-nociceptive activity was determined by investigating the effect of GR79236 on normal mechanical paw withdrawal thresholds. Statistical analysis was carried out to determine whether there was a significant difference between the vehicle treated group and the drug treated group using unpaired student t test. ($p<0.05$).

GR79236 inhibited the carrageenan induced decrease in weight bearing (ED_{50} 0.02mgkg^{-1} s.c). and thermal paw withdrawal latency (ED_{50} 0.01mgkg^{-1}s.c). GR79236 also reduced the associated paw oedema, ED_{50} 0.07mgkg^{-1}s.c. Similarly GR79236 produced a dose related inhibition of the FCA induced decrease in weight bearing after 6 hours (ED_{50} 0.02mgkg^{-1} s.c. In a model of chronic inflammatory hyperalgesia (2 days), GR79236 produced a dose related reversal of the FCA induced decrease in weight bearing (ED_{50} 0.19mgkg^{-1} s.c.). GR79236 (10mgkg^{-1} p.o) increased the normal mechanical nociceptive thresholdsby 32%.

In conclusion, GR79236 has a small but significant anti-nociceptive effect. GR79236 inhibits acute and reverses chronic inflammatory hyperalgesia. GR79236 also exhibited significant anti-inflammatory activity in the model of acute inflammation (carrageenan). The anti-hypersensitivity action of GR79236 are indicative of possible clinical utility for adenosine A1 agonists in clinical inflammatory pain states.

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76P THE EFFECT OF GR79236, A HIGHLY SELECTIVE ADENOSINE A1 RECEPTOR AGONIST, ON THE TREATMENT OF NEUROPATHIC PAIN IN THE RAT

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Adenosine has been reported to possess analgesic properties in the treatment of neuropathic pain in man (Sollevi et al., 1995). More specifically adenosine A1 receptor agonists have been shown to have a role in modulation of both acute and inflammatory nociception in the spinal cord (Reeve et al., 1995). We have tested the highly selective A1 agonist GR79236, N-[(1S, trans)-2-hydroxycyclopentyl]adenosine, (Gurden et al., 1993), in the Chronic Constriction Injury (CCI) model of neuropathic pain in the rat (Bennett & Xie, 1988). Briefly, under isoflurane anaesthesia, the common left sciatic nerve of male Random Hooded rats (180-200g) was exposed at mid-thigh level. Four ligatures of chromic gut (4.0) were tied loosely around the nerve with a 1mm spacing between each. The wound was then closed and secured with suture clips. The surgical procedure was identical for the sham operated animals except the sciatic nerve was not ligated. The rats were allowed a period of seven days to recover from the surgery before behavioural testing began. The effect of GR79236 on CCI-induced decrease in mechanical paw withdrawal threshold was measured using an algesymeter (Randall & Selitto, 1957). The presence of mechanical allodynia was assessed using Von Frey Hair monofilaments (range: 4.19-84.96g). The rats were lightly restrained and placed upon a metal grid floor. The monofilaments were applied to the plantar surface of the hindpaws from below the grid. The lowest monofilament to produce a withdrawal was the response recorded. Additional studies to examine the possible site of action of GR79236 in neuropathic pain were undertaken using the peripherally restricted and centrally penetrating adenosine receptor antagonists, 8-sulphophenyltheophylline (8-SPT, Daly et al., 1985) and 8-phenyltheophylline (8-PT, Baumgold et al., 1992) respectively. The relevant antagonist was administered by the i.p. route, 30 minutes prior to a single dose of GR79236. In these studies, behavioural testing was performed 5 hours after dosing with GR79236. In each study, GR79236 was dosed when the mechanical hypersensitivity had reached a stable maximum. Statistical analysis was carried out to compare the difference between the drug treated group and vehicle treated group using unpaired Student's t test ($P<0.05$ considered significant).

Chronic dosing with GR79236 (3mgkg^{-1} t.i.d. p.o. days 11-15 post-surgery) produced a rapid reversal (5hrs) of the CCI-induced decrease in paw withdrawal threshold to that of sham operated animals (112 ± 6g vs 110 ± 4g respectively, both n=10; $P>0.05$). A highly significant reversal (55 ± 15%, $P<0.01$) of the mechanical allodynia was achieved following 2 days of chronic dosing with GR79236 (3mgkg^{-1} t.i.d. p.o.). The antagonist studies revealed that 8-PT completely blocked the analgesic activity of GR79236 (76 ± 5g vs 143 ± 23g respectively, both n=10; $P<0.01$) whereas, 8-SPT had no effect (106 ± 10g vs 112 ± 12g respectively, both n=10; $P>0.05$). In conclusion, GR79236 reverses the hypersensitivity associated with neuropathic pain through a central site of action. These studies demonstrate that adenosine A1 agonists may be of utility in the treatment of neuropathic pain in man.

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77P DESENSITISATION AND DOWN-REGULATION OF HUMAN RECOMBINANT NOCICEPTIN RECEPTORS EXPRESSED IN CHINESE HAMSTER OVARY CELLS

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Nociceptin (NC) is the endogenous ligand for the nociceptin receptor (NCR). Activation of NCR results in reduced cAMP formation (Meunier, 1997). Using Chinese hamster ovary cells expressing recombinant human nociceptin receptors (CHO_{hNCR}) we have previously shown that pre-treatment with nociceptin (NC) results in a rightward shift in the concentration response curve for inhibition of cAMP formation. Pre-treatment times of $\geq$24hrs were required and we suggested that this (along with no depression of the maximum response) was due to high levels (~2pmol/mg protein) of NCR expression (Hashimoto & Lambert, 1999). In this study we have repeated our 48hour pre-treatment in the absence and presence of a novel competitive NCR antagonist [$Nphe^1$]NC(1-13)NH_2 (pA_2 6.0, Calo et al 1999). In addition, we have examined the time course for loss of cell surface receptors and the effects of [$Nphe^1$]NC(1-13)NH_2 ([$Nphe^1$]) on this response.

Cells were maintained as described previously (Okawa et. al., 1999). Cultures were pre-treated with NC (1nM) for various times in the presence of 30μM amastatin, bestatin, captopril and phosphoramidon. In some experiments [$Nphe^1$] (100nM) was included in the pre-treatment. cAMP was measured in whole CHO_{hNCR} cells and [^{125}I]Y^{14}NC binding was measured in freshly prepared membranes as descried previously (Okawa et. al., 1999). All data are presented as mean±s.e.mean (n=6).

NC produced a concentration dependent inhibition of forskolin (1μM) stimulated cAMP formation. This concentration response curve was shifted to the right following NC 24 and 48 hours pre-treatment (Hashimoto & Lambert, 1999). At 48 hours pre-treatment the rightward shift was reversed by 100nM [$Nphe^1$] (pIC_{50} control 9.40±0.07, 48hrs 8.73±0.04, 48hrs+[$Nphe^1$] 9.55±0.02). In addition, there was a time dependent loss of NCR, which at 48 hours was fully reversed by [$Nphe^1$] (Table 1).

Table 1. Time dependent loss of cell surface NCR (B_{max}, fmol.mg $protein^{-1}$) following NC pre-treatment and reversal by [$Nphe^1$].

Time (h)	B_{max}	%reduction	pK_d
0	2016±133	-	9.63±0.11
2	1860±178	7.7	9.75±0.10
6	1704±155	15.5	10.00±0.10
24	1491± 90	26.1	9.69±0.10
48	1264± 40	47.4	9.83±0.13
48+[$Nphe^1$]	2011±207	0.2	9.58±0.08

These data show that NC pre-treatment produces a functional desensitisation measured at the level of adenylyl cyclase and that there is an attendant loss of cell surface receptors. Importantly, we have demonstrated that a novel peptide antagonist [$Nphe^1$] fully reverses both the rightward shift in the cAMP concentration response curve and the loss of cell surface receptors and represents an important tool for use in studies of NC/NCR signalling.

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78P μ-OPIOID RECEPTOR DESENSITISATION AND DOWN-REGULATION INDUCED BY ENDOMORPHIN-1

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Prolonged treatment with opioids leads to receptor desensitization and down regulation (Koch *et al*, 1988). In a previous study using Chinese hamster ovary cells expressing recombinant μ-opioid receptors (CHOμ), we showed that following 18h pretreatment with endomorphin-1 (E1), the response desensitized i.e., maximum inhibition of cAMP formation was depressed (Harrison *et al*, 1999b). In this study we have probed this phenomenon further.

CHOμ cells (grown in supplemented Hams F12 medium) were pre-treated with E1 (10μM) in serum free medium containing BSA (0.5%) and peptidase inhibitors; amastatin, bestatin, captopril, phosphoramidon (each 10μM) for various times then washed 5x at 4°C with Krebs-HEPES (KH) buffer prior to experiments. cAMP experiments were performed on adherent CHOμ cells in 12-well plates for 5min at 37°C in 0.6ml volumes of KH buffer containing forskolin (10μM), IBMX (1mM) 0.5%BSA, 10μM peptidase inhibitors and varying concentrations of E1 (10^{-10}-10^{-5}M). Reactions were terminated and cAMP assayed as described previously (Harrison *et al*, 1999a) following detatchment of cells. Radioligand binding studies were performed at 4°C for 3h on adherent CHOμ cells in 12-well plates in 1ml volumes of KH buffer containing a saturating concentration of [^{3}H]-diprenorphine ([^{3}H]-DPN) (~2.5nM). Non specific binding was defined using 10μM naloxone. Reactions were quenched by removal of the buffer (and 2x wash) and incubation with 0.4M PCA. Following detatchment of cells, radioactivity measured as described previously (Harrison *et al*, 1999a). [^{3}H]DPN displacement experiments were performed in CHOμ membranes in 1ml volumes of Tris buffer, 20°C for 1h using [^{3}H]DPN (~0.3nM), peptidase inhibitors (10μM) and increasing concentrations of E1 (10^{-11}-10^{-5}M) in the presence or absence of GTPγS (50μM). Non specific binding was defined using 10μM naloxone. Bound radioactivity was measured as described previously (Harrison *et al*, 1999a). Data are expressed as mean±s.e.mean (n=3-18).

E1 produced a concentration-dependent inhibition of forskolin stimulated cAMP formation in untreated cells and cells that had been pre-treated with E1 for 18h (Untreated pIC_{50} = 7.8±0.3, I_{max}= 67.0±8.1, 18h E1 pre-treatment pIC_{50} = 7.3±0.2, I_{max} = 27.3±12.0). In E1 pre-treated cells there was a significant increase (2.46fold) in forskolin stimulated cAMP levels. Radioligand binding experiments revealed that E1 pre-treatment produced a time-dependent loss of cell surface receptors (expressed as % control=100%, 5min=78.5±4.6, 10min=74.1±4.7, 20min=71.8±3.7, 30min=69.5±3.5, 1h=73.2±4.7, 2h=67.5±5.9, 3h=70.0±4.1, 4h=64.1±3.2, 5h=67.4±5.9, 18h=69.4±4.2 (all significantly different compared to control, $p \leq 0.05$). GTPγS produced a small rightward shift in the E1 displacement curve in CHOμ membranes prepared from cells that had been pre-treated with E1 for 0h and 30min but not in membranes prepared from cells that had been pre-treated with E1 for 18h.

These data indicate constitutive activity of the μ-receptor expressed in CHO cells in that pre-treatment with E1 enhances forskolin stimulated cAMP formation. In addition, E1 pre-treatment also causes a rapid loss of cell surface receptors. However, the time course for the latter does not mirror that of the former and may result from transfection of a receptor reserve (B_{max} ~600fmol mg $protein^{-1}$). Moreover, our data suggest receptor G protein coupling is lost following long pre-treatment protocols.

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79P GABAPENTIN AND THE NK_1 RECEPTOR ANTAGONIST CI-1021 ACT SYNERGISTICALLY TO BLOCK ALLODYNIA INDUCED IN A RAT MODEL OF NEUROPATHIC PAIN

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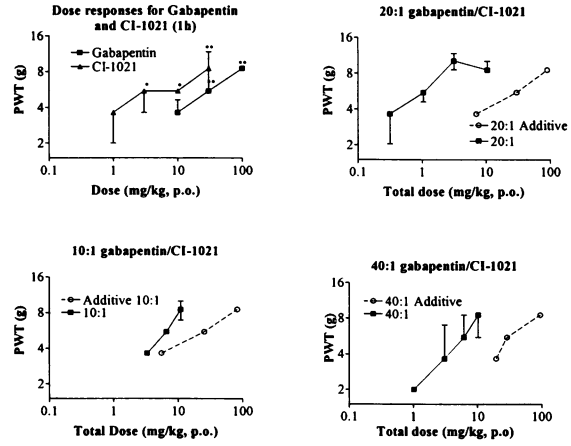
Conventional analgesics, such as opiates and NSAIDS have limited therapeutic value in the management of neuropathic pain. This has led to the use of adjuvant analgesics for the management of these conditions. However, no agent is fully effective in all patients and undesirable side effects are common. Recent studies have demonstrated that gabapentin is an effective treatment of neuropathic pain (Backonja et al., 1998; Rowbotham et al., 1998). Preclinical data suggest that NK_1 receptor antagonists, such as CI-1021 (Singh et al., 1997), are active in animal models of neuropathic pain. We have previously reported that when administered alone, both gabapentin and CI-1021 block the maintenance of streptozocin-induced static allodynia in the rat (Field et al., 1998; Field et al., 1999). In the present study we examined the effects of combinations of gabapentin and CI-1021 in the streptozocin model of neuropathic pain.

Male Sprague Dawley rats (200-250g), obtained from Bantin and Kingman, (Hull, U.K.) were housed in groups of 6. All animals were kept under a 12h light/dark cycle (lights on at 07h 00min) with food and water *ad libitum*. Diabetes was induced by an i.p. injection of streptozocin (50mg/kg). Control animals received a similar administration of isotonic saline. Static allodynia was measured as previously described (Field *et al.*, 1999). Dose responses to gabapentin and CI-1021 were first performed alone in the streptozocin model. The dose response data for both compounds were used to determine theoretical additive lines using the method described by Berenbaum 1989. Combinations of gabapentin and CI-1021 were determined following a fixed ratio design. A dose response to each fixed dose ratio of the combination was performed and compared to the theoretical additive line. The data is expressed as paw withdrawal thresholds (PWT) at the 1h time point as this time represented the peak antiallodynic effect.

Gabapentin and CI-1021, administered alone, dose-dependently blocked the maintenance of static allodynia, with respective doses of 100 and 30mg/kg producing a complete blockade (Figure 1). Combinations of gabapentin:CI-1021 were administered at fixed dose ratios of 1:1, 10:1, 20:1, 40:1 and 60:1. The fixed dose ratios of 1:1 and 60:1 produced data close to the theoretical additive line indicating an additive response (data not shown). However, following fixed dose ratios of 10:1 20:1 and 40:1 static allodynia was completely blocked by respective total doses of 11, 3.15 and 10.25mg/kg compared to respective theoretical additive total doses of 82.5, 89.9 and 94.6mg/kg (Figure 1). The 10:1, 20:1 and 40:1 ratios were approximately 8, 30 and 10 fold more potent respectively than the theoretical additive line (Figure 1) indicating synergy at these dose ratios.

FIGURE 1.



These data suggest a synergistic interaction between gabapentin and CI-1021 in an animal model of neuropathic pain. The reduction in dose of gabapentin may lead to an increased therapeutic window and enhance its clinical utility.

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80P CHARACTERISATION OF A CHO CELL LINE STABLY CO-EXPRESSING $GABA_BR1b$ AND $GABA_BR2$ RECEPTORS

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GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter in the mammalian central nervous system. Evidence from a number of studies suggests that functional metabotropic $GABA_B$ receptors exist as heterodimers consisting of $GABA_BR1$ and $GABA_BR2$ subunits (reviewed by Marshall *et al.*, 1999). In the present study we describe the generation and functional characterisation of a cell line which stably expresses both $GABA_B$ receptor subunits.

CHO cells were transfected with equimolar amounts of human $GABA_BR1b$-myc and human $GABA_BR2$ cDNA in pcDNA3.1-neo and pcDNA3.1-hygro expression vectors, respectively, using Lipofectamine Plus. Following dual selection with hygromycin and neomycin, for ten days, the mass culture was labelled with a FITC-labelled anti-c-myc monoclonal antibody and single cells sorted using flow cytometry. Twenty one clones were screened by [^{3}H]CGP54626 binding and one of them, 1E4, was chosen for further characterisation. The cell line was maintained in minimal essential medium containing 10% fetal bovine serum, 2 mM L-glutamine, 800 μg ml^{-1} G418 and 400 μg ml^{-1} hygromycin. Cells for radioligand and [^{35}S]GTPγS binding studies or cAMP accumulation experiments were processed as previously described (Watson *et al.*, 1996). Data are mean ± s.e.mean, n = 3-5.

Double immunofluorescence microscopy, using anti-myc antibodies to label the myc-tagged $GABA_BR1b$ subunit and anti-$GABA_BR2$ antisera confirmed that, when expressed together, both receptors are located at the cell surface. In equilibrium binding studies [^{3}H]CGP54626 (Froestl *et al.*, 1996) labelled a single binding site with a K_D of 4.2 ± 0.8 nM and B_{max} of 22.2 ± 2.5 pmol mg^{-1} protein. In competition binding assays GABA, baclofen, 3-aminopropylphosphinic acid (3-APPA) and CGP62349 (Froestl *et al.*, 1996) all displaced [^{3}H]CGP54626 binding, pK_i values of 3.83 ± 0.08, 3.47 ± 0.06, 5.05 ± 0.10 and 9.06 ± 0.12, respectively. GABAergic agonists produced a concentration-dependent stimulation of [^{35}S]GTPγS binding and inhibited forskolin stimulated cAMP levels. CGP62349 was a competitive antagonist in both functional assays (data summarised in Table 1).

Table 1. Summary of functional data in 1E4 $GABA_B$ receptor cell line.

	[^{35}S]GTPγS binding (pEC_{50})	cAMP accumulation (pIC_{50})
GABA	4.67 ± 0.05	6.31 ± 0.07
Baclofen	5.19 ± 0.05	6.05 ± 0.09
3-APPA	5.42 ± 0.15	7.17 ± 0.07
CGP62349	8.58 ± 0.10*	8.54 ± 0.35*

*apparent pK_B values

In conclusion we have generated and characterised a cell line stably expressing functional $GABA_B$ receptors. This will be a useful tool in elucidating the pharmacology and biochemistry of this receptor.

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81P BIPHASIC AND DOSE-DEPENDENT *IN VIVO* TIME-COURSE OF $GABA_A$ RECEPTOR-MEDIATED EEG EFFECTS OF THE NEUROSTEROID ALPHALAXONE IN RATS

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Neurosteroids can modulate $GABA_A$ receptor function via a specific, allosteric binding site. It is well established that neurosteroids and other allosteric modulators show brain-region-dependent heterogeneity of binding and function at the $GABA_A$ receptor complex (Sapp *et al.*, 1992). Previously, we have shown that pharmacokinetic-pharmacodynamic (PK-PD) analysis of changes in the electroencephalogram (EEG) can be used to quantify $GABA_A$-receptor-mediated effects of benzodiazepines and barbiturates *in vivo* (see Mandema *et al.*, 1991). The aim of the present study was to characterize the *in vivo* time-course of the $GABA_A$-receptor-mediated EEG effects of the neurosteroid, alphaxalone, using the *in vivo* model developed in our laboratory (Mandema *et al.*, 1991).

Four groups (A-D) of eight male Wistar rats (~300 g) were given (A) 2, (B) 5 or (C) 10 mg/kg alphaxalone in a 5 min intravenous infusion or (D) 5 mg/kg in a 15 min infusion. The EEG was recorded continuously from 45 min before until 150 min after infusion. Off-line fast-Fourier transformation (FFT) was used to quantify the drug-mediated EEG effect in the range from 12-30 Hz (see Mandema *et al.* for details). Serial plasma samples were drawn at regular time intervals and alphaxalone concentrations were determined using HPLC.

The pharmacokinetics of alphaxalone could be described by a standard two-compartment model and parameter estimates (mean±s.e.mean) for half life ($t_{1/2}$;min), clearance (Cl; ml/min/kg), and volume of distribution at steady state (V_{ss}; ml/kg) were 13.7±2.8, 50±5 and 412±55 for group A; 13.2±1.3, 72±4 and 795±62 for group B; 13.6±0.9, 83±12 and 1000±171 for group C; 13.4±1.8; 87±6 and 846±118 for group D, respectively. No significant differences for half life were observed between the groups, however significant differences were observed for clearance and volume of distribution at steady state ($p<0.05$, ANOVA). At present we have no explanation for this dose dependency.

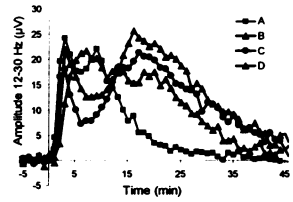

Fig. 1: Average (n=8) time-effect curves for treatment groups A, B, C and D (see text).

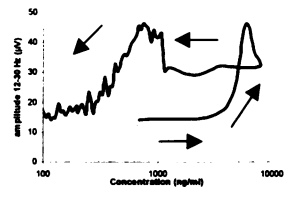

Fig. 2: Concentration-effect curve for typical rat which received treatment B (see text).

The time-effect profiles were biphasic and dose and infusion-rate dependent. The first phase (~0-10 min) consisted of a rapid increase and an immediate, partial decrease of the EEG amplitude. Subsequently, the amplitude increased again and gradually returned to baseline within 45 min (Fig. 1). The maximal observed effect (25.9±1.1 µV, group B) was 1.7 times higher than for the full agonist at the benzodiazepine binding site of the $GABA_A$ receptor complex zolpidem (14.9±1.6 µV (mean±s.e.mean, n=8, $p<0.05$).

The biphasic concentration-effect relationship (Fig. 2) suggest that alphaxalone exerts inhibitory and stimulatory effects on the EEG activity *in vivo* at high and low concentrations, respectively.

In conclusion, we have developed a novel approach to study the *in vivo* time-course of the $GABA_A$-mediated-EEG effects of neurosteroids. The concentration-effect relationship of alphaxalone showed dose-dependent delay and a biphasic pattern. Whether this complexity is related to heterogeneity of steroid recognition sites at $GABA_A$ receptor complexes remains to be clarified (Morrow *et al.*, 1990).

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82P PHARMACOLOGICAL ANALYSIS OF HUMAN $GABA_B$ (1a,2) AND $GABA_B$ (1b,2) HETERODIMERS EXPRESSED IN CHO CELLS: ALLOSTERIC MODULATION OF AGONISTS AND ANTAGONISTS BY Ca^{2+}

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The $GABA_B$ receptor is a functional heterodimer composed of the R1 and R2 receptor proteins (White *et al*, 1998; Kaupman, *et al*, 1998; Jones *et al*, 1998). Two variants of the R1 receptor protein exist, named R1a and R1b, which differ at the N-terminus and which may confer heterodimer-specific pharmacology. Indeed, in rat brain, specific distributions of pharmacologically distinct $GABA_B$ receptors have been described (Yamada *et al*, 1999). However, data reported for $GABA_B$ receptor-selective ligands have sometimes been conflicting. The observation that the potency of GABA can be increased by calcium in a manner compatible with allosteric enhancement (Wise *et al*, 1999) adds further complexity. We have compared the pharmacology of human $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors stably expressed in CHO cells by measuring [^{35}S]-GTPγS accumulation with and without calcium.

GTPγS assays were performed in a 100µl total volume at room temp. The reaction mixture contained: membranes (8µg/well), [^{35}S]-GTPγS (0.19nM), agonist (0.1µM–3mM), GDP (10µM), ± $CaCl_2$ (1mM). Reactions were started by addition of membranes. SPA beads (0.5mg/well) were added 30min later. After 4h incubation, plates were counted for radioactivity. Single [antagonist]s (10µM) were used to calculate apparent pA_2 values using the Schild equation.

The rank orders of agonist potencies differed between $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors (Table 1), suggesting that these subtypes may posess distinct pharmacology. However, the rank orders of antagonist potency were the same for both receptors. In both cell lines, Ca^{2+} increased the potency of all agonists except baclofen. Futhermore, Ca^{2+} increased the potency of antagonists vs GABA, particularly for the $GABA_{B(1a,2)}$ receptor. The ability of Ca^{2+} to enhance potency may be allosteric i.e. Ca^{2+} binds to a site distinct from agonist/antagonist binding site causing a conformational change and resulting in increased affinity of agonists/antagonists for their site. A possible reason for the lack of potency-enhancing effects of Ca^{2+} in the presence of baclofen is that baclofen may, by steric hindrance or another mechanism, block the access of Ca^{2+} to its site. We conclude that the $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ heterodimers do posess distinct pharmacologies. Due to Ca^{2+} modulating effects, care must be taken when interpreting receptor characterisation data.

Table 1. Agonist and antagonist potencies at $GABA_B$ receptors.
Curve parameters were estimated by fitting a four-parameter logistic function to quench-corrected cpm data. Values are mean ± s.e.m. (n).

	$GABA_{B(1a,2)}$		$GABA_{B(1b,2)}$	
	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}
Agonists	**(values are mean pEC_{50})**			
GABA	4.9±0.1 (4)	5.5±0.2 (4)	4.2±0.03 (12)	5.5±0.02(13)
Baclofen	5.1±0.1 (4)	5.3±0.1 (4)	5.0± 0.04 (9)	4.9±0.03 (9)
*SKF97541	4.8±0.2 (4)	5.7±0.2 (4)	4.9±0.1 (7)	6.2 ±0.1 (8)
*3-APPA	4.7±0.1 (4)	5.7±0.1 (4)	5.3± 0.1 (5)	6.5±0.1 (6)
* For structures refer to Brauner-Osborne & Krogsgaard-Larsen (1999)				
Antagonists	**vs GABA (values are mean apparent pA_2)**			
SCH50911	5.6±0.2 (4)	6.5±0.2 (4)	5.7±0.1 (5)	7.0±0.2 (5)
CGP36742	<5.2±0.2 (4)	6.1±0.1 (6)	<5.0±0.01 (2)	4.8±0.1 (3)
CGP54232	6.5±0.1 (4)	7.2±0.1 (5)	6.2±0.1 (5)	6.9±0.1 (5)
CGP35348	<5.1±0.1 (2)	5.7±0.1 (5)	<5.4±0.2 (4)	4.9±0.1 (5)
Antagonists	**vs baclofen (values are mean apparent pA_2)**			
SCH50911	5.7±0.1 (5)	5.7±0.1 (4)	5.9±0.1 (5)	6.1±0.1 (5)
CGP36742	<5.0±0.1 (4)	<5.0±0.1 (5)	<5.2±0.1 (5)	<5.1±0.1 (4)
CGP54232	6.7±0.1 (5)	6.6±0.2 (3)	6.29±0.1 (5)	6.9±0.1 (5)
CGP35348	<5.3±0.2 (4)	5.4±0.2 (4)	<5.1±0.1 (4)	4.6±0.2 (3)

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83P BINDING AND ENDOCYTOSIS OF SOMATOSTATIN$_2$ AGONISTS AND RECEPTORS

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Somatostatin agonists are rapidly and efficiently endocytosed with the somatostatin sst$_2$ receptor (Koenig *et al.*, 1998). Our aim was to investigate the effect of agonist affinity on the amount of agonist endocytosis using a simple model which describes the association (k_{+1}), dissociation (k_{-1}) and endocytosis (k_e) of agonists and their receptors, where R_s is the number of receptors at the cell surface, D is the drug concentration, DR_s is the drug receptor complex at the cell surface and DR_e is endocytosed drug-receptor complex:

$$D + R_s \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} DR_s \xrightarrow{k_e} DR_e$$

To date, values for k_{-1} and k_{+1} have been obtained from experiments performed under non-physiological conditions (Wu-Wong *et al.*, 1995; Koenig *et al.*, 1997). We have used Chinese hamster ovary cells which have been transfected with the N-terminal epitope tagged human somatostatin$_2$ receptor. Radioligand binding studies were performed in the presence of 0.1nM [^{125}I]-Tyr^{11}-somatostatin-14 at 37°C on cell membranes prepared in phosphate-buffered saline (PBS), pH 7.4. Non-specific binding was defined by the inclusion of 1μM somatostatin-14 and the reaction was terminated by filtration. All results are quoted as mean ± s.e.m. The association rate constant of [^{125}I]-Tyr^{11}-somatostatin-14 was 3.8±1.3 x 10^9 $M^{-1}min^{-1}$ (n=4) and equilibrium was reached within 30min. The dissociation rate constant was determined by incubating membranes with radioligand for30min, followed by addition of 2μM somatostatin-14 either in the presence k_{-1}>5min^{-1} (n=5) or absence k_{-1}=0.3±0.1min^{-1} (n=3) of 10μM GTPγS.

Confocal microscopy using a fluorescein-derivative of somatostatin-14 (fluo-somatostatin) was employed to estimate the dissociation rate constant in intact cells. Competition ligand binding studies in cell membranes showed that the binding affinity of fluo-somatostatin (pK_i = 8.61 ± 0.11, n=3) was slightly lower than that of somatostatin-14 (pK_d = 9.31 ± 0.18, n=4). Confocal images were obtained with a Zeiss LSM510 confocal microscope using an Ar-Kr laser to provide excitation at 488nm. Emitted light was collected using a long pass filter (>505nm). Under the scanning conditions used, photobleaching of the fluo-somatostatin was less that 10% and the pixel intensity was linearly proportional to the concentration of fluorescein. Cells were mounted in Dulbecco's Modified Eagle's Medium buffered with HEPES, pH7.4 onto a microscope stage heated to 37°C. Fluo-somatostatin (40nM) was added and scans were obtained every 20 sec. After 5 min, a saturating concentration of the somatostatin antagonist Cyanamid 154806 (2μM) was added (Bass *et al.*, 1996). This caused a decrease in the pixel intensity of individual cells with time at an estimated dissociation rate constant of 3 ± 1 min^{-1} (n=5).

The internalisation of receptors was measured using an enzyme-linked immunoassay to detect the change in the number of epitope-tagged receptors at the cell surface (Koenig *et al.*, 1998). The rate constant for endocytosis (k_e=0.02 min^{-1}) in the presence of 10nM somatostatin was very much smaller than the rate constant for dissociation.

In conclusion, we have demonstrated that binding of somatostatin with its receptor occurs rapidly under physiological conditions but endocytosis occurs slowly. This suggests that only a small proportion of the cell surface agonist-receptor complex is endocytosed.

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84P CLONING, EXPRESSION AND PHARMACOLOGICAL CHARACTERISATION OF THE MOUSE SOMATOSTATIN sst$_5$ RECEPTOR: COUPLING TO LUCIFERASE ACTIVITY VIA THE SERUM RESPONSIVE ELEMENT

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The mouse somatostatin (SRIF) sst$_5$ receptor was cloned from a mouse BALB/c genomic library (EMBL accession # AF004740). It has respectively 97 % and 81 % homology with rat and human receptors. The msst$_5$ receptor was stably expressed in the hamster fibroblast cell line CCL39-SRE-Luci, which harbours the luciferase reporter gene driven by the serum responsive element.

Characterisation of the msst$_5$ receptor was performed in radioligand binding studies with: [^{125}I]LTT-SRIF$_{28}$ ([Leu^8, D-Trp^{22}, ^{125}I-Tyr^{25}]-SRIF$_{28}$), [^{125}I]Tyr^{10}-cortistatin, [^{125}I]CGP 23996, and [^{125}I]Tyr^3-octreotide labelled msst$_5$ receptors with high affinity (pK_d values: 11.0, 10.15, 9.75 and 9.43) and in a saturable manner, but defined different Bmax values: 697, 495, 540 and 144 fmoles/mg, respectively. SRIF analogues displayed the following rank order of pKd's for msst5 [^{125}I]LTT-SRIF$_{28}$-labelled sites: SRIF-28 (9.8, pK_d, n ≥ 3)> BIM-23052 (8.9)> rCST-14 (8.7)> RC160 (8.4)> CGP-23996 (8.0)> SRIF-14 (7.7)= Octreotide > BIM-23056 (7.5)> Seglitide (6.7). However, [^{125}I]Tyr^3-octreotide- labelled sites displayed a different profile with higher affinity for a number of, but not all, peptides tested: Octreotide (9.9)= BIM-23052 (9.8)= SRIF-28 > RC160 (9.5)> rCST-14 (9.4)> SRIF-14 (9.1)> CGP-23996 (8.9)> L 362,855 (8.7)> Seglitide (8.5)> BIM-23056 (7.9) (abbreviations see Siehler et al, 1998). [^{125}I]LTT-SRIF$_{28}$, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin binding correlated highly significantly (r^2 = 0.88-0.99), less so for [^{125}I]Tyr^3-octreotide binding (r^2 = 0.77).

The msst$_5$ and hsst$_5$ homologue both expressed in CCL39 cells show only a limited correlation, e.g. r^2 = 0.385 for [^{125}I]Tyr^{10}-cortistatin- and r2 = 0.323 for [^{125}I]LTT-SRIF$_{28}$-labelled sites further illustrating species differences between sst$_5$ receptors.

Stimulation of msst$_5$ with SRIF induces expression of luciferase reporter gene. The rank order of potency was consistent with a msst$_5$ receptor-mediated effect, although apparent potency (pEC_{50}) in the luciferase assay was 10-100 fold less than affinity values determined in radioligand binding: BIM-23052 (8.0)> octreotide (7.6)= SRIF-28 > RC 160 (7.1)> rCST-14 (6.9) > SRIF-14 (6.7) = L 362,855 = BIM-23056 (6.6) = CGP-23996 > Seglitide (6.3). SRIF$_{28}$, BIM23052 and octreotide behaved as full or nearly full agonists compared to SRIF$_{14}$, whereas the other compounds tested had relative efficacies of 40 to 70%.

Thus, agonist radioligands, define apparently different receptor populations in terms of number of sites and pharmacological profile in CCL39 cells expressing recombinant msst$_5$ receptors. Human and mouse sst$_5$ receptors have different pharmacological profiles. The msst$_5$ receptor which is primarily coupled to effector systems via Gi/Go proteins, is able to stimulate luciferase gene expression driven by the serum responsive element, as reported for other G-protein-coupled-receptors and tyrosine kinase receptors (Treisman, 1995). Whether the MAP kinase pathway is involved, remains to be investigated.

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85P FUNCTIONAL STUDIES WITH A NOVEL NEUROPEPTIDE Y Y_2 RECEPTOR ANTAGONIST, BIIE0246, IN ISOLATED MUCOSAL PREPARATIONS FROM THE RAT GASTROINTESTINAL TRACT

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Three of the six different neuropeptide Y (NPY) Y receptor types characterised to date (see review; Michel *et al.*, 1998) are most commonly expressed in the mammalian gastrointestinal tract, namely, Y_1, Y_2 and Y_4 (Cox, 1998). Isolated preparations of rat jejunum are sensitive to NPY, peptide YY (PYY) and their C-terminal fragments, all of which inhibit basal and stimulated anion secretion (Cox *et al.*, 1988; Cox & Cuthbert, 1990). Using agonists alone we and others have concluded that Y_2 receptors mediate the inhibitory responses in rat jejunum, while colonic mucosa also expresses Y_1 receptors (Tough & Cox, 1996). Selective Y_1 (Wieland *et al.*, 1998) or Y_5 (Criscione *et al.*, 1998) receptor antagonists have been developed but no Y_2 receptor blockers have been available until recently and here we present data with the first, selective Y_2 receptor antagonist, BIIE0246 (Doods *et al.*, 1999).

Mucosal preparations of rat (Sprague-Dawley, male, 250-300g) jejunum and descending colon were prepared as described previously (Tough & Cox, 1996). Tissues (area 0.6 cm^2) were placed in Ussing chambers and voltage-clamped at 0 mV, recording the resultant short-circuit current (I_{sc}). Once the I_{sc} levels had stabilised, BIIE0246 (or vehicle) was added (with BIBP3226, 1 μM to colonic tissue only) and 20 min later cumulative additions of (3-36)PYY (0.3 - 444.3 nM) were made to the basolateral reservoir. The reductions in I_{sc} to (3-36)PYY were pooled and EC_{50} and pK_B values were calculated using GraphPad Prism (version 2.0).

Basal I_{sc} levels were 35.0 ± 3.3 $\mu A.cm^{-2}$ (n=28) in rat jejunum and 22.6 ± 1.8 $\mu A.cm^{-2}$ (n=27) in rat colon mucosa. Neither BIBP3226 nor BIIE0246 (10 nM - 1 μM) altered I_{sc}, however subsequent responses to (3-36)PYY were attenuated in both preparations by BIIE0246. In jejunal mucosa the Y_2 antagonist alone (10 nM) displaced the (3-36)PYY concentration-response curve to the right without a reduction in the maxima, but higher BIIE0246 concentrations (30 nM - 1 μM) reduced agonist responses (244 nM) by between 53 - 86%. A pK_B of 9.1 was obtained in jejunum mucosa.

In rat descending colon mucosa a more gradual displacement to the right of the (3-36)PYY response curve was observed in the presence of BIIE0246 without any significant reduction in maxima (pK_B 8.0 ± 0.2, n=4). Addition of PYY (100 nM) after (3-36)PYY did not further reduce I_{sc} in jejunum but residual responses were obtained in colonic preparations to PYY. Subsequent responses to the α_2-agonist UK14,304 (1 μM) were not significantly altered by BIIE0246 (1 μM) in either preparation (in $\mu A.cm^{-2}$; jejunum controls, -15.3 ± 3.8, n = 6 and plus BIIE0246, -14.3 ± 3.5, n = 4: colon controls, -39.5 ± 12.1, n = 6 and plus BIIE0246, -35.8 ± 7.1, n = 4).

We conclude that BIIE0246 attenuates epithelial Y_2 receptors in both rat jejunum and descending colon mucosae. In the former tissue Y_2 receptors alone are responsible for Y agonist-induced antisecretory responses, while Y_1 and Y_2 receptors contribute to inhibitory PYY responses in rat colon mucosa.

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86P USE OF AN INDUCIBLE EXPRESSION SYSTEM TO EXAMINE THE EFFECTS OF ALTERING HUMAN $sst_{2(A)}$ RECEPTOR EXPRESSION LEVELS ON RECEPTOR ACTIVATION

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Previous reports have suggested that high levels of receptor expression, a key feature of heterologous expression systems, may be unsuitable for predicting receptor signalling in whole tissue (Kenakin, 1995). We have created and used an inducible expression system to examine the consequence of modulating the level of human recombinant somatostatin (SRIF) type 2 (sst_2) receptor expression on the effects on receptor activation. Stable expression of the sst_2 receptor was achieved in Chinese Hamster Ovary (CHO) cells using an ecdysone inducible expression system (No et al., 1996).

Following application of an inducing agent, muristerone A (MuA), increases in surface HA-tagged human sst_2 receptor expression were determined by use of an immunoassay. Radioligand binding, using [^{125}I]-[Tyr^{11}]-SRIF radioligand, was employed to determine the density of specific binding sites (Bmax) for individual cell lines exposed to a range of concentrations of MuA. SRIF-induced increases in extracellular acidification rates (EAR) were measured by microphysiometry, as previously described (Schindler et al., 1998).

Using an immunoassay, a concentration-dependent induction of sst_2 receptor expression was attained by treatment of the cells with increasing concentrations of MuA for 24h (Table 1).

Radioligand binding studies were performed on membrane homogenates of stably transfected CHO cells. In CHO cells where sst_2 receptor expression was induced, SRIF caused a concentration-dependent inhibition of specific [^{125}I]-[Tyr^{11}]-SRIF binding (Table 1). No specific SRIF binding was detected in uninduced cells.

Using microphysiometry, SRIF caused concentration-dependent increases in EAR in MuA-induced CHO-sst_2 cells. Although the magnitude of the SRIF-induced response was dependent on the level of sst_2 receptor expression, pEC_{50} values did not differ significantly (Table 1).

Table 1. All values shown are mean ± sem from 3-5 experiments.

[MuA] (μM)	% increase in surface sst_2 labelling (over basal)	Bmax (fmol/mg^{-1})	Microphysiometry Response to SRIF	
			Max. response (% over basal)	pEC_{50}
1	334 ± 6	72 ± 9	20 ± 4	8.7 ± 0.06
2	811 ± 144	183 ± 14	30 ± 6	9.3 ± 1.36
7	3902 ± 381	460 ± 48	49 ± 11	8.8 ± 0.03

To conclude, with the use of an inducible expression system we have achieved titration of human sst_2 receptor expression in a CHO cell line. We have demonstrated that the maximum response to SRIF is dependent on the level of receptor expression although no corresponding change in the potency of SRIF was observed. It is suggested that, using microphysiometry, the magnitude of the agonist response generated is highly dependent on the receptor density, with no evidence for receptor reserve. The effects of receptor density on other cellular responses are currently under investigation.

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87P EFFECTS OF SOMATOSTATIN ON THE ACTIVITY OF RAT MESENTERIC AFFERENT NERVES *IN VIVO*

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The somatostatin analogue, octreotide, reduces visceral hypersensitivity in humans (Hasler *et al*, 1993) but the mechanism underlying this effect is unknown. Possible sites of action include the peripheral terminals of afferent nerves. Indeed, we have recently demonstrated an inhibitory action of octreotide on rat jejunal high-threshold mechanosensitive afferents (Booth *et al*, 1999). A subpopulation of mesenteric afferents are chemosensitive, responding to capsaicin and to the pain-producing peptide, bradykinin (Maubach & Grundy, 1999). We therefore examined the effect of octreotide and the selective sst_2 receptor agonist, BIM 23027, on both baseline and bradykinin-evoked mesenteric afferent fibre activity in the rat *in vivo*.

Extracellular recordings of mesenteric afferent nerve activity were obtained from pentobarbitone-anaesthetised (60mg.kg^{-1}, i.p.) male Wistar rats (330-450g) as previously described (Kirkup *et al*, 1999). Data are the mean±s.e.mean from 3-14 animals and statistically analysed using the Mann-Whitney U test.

Octreotide and BIM 23027 (0.001-100μg.kg^{-1} i.v.) evoked a dose-dependent inhibition of baseline afferent nerve activity with -$\log_{10}$ ED_{50} (μg.kg^{-1}) values of 6.37±1.01 and 7.13±0.39 respectively. Bradykinin, at doses of 0.1, 0.3 and 1μg.kg^{-1} (i.a.), induced dose-dependent increases in afferent nerve activity by 76±15%, 162±35 % and 314±64 % ($P<0.05$) respectively, from a baseline firing rate of 30.2±4.1 spikes.s^{-1}. No desensitisation was observed when the agonist was administered every 15 min. Treatment with 10μg.kg^{-1} and 100μg.kg^{-1} octreotide (i.v.) induced a reversible, dose-dependent inhibition of the mean peak responses to bradykinin.

Table 1 Effect of octreotide on the bradykinin (BK)-induced increases in mesenteric nerve activity.

	% of response prior to octreotide/saline treatment		
Dose of octreotide	**0.1 μgkg^{-1} BK**	**0.3 μgkg^{-1} BK**	**1 μgkg^{-1} BK**
Time control (saline, n=4)	104.7±11.9	118.8±13.8	116.4±24.6
10μgkg^{-1} (n=4)	53.5±19.6	57.5±11.2*	92.1±22.5
100μgkg^{-1} (n=6)	33.1±9.5*	28.1±8.7*	41.9±9.4*

(*=$P<0.05$ *cf* time match control)

In conclusion, octreotide and BIM 23027 each induce a dose-dependent inhibition of baseline afferent nerve activity, which is probably mediated by sst_2 receptors. Moreover, octreotide produces a dose-dependent reduction of the bradykinin-evoked excitation of mesenteric afferent nerves. Studies are in progress to determine the receptor type(s) involved in this effect. These effects of octreotide could contribute to its ability to modulate the perception of visceral sensations in humans.

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88P SYSTEM AND ASSAY-DEPENDENT EFFICACY OF DOPAMINE D_4 RECEPTOR LIGANDS AT HUMAN DOPAMINE $D_{4.4}$ RECEPTORS

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We recently reported the agonist activities of the dopamine D_4 receptor ligands U-101958, L-745,870 and NGD 94-1 at human recombinant dopamine $D_{4.4}$ receptors expressed in HEK293 cells (Gazi et al., 1998, 1999a). As an extent of that work, we demonstrated that the efficacy of these ligands is governed by receptor density in CHO cells (Gazi et al., 1999b). These findings prompted us to investigate these ligands in other models. In the absence of recognised functional model for native dopamine D_4 receptors, we used four different expression systems (HEK293, CHO, HeLa and CCL39 cells) and three functional readouts (inhibition of cyclic AMP accumulation, stimulation of [^{35}S]-GTP-γ-S binding and of extracellular acidification rate).

The cloning of the human dopamine $D_{4.4}$ receptor cDNA and the transfection into HEK293 and CHO cells have been described (Gazi et al., 1998, 1999b). HeLa and CCL39 cells were transfected with the same expression construct by the lipofectamine transfection method and by the electroporation method, respectively. Receptor density (B_{max}) was determined by [^{3}H]-spiperone saturation binding. Cyclic AMP data were obtained as previously (Gazi et al., 1998), [^{35}S]-GTP-γ-S binding was performed using a scintillation proximity assay (Amersham RPNQ0210) and the extracellular acidification rate was measured with a Cytosensor microphysiometer. Results are given as mean ± s.e.mean.

B_{max} and efficacy values (relative to dopamine) for inhibition of cyclic AMP accumulation are given in Table 1. Whereas quinpirole and 7-OH-DPAT were full agonists in all four cell lines, apomorphine and PD168077 tended to be less efficacious, particularly in CHO and CCL39 cells. U-101958 behaved as a quasi-full agonist in HEK293 cells and as a partial agonist in the other cell lines. L-745,870 was a partial agonist in HEK293 and HeLa cells, had a very small efficacy in CCL39 cells and was silent in CHO cells. NGD 94-1 acted as a partial agonist in all four cell lines. No agonist effects were found in the corresponding non-transfected cells. Spiperone and clozapine were silent in all cases.

Table 1. B_{max} and efficacy values of dopamine D_4 receptor ligands (relative to dopamine) for inhibition of cyclic AMP accumulation

Cell line	HEK293/D_4	CHO/D_4	HeLa/D_4	CCL39/D_4
B_{max}	505±90	240±30	621±165	1080±240
Efficacy				
Dopamine	100	100	100	100
Quinpirole	98±4	99±11	98±1	102±2
7-OH-DPAT	100±3	94±2	98±1	96±5
Apomorphine	85±4	81±7	95±2	80±3
PD168077	84±4	55±10	90±2	78±7
U-101958	93±4	39±7	65±5	49±6
L-745,870	71±3	0	44±4	24±4
NGD 94-1	79±6	40±7	79±5	42±8

Efficacy values are E_{max} values of ligands expressed as percentage of the E_{max} value of dopamine. B_{max} are in fmol.mg^{-1} protein. $n \geq 3$.

Measurements of stimulation of [^{35}S]-GTP-γ-S binding and of extracellular acidification rate, when applicable, yielded reduced efficacy values for all compounds (relative to dopamine) as compared to the cyclic AMP data. In particular, U-101958, L-745,870 and NGD 94-1 displayed very little, if any, intrinsic activity in these assays.

These results show that the efficacy of dopamine D_4 receptor ligands can vary according to the assay type and the expression system. The efficacies of U-101958, L-745,870 and NGD 94-1 were subject to large variations under these conditions. These three compounds, however, were distinct from spiperone and clozapine which were always silent.

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89P PHOTOSENSITISATION OF PANCREATIC TUMOUR CELLS BY A SERIES OF δ-AMINOLAEVULINIC ACID (ALA) ESTERS

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Photodynamic therapy (PDT) is being used increasingly for the treatment of tumours, in particular by administration of the precursor δ-aminolaevulinic acid (ALA) which results in the photosensitisation of target cells by the formation of protoporphyrin IX (PpIX). However, ALA-PDT is restricted by the hydrophilic nature of ALA which limits its cellular uptake. The lipophilicity of ALA and its ability to cross cell membranes can be increased by esterification. Once inside the cell, the esteratic group is cleaved by non-specific esterases yielding free ALA. The aim of this study was to systematically investigate the use of ALA esters, identifying any similarities or differences when compared to ALA. We have used an *in vitro* system of cultured AR4 2J rat pancreatoma cells at 37°C, since these cells have previously been shown to be highly responsive to photosensitisation by PpIX (Ratcliffe & Matthews, 1995).

Photo-active species were detected spectrofluorimetrically in both cell monolayers and extracellular media (λE_x:405 nm and λE_m:500 nm-700 nm) using AR4 2J cells which had been mounted on cover slides and incubated with ALA (≤500 μM) or n-pentyl ALA ester (n-5) (≤100 μM) for 24 h. The main cellular species detected in both cases was PpIX (λE_m: 635 nm), the amount of which was dependent on the concentration of ALA or ALA ester. It was shown that ALA, 500 μM, was required to generate the same amount of intracellular PpIX as n-5, 50 μM. Seven different ALA esters tested (10 μM) produced between 4.03 ± 0.55 and 8.46 ± 0.47 fluorescence units (FU) of intracellular PpIX compared to only 0.32 ± 0.05 from ALA. Considerably lower levels of PpIX were produced by 10 μM iso-propyl (0.22 ± 0.05, n=6) and cyclo-pentyl (1.52 ± 0.21, n=6) esters.

Parallel studies of photocytotoxicity were made by incubating cells with ALA or n-5 for 2, 4 or 24 h before 15 min illumination (0.46 mW/cm^2) and cell survival determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Comparison of EC_{50} values showed that n-5 was most effective after 2 h (4.53 ± 0.96 x 10^{-7}M, n=4) and ALA after 24 h (1.96 ± 0.21 x 10^{-5}M, n=9). In order to investigate the kinetics of PpIX production from ALA and ALA esters, PpIX fluorescence was measured after incubation with 250 μM ALA or n-5 every 2 h for 8 h and at 24 h. It was shown that n-5 generated intracellular PpIX more rapidly than ALA, n-5-induced PpIX being greater than ALA-induced PpIX after only 2 h, with the difference becoming significant at 6 h (P<0.05, n=3) and indicating separate access routes through the cell membrane. By blocking PEPT1, which transports ALA into the cell (Döring *et al.*, 1998), with 5 mM AMBA (Meredith *et al.*, 1998) ALA-induced PpIX was decreased to 48 ± 2.4%, compared to a decrease to only 85 ± 3.9% for n-5-induced PpIX (P<0.001, n=3). Hence ALA, but not n-5, is actively transported across the cell membrane by PEPT1.

From these experiments it can be concluded that the photodynamic effectiveness of ALA esters is due to differences in access through the cell membrane compared to ALA, allowing the generation of the same porphyrin species and similar levels of intracellular PpIX as for ALA but with much lower ALA ester concentrations required for shorter incubation periods. The small amount of PpIX formed from the iso-propyl and cyclo-pentyl esters is probably due to a structural branch point next to the site of esteratic cleavage which limits access of these esters to the active site of the intracellular esterase. This study provides evidence for the potential advantages of using ALA esters, instead of ALA, for PDT in the future.

C.J.W. holds a BBSRC Studentship

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90P AGONIST-MEDIATED TRANSLOCATION AND DOWN-REGULATION OF PHOSPHOLIPASE C ISOFORMS IN THE HUMAN NEUROBLASTOMA, SH-SY5Y

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A variety of agonists, acting through either G protein-coupled receptors (GPCR) or receptor tyrosine kinases (RTK), elevate intracellular [Ca^{2+}] through a phospholipase C (PLC) and Ins(1,4,5)P_3-dependent pathway. Although presumptions are often made regarding which of the ten identified PLC isoforms are activated for a given receptor, this is largely based on indirect evidence from known *in vitro* differences in their activation and regulation. Furthermore, it is unknown if receptor-specific signalling patterns result from the recruitment of different PLC isoforms. In this study we sought to determine directly the nature of the PLCs activated in response to agonist occupation of GPCRs (muscarinic M_3) or RTKs (PDGF) in human neuroblastoma SH-SY5Y cells by measuring receptor-mediated translocation and down-regulation of specific isoforms.

SH-SY5Y cells were cultured as described previously (Willars & Nahorski 1995) and plated out in 6-well plates before being serum-starved for 20 hours to enhance PDGF-mediated Ca^{2+} signalling (Wheldon *et al* 1999). PLC isoforms were identified by Western blot and quantified by densitometric analysis following SDS-PAGE of cytosolic and membrane preparations from lysed cells (translocation) or whole cell extracts (down-regulation). All data are mean±sem (n=4). Translocations are expressed as percentage of total PLC isoform immunoreactivity (membrane plus cytosol) at the membrane.

PLC β1, β2, β3, γ1 and δ2 but not PLC β4, γ2 and δ1 were detected in SH-SY5Y cells. Following methacholine treatment (1 mM), a number of PLC isoforms translocated from cytosol to membrane but with differing temporal profiles. The translocations of PLC β1 and β2 were maximal at 10 and 5 min respectively (PLC β1: 55±22% to 90±1%; PLC β2: 32±10% to 84±4%) and relatively sustained. PLC β3 did not translocate (61±4% (basal)). There was a transient translocation of PLC γ1 peaking at 5 min (41±6% to 53±7%) and returning to the basal distribution by 10 min. PLC δ2 remained almost exclusively at the membrane. In contrast, stimulation with PDGF-BB (200 ng.ml^{-1}) resulted only in redistribution of PLC γ1, which continued to translocate to the membrane up to 30 min (25±2% to 49±4% at 30 min). Chronic muscarinic receptor stimulation (1 mM methacholine, 24h) caused a marked reduction in all the PLC isoforms present (PLC β1: 23±6%; PLC β2: 42±31%; PLC β3: 33±9%; PLC δ2: 53±18%) except PLC γ1 (93±29%) (all % of unstimulated). In contrast, chronic treatment with PDGF-BB (200 ng.ml^{-1}, 24h), caused increases in PLC β3, γ1 and δ2 (340±101%; 209±21%; 224±52%, respectively) but no changes in PLC β1 or β2 (107±7%; 139±33%, respectively).

Both the down-regulation and translocation experiments suggest that multiple isoforms of PLC may be recruited following activation of GPCRs. In contrast, the translocation data suggest that RTKs may recruit only PLCγ. Interpretation of the effects of chronic PDGF treatment is complicated by its mitogenic properties in these cells. Importantly, the temporal profiles of translocation induced by GPCR or RTK activation do not match that of phosphoinositide and Ca^{2+} signalling in these cells (Wheldon *et al.*, 1999) suggesting that whilst translocation may indicate recruitment, this may not be indicative or necessary for this signalling.

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91P THE EFFECTS OF PCMBS ON S4 CYSTEINE MUTANTS OF THE HUMAN POTASSIUM CHANNEL hKv2.1

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We have previously shown (Yusaf *et al.*, 1996) that outward movement of the fourth transmembrane segment (S4) of the voltage-sensitive *Shaker* K^+ channel occurs during depolarisation. In this study, we have used a slowly activating human potassium channel, hKv2.1, which has a shorter S3-S4 linker. The S3-S4 linker of the *Shaker* K^+ channel plays an important role in setting activation and deactivation kinetics (Mathur et al., 1997; Gonzalez *et al.*, 1999).

Cysteine residues were introduced into the S4 segment at positions V296, I298, F299, I301 and M302 which align with *Shaker* residues L361, V363, I364, L366 and V367 respectively. Wild type and mutant channels were expressed in *Xenopus* oocytes by cRNA injection, and currents recorded 24-48hrs later using the two-electrode voltage clamp technique. The oocytes were held at -80 mV and repetitively depolarised to +40 mV (500 ms pulses, 0.1 Hz) to examine the effects of parachloromercuribenzene-sulphonate (PCMBS), a membrane impermeable sulphydryl reagent.

For the hKv2.1 channel, we observed shifts (relative to wild type) in the current-voltage curves in the hyperpolarising direction for mutants F299C and I301C and in the depolarising direction for mutants V296C and M302C, but no shift was observed for mutant I298C. PCMBS had no effect on wild type channel currents. For mutant channels, PCMBS (100μM) inhibited K^+ currents for V296C, I298C, F299C and I301C, but not those for M302C, demonstrating exposure up to residue I301C but not M302C, which parallels similar results for *Shaker*. The time taken to reach 50% inhibition of the current by PCMBS was 3.3 ± 0.4 min (V296C) n = 5, 19.2 ± 1.3 min (I298C) n = 5, 11.1 ± 0.9 min (F299C) n = 4 and 8.7 ± 1.1 min (I301C) n = 4. In the same experiments, the extent of inhibition of the current amplitudes by PCMBS was 73.6 ± 4.2% (V296C), 56.4 ± 2.5% (I298C), 77.3 ± 4.4% (F299C) and 85.5 ± 3.5% (I301C).

Inhibition of these hKv2.1 mutant K^+ channel currents by PCMBS was slower than observed in corresponding *Shaker* K^+ channel mutants, suggesting reduced accessibility. The slow and incomplete effect on residue I298C is particularly striking, and suggests that this residue is less accessible than the deeper residues F299C and I301C. These effects may arise because of the shorter S3-S4 linker and slower activation properties of the hKv2.1 potassium channel.

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92P GRK6 SELECTIVELY REGULATES SECRETIN RECEPTOR RESPONSIVENESS

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We have previously shown that G_s-coupled secretin receptors endogenously expressed in NG108-15 mouse neuroblastoma x rat glioma cells undergo rapid agonist-induced desensitization (Mundell *et al.* 1997). Furthermore, we have shown that endogenous secretin receptor desensitization does not appear to involve G protein-coupled receptor kinase 2 (GRK2; Mundell *et* al. 1997, 1998). However, NG108-15 cells express not only GRK2, but also GRKs 3 and 6 (Willets *et al.* 1999). To further investigate the mechanism of secretin receptor desensitization, we transfected wild type NG108-15 cells with human GRK6. Three clones which overexpressed GRK6 as determined by Western blotting were expanded into cell lines (named GRK6/7, GRK6/14, and GRK6/16, overexpressing GRK6 by at least 20-, 10- and 5-fold over endogenous levels, respectively).

GRK6 or plasmid (pcDNA3) transfected control cells (two of the latter being named P1 and P3) were grown in 24-well plates in DMEM containing 6% fetal calf serum supplemented with geneticin (300 μg/ml). When confluent, the medium was exchanged for 0.5 mL of fresh medium 1 hr prior to experimentation. With the exception of basal, cells were challenged with either secretin (100 nM), NECA (5′-(N-ethylcarboxamido)-adenosine; 10 μM, adenosine receptor agonist), iloprost (1 μM, IP-prostanoid receptor agonist), or forskolin (10 μM) for up to 2 hours at 37°C in the presence of the phosphodiesterase inhibitor Ro201724 (250 μM). At appropriate time points trichloroacetic acid was added to terminate the reaction and cAMP concentrations determined by a protein binding assay (Mundell *et al.* 1997). Data are expressed as pmol cAMP mg^{-1} protein. Secretin-stimulated cAMP formation was less in GRK6-overexpressing cells as compared to plasmid controls (Table 1, similar data were obtained with both P1 and GRK6/7, and wild type and GRK6/16 cells). The reduction in secretin-stimulated cAMP accumulation appeared to correlate with the degree of GRK6 overexpression. In contrast, cAMP accumulation stimulated by activation of A_2 adenosine receptors, IP-prostanoid receptors, or directly by forskolin, was not reduced in GRK6-overexpressing as compared to plasmid control cells.

Table 1 Secretin-stimulated (100 nM) cAMP accumulation (pmol mg^{-1} protein) in plasmid control (P3) and GRK6 overexpressing (GRK6/14) cells. Values are means ± s.e. mean; cAMP accumulation was less in GRK6/14 cells ($p<0.05$; 2-way ANOVA).

Time (min)	P3 (n =4)	GRK6/14 (n =4)
Basal	37 ± 2	35 ± 1.8
2.5	86 ± 8	52 ± 5.5
5	113 ± 18	66 ± 3.7
10	213 ± 39	85 ± 7.5
20	496 ± 56	170 ± 18
30	591 ± 61	199 ± 26

These data show that overexpression of GRK6 in NG108-15 cells leads to a selective decrease in secretin-stimulated cAMP formation, suggesting that secretin receptor responsiveness is regulated by GRK6. Whether endogenous GRK6 in NG108-15 cells regulates secretin responsiveness remains to be determined.

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93P EFFECTS OF TETRAETHYLAMMONIUM ON KCNQ1-4 AND ON HETEROMERIC KCNQ2/KCNQ3 AND KCNQ2/KCNQ3(T323Y) POTASSIUM CHANNELS

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The KCNQ family of channels includes the cardiac slow delayed rectifier KCNQ1 (KvLQT1), the proposed M-channel components KCNQ2 and KCNQ3, and KCNQ4, whose mRNA is expressed in the cochlea. The TEA sensitivity of KCNQ2 is higher than that of the M-channel, that of KCNQ3 is lower, and that of the KCNQ2/3 heteromultimer closely matches that of the M-current (Yang *et al.*, 1998; Wang *et al.*, 1998). The high TEA sensitivity of KCNQ2 might result from the presence of a tyrosine residue in the pore loop of the channel (see MacKinnon & Yellen, 1990; Kavanaugh *et al.*, 1991), since KCNQ3 has a threonine and KCNQ1 and KCNQ4 have a valine and a threonine, respectively, in the corresponding pore positions (Kubisch *et al.*, 1999).

Using a CHO cell expression system and the perforated-patch configuration of the patch-clamp (see Selyanko *et al.*, 1999), we have assessed the blocking effect of TEA on currents generated by homomeric wild-type KCNQ1-4 channels, on heteromeric wild-type KCNQ2/3 channels and on heteromeric KCNQ2/3 channels incorporating a mutated KCNQ3 subunit in which tyrosine replaced threonine at position 323.

IC_{50} values (measured as fractional reduction of deactivation tail-currents recorded at –50 mV) were (mM, mean ± s.e.m.): KCNQ1, 5.0 ±0.2 (n=4); KCNQ2, 0.3 ±0.02 (n=4); KCNQ3, >30 (n=4); and KCNQ4, 3.0 ±0.3 (n=4). EC_{50} values for the heteromeric channels were: KCNQ2 + KCNQ3, 3.8 ± 0.2 mM (n=4); KCNQ2 + KCNQ3(T323Y), 0.5 ± 0.05 mM (n=3).

Thus, substitution of tyrosine for threonine at position 323 in KCNQ3 increases the TEA sensitivity of the heteromeric KCNQ2/3 channel, as predicted. Nevertheless, KCNQ1 and KCNQ4, lacking a tyrosine in this position, have a higher TEA sensitivity than KCNQ3, suggesting that other residues are also important in determining TEA block of KCNQ channels.

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94P MECHANISM OF AIRWAY HYPERREACTIVITY TO ADENOSINE INDUCED BY LPS CHALLENGE IN BROWN NORWAY RATS

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We have shown a marked and selective augmentation of the bronchoconstrictor response to adenosine following allergen challenge in actively sensitised, Brown Norway (BN) rats; the response occurs against a background of pulmonary inflammation, is mast cell dependent and mediated by A_{2B} receptors (Hannon et al., 1999a; 1999b). In an attempt to define the specificity of the inflammatory stimulus, we have investigated the consequence of inducing pulmonary inflammation with lipopolysaccharide (LPS; from *Salmonella typhosa;* L-7136; Sigma) on the bronchoconstrictor response to adenosine in the BN rat.

Male BN rats weighing approximately 250g were used. For measurement of lung function, animals were anaesthetised (pentothal 70 $mgkg^{-1}$, i.p., a dose sufficient to maintain full anesthesia throughout the experiment), ventilated, immobilised (vercuronium bromide (12 $mgkg^{-1}$)) and cannulated for measurement of cardiovascular parameters (carotid artery) and drug administration (jugular vein). Airway resistance (R_L) was calculated from measurement of airflow and transpulmonary pressure by use of a digital electronic respiratory analyzer (PMS 8000, Mumed Ltd., U.K.) (Hannon *et al.*, 1995). In separate groups of animals (n=10), total leukocyte numbers and differential cell counts in bronchoalveolar lavage (BAL) fluid were obtained using an automated cell analysing system (Cobas Helios, Axon Lab, Switzerland). Eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities and TNF_α and protein concentrations in BAL fluid were determined using standard assays.

In naïve animals adenosine (0.3 & 1 $mgkg^{-1}$ i.v.) was only a weak bronchoconstrictor (incremental increase in R_L (mean±s.e.mean): 14.5±0.9 and 107.9±19.9 $cmH_2O/l/s$ n=5, respectively). The response of the airways to adenosine was markedly affected by LPS. For instance, 1 h following i.t. administration of LPS (0.3 $mgkg^{-1}$), which did not *per se* affect basal R_L, the increases in R_L to the 0.3 and 1$mgkg^{-1}$ doses of adenosine were 38.9 ± 13.4 and 312.1±69.4 $cmH_2O/l/s$, n=4, $p<0.05$, respectively. In contrast, responses to methacholine (3-10 μgkg^{-1} i.v.) were unaffected by LPS and those to bradykinin (30-100μgkg^{-1}, i.v.), although significantly greater following LPS, were enhanced to a lesser extent than those of adenosine. When measured 3h post LPS challenge, airway responsiveness to all three spasmogens was similar to that in naïve animals and after 24h a significant hyporesponsiveness to adenosine, methacholine and bradykinin was observed (mean decreases relative to vehicle - treated controls, 76, 34 and 49% for the highest doses of each spasmogen, respectively).

One hour following LPS (0.3 $mgkg^{-1}$ i.t.) there were no signs of pulmonary inflammation as monitored by leukocyte numbers, EPO/MPO activities or protein concentration in the BAL fluid. TNF_α concentrations were, however, increased from 85±20 to 1315±31$pgml^{-1}$ ($p<0.001$). At 24 h, significant increases in the numbers of eosinophils (2.4 fold, $p<0.001$), macrophages (2.1 fold, $p<0.01$) and neutrophils (56 fold, $p<0.001$), the activities of EPO/MPO (5 and 7 fold, $p<0.001$ and $p<0.001$ respectively) and the concentrations of protein (1.5 fold, $p<0.05$) and TNF_α (2.6 fold, $p<0.01$) were evident in the BAL fluid.

The selective adenosine receptor antagonists, DPCPX (A_1, 100 μgkg^{-1} i.v.) and ZM 243185 (A_{2A}, 30 μgkg^{-1} i.v.), were without effect on the response to adenosine augmented following LPS challenge despite blocking the A_1 and A_{2A} receptor mediated components of the cardiovascular response to the corresponding selective agonists, respectively. In contrast, the broad spectrum (A_1, A_{2A}, A_{2B}) adenosine receptor antagonist, 8-SPT (40 $mgkg^{-1}$ i.v.), induced significant suppression of the augmented response to adenosine (mean inhibition 72%). Mast cell involvement was implicated by the dose-dependent attenuation of the response to adenosine by pretreatment with sodium cromoglycate (20 – 40 mg kg^{-1} i.v; mean inhibition 55 and 62%, respectively) and essentially complete inhibition by the 5-HT_2 receptor antagonist, methysergide (30 μgkg^{-1}.i.v.).

Thus, challenge with LPS induces significant, selective but transient augmentation of the response to adenosine in BN rats. The response is associated with a marked increase in the BAL fluid TNF_α concentration but does not correlate with the degree of pulmonary inflammation as monitored by changes in BAL fluid leukocyte numbers, EPO/MPO activities or protein concentrations. The response appears to be mast cell mediated and to involve A_{2B} receptors and in these respects bears similarity to the response to adenosine augmented following antigen challenge in sensitised animals (Hannon et al., 1999a;1999b).

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95P THE INVOLVEMENT OF THE NK_1 RECEPTOR IN NEUTROPHIL ACCUMULATION: A STUDY IN WILDTYPE AND NK_1 RECEPTOR KNOCKOUT MICE

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We have studied NK_1 receptor mediated oedema formation through use of NK_1 knockout and wildtype mice (Cao et al, 1999). We have now used the same strain, of mice (SV129 + C57BL/6) to study the involvement of the NK_1 receptor in neutrophil accumulation.

Mice (male and female, 30-35 g) were anaesthetised with halothane (2%) in gas (95% O_2, 5% CO_2) and the dorsal skin shaved. Test agents (50 µl/site intradermally) were given at 0h and 3h where required, and animals allowed to recover. Animals were killed by cervical dislocation at 5h. Neutrophil accumulation was determined by myeloperoxidase (MPO) activity and quantified by comparing results with those of neutrophil standards. Statistics was by ANOVA followed by Bonferroni's modified t-test. Results are shown in Table 1.

Substance P (SP) when injected alone had no effect on neutrophil accumulation. Mouse recombinant interleukin-1ß (IL-1β) induced similar accumulation in NK_1 knockout and wildtype mice. However, co-administration of IL-1β (0.3 pmol) and SP (300 pmol given at 0 and 3 h) produced potentiation of neutrophil accumulation in wildtype but not knockout mice. Carrageenin (500 µg) and zymosan (20 µg) both induced substantial neutrophil accumulation. The effect of zymosan was similar in both wildtype and knockout mice but the effect of carrageenin was attenuated in knockout mice. Pretreatment of wildtype mice with a selective NK_1 antagonist SR 140333 also significantly reduced carrageenin-induced neutrophil accumulation compared to untreated wild-type mice (results not shown).

Table 1. Effect of agents on neutrophil accumulation in mice, mean ± s.e.mean, with (n). *=P<0.05 compared to corresponding control, †=P<0.05 compared to corresponding IL-1β value, ***=P<0.001 compared to corresponding control (not shown),††=P<0.01, compared to carrageenin.

Treatment (n)	+/+ wildtype (x10^6cells/site)	-/- knockout (x10^6cells/site)
Control (Tyrodes)	1.15± 0.21 (10)	1.12± 0.0.33 (8)
SP (300 pmol)	1.37± 0.72 (4)	1.32± 0.19 (4)
IL-1β (0.3 pmol)	2.75± 0.48* (10)	2.89± 0.42* (8)
Control (Tyrodes)	1.58± 0.14 (7)	1.49± 0.26 (9)
IL-1β (0.3 pmol)	3.20± 0.26 (13)	3.07± 0.28 (8)
IL-1β + SP	4.21± 0.35† (13)	3.19± 0.29 (8)
Carageenin (500 µ g)	6.64± 1.41*** (10)	2.13± 0.49†† (10)
Zymosan (20 µg)	6.55± 1.27*** (7)	5.33± 0.54*** (7)

The results show that, in naive skin, the NK_1 receptor does not seem to have a significant role in mediating neutrophil accumulation, either in the presence or absence of IL-1β. However, the addition of SP potentiates neutrophil accumulation induced by IL-1β. This is in keeping with findings by Ahluwalia et al., (1998) that the NK_1 receptor has a role in IL-1β-induced neutrophil accumulation in an inflammatory model (the murine air pouch). Furthermore, the present results suggest an NK_1 mediated mechanism in carrageenin but not zymosan mediated neutrophil accumulation.

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96P EFFECT OF LIPOCORTIN 1 ON LPS-INDUCED NEUTROPHIL ACCUMULATION IN THE RAT

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Administration of bacterial lipopolysaccharide (LPS) causes tissue injury and multiple organ failure. LPS-induced tissue injury may be due, in part, to infiltration of leucocytes. Annexin 1, also known as lipocortin 1 (LC1), can reduce leucocyte migration. However, its effectiveness as an anti-inflammatory agent against an inflammatory stimulus as strong as LPS is unknown. Thus, we have examined whether LC1 can modulate LPS induced neutrophil accumulation in the rat, as assessed by intravital microscopy and by myeloperoxidase (MPO) assay. Furthermore, dexamethasone (DEX) was also studied in these models for comparison.

Male Sprague-Dawley rats (170-220 g) received LPS (1 mg/kg i.p., serotype 0127:B8) or sterile saline (i.p.) for 6 h. In additional groups of animals, DEX (0.1-1 mg/kg, s.c.) was given 1 h prior to intraperitoneal administration of LPS (1 mg/kg) or, alternatively, LC1 (100 µg/kg, s.c.) given concomitantly with the LPS. After 6 h, animals were exsanguinated and sterile saline perfused through the animal to flush blood from the organs. Tissues (kidney, ileum, lung and liver) were then harvested, snap frozen and kept at –80 °C until ready for the MPO assay. The MPO assay was performed as previously described (Mullane *et al.*, 1985). Briefly, 50 mg of tissue was homogenized in 1 ml of 0.5 % hexa-decyl-trimethyl ammonium bromide in MOPS (10mM, pH 7) buffer. Samples were centrifuged (4000 g) for 20 min and then the supernatant was removed. The sample supernatant was allowed to react with a solution of tetra-methyl benzidine (1.6 mM) and 0.1 mM H_2O_2. The change in absorbance was measured with a spectrophotometer at 650 nm and compared to a standard curve of MPO

For intravital microscopy, the rat mesenteric preparation was performed as previously described (Tailor *et al.*, 1997). Rats (170-220 g) were fasted 24 hours prior to use. Animals were anaesthetised (Inactin 120 mg/kg i.p.) and the jugular vein and trachea cannulated. A midline laparotomy was performed, the mesenteric vascular bed exteriorised and placed on a viewing stage where it could by viewed by a light microscope with an immersion lens. Images were acquired by camera and captured on video cassette for subsequent off-line analysis. An optical doppler velocimeter was attached to the camera to record blood flow. The mesentery was superfused with gassed (5% CO_2/95 % N_2) bicarbonate-buffered solution (37 °C, pH 7.4) at a rate of 2 ml/min. A suitable mesenteric venule (25-40 µm diameter and less than 8 adherent cells) was found and preparation allowed to stabilise for 20 min before the mesentery was then superfused for 2 h with or without LPS (1 µg/ml) in the buffer. Animals were either treated with LC1 (100 µg/kg, i.v.) 20 min after the LPS superfusion was started, or DEX (1 mg/kg s.c.) 1 h prior to the LPS superfusion.

MPO activity in the lung (15.4 ± 2.2 U/g tissue), kidney (1.0 ± 0.1 U/g tissue) and ileum (3.8 ± 2.7 U/g tissue) was elevated after exposure to LPS for 6 hours (lung, 40.7 ± 6.1 U/g tissue; kidney, 1.4 ± 0.1 U/g tissue; ileum, 13.9 ± 2.0 U/g tissue. n=6-12). DEX attenuated the LPS-induced increase in MPO in the ileum (5.9 ± 2.4 U/g tissue) but had no effect on MPO in the lungs or kidneys. LC1 had no effect on the LPS-induced increase in MPO activity in any organ. Intravital microscopy of the rat mesentery was utilised to study the effects of LC1 and DEX on LPS-induced on leucocyte-endothelial interactions. Prior to the onset of the LPS superfusion, there was no difference between groups of animals in the amount of adherent and emigrated leucocytes (n=5-6). LPS superfusion induced adhesion of leucocytes to the endothelium such that after 120 min there were 15.3 ± 3.2 cells/100 µm in LPS treated rats venules (compared to 6.0 ± 1.1 in controls). LPS also caused an increase in leucocyte emigration (39.6 ± 9.2 cells/field LPS vs 8.0 ± 1.4 controls at 120 min, P<0.05). LC1 attenuated LPS-induced adhesion (8.5 ± 2.5 cells/100µm) and emigration of leucocytes (20.7 ± 5.3 cells/field) at 120 min. DEX also reduced the number of adherent (4.0 ± 11.4) and emigrated (6.0 ± 1.2) cells after 120 min. LPS had no effect on leucocyte rolling velocity and no change in blood flow between treated groups was observed throughout the experiment.

Thus, from these data, LC1 appears to reduce leucocyte adhesion and emigration induced by a short term (2 h), but not a longer (6 h) exposure to LPS. DEX was able to attenuate leucocyte adhesion and emigration in the mesenteric post capillary venules (2 h) and neutrophil accumulation in the ileum (6 h). However, DEX was unable to reduce MPO activity, a marker for neutrophils, in the lung and kidney at 6 h suggesting that the underlying mechanisms by which neutrophils accumulate in the ileum is different from the other organs.

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97P LIPOCORTIN 1 INHIBITS MYOCARDIAL INFARCT BY REDUCING LOCAL NEUTROPHIL INFILTRATION

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The glucocorticoid-inducible protein lipocortin 1 (LC1; also termed annexin I) and LC1-derived N-terminus peptides have been shown to reduce neutrophil trafficking in several models of acute inflammation, including an experimental model of ischemia-reperfusion in the rat mesenteric compartment (Cuzzocrea *et al.*, 1997). Here, we have assessed the effect of LC1 in a rat model of myocardial injury, with the scope to widen the potential applications for this protein and its mimetics.

Male Sprague-Dawley rats (200-220 g, Banton and Kingsman, UK) were anesthetised with inactin™ (120 mg kg^{-1}, i.p.) and subjected to the following procedure: cannulation of the left jugular vein (for drug administration); tracheotomy; cannulation of the right carotid artery for blood pressure measurement; thoracotomy and exposure of the heart; placement of a silk ligature around the left anterior descending coronary artery (LADCA) close to its origin. Rats were kept under artificial ventilation (Harvard, 50-1718, Edenbridge, Kent, UK) with room air at a rate of 54 strokes min^{-1}, a stroke volume of 1.0 to 1.5 ml 100 g^{-1} and a positive end expiratory pressure of 0.5 to 1 cm H_2O. After a 30 min stabilisation period, LADCA was occluded for 25 min, and then a 2 h reperfusion period allowed. The LADCA was then re-occluded, and Evans blue dye (1 ml of 2% wv^{-1}) injected i.v. to stain the area at risk (AR). The AR was calculated and expressed as per cent of the total left ventricular weight. The extent of tissue necrosis was then assessed by staining with *p*-nitro-blue tetrazolium (0.5 mg ml^{-1}, 20 min at 37°C). The infarct size (IS), necrotic tissue, as a function of the AR mass, and the IS as a function of the total left ventricular weight (IS/LV) were calculated. In selected experiments, the staining procedure to determine IS, and injured tissues were collected to measure myeloperoxidase activity (MPO) (Cuzzocrea *et al.*, 1997), and the levels of TNF-α or MIP-1α using specific ELISAs (R&D Systems, Abingdon, UK). Blood aliquots (0.5 ml) were also taken from selected animals, and CD11b expression on circulating neutrophils monitored by flow cytometry as described (Harris *et al.*, 1995). The following drugs were given i.v. at the end of the ischemic period: dexamethasone (DEX, 0.1 mg/kg); annexin V (50 μg per rat); LC1 (0.5-50 μg) and boiled LC1. Finally, some animals were pre-treated with an anti-LC1 rabbit serum (LCS4) or a normal rabbit serum (RbS; 0.2 ml in both cases). Statistical differences were assessed by ANOVA followed by Bonferroni test.

In control animals, occlusion and reopening of LADCA produced a 2 h an IS/AR ratio of 58 ± 4% (n=6). This figure was reduced by LC1 treatment (42 ± 2% and 32 ± 4% for the 5 and 50 μg doses, respectively, n=6, $P<0.05$ vs. control) as well as in the rats treated with DEX (42 ± 4%, n=6, $P<0.05$), but not in those receiving annexin V or the denatured preparation of LC1 (not shown). A sharp increase in MPO activity was measured in the tissue myocardium 2 h post-reperfusion (11.6 ± 0.8 mU mg^{-1}), with only 1.4 ± 0.2 mU mg^{-1} being measured in sham operated rats. LC1 reduced MPO values with a significant effects again at 5 and 50 μg doses (38% and 58% of inhibition, respectively, n=6, $P<0.05$), whereas denatured LC1 (50 μg) was inactive. Local formation of TNF-α or MIP-1α was also suppressed by LC1 (e.g. TNF-α: from 6.2 ± 0.8 pg mg^{-1} in vehicle treated rats to 2.4 ± 0.1 in LC1 50 μg, n=6, $P<0.05$) and DEX. In contrast neither treatment was effective in modifying the peripheral blood neutrophil counts and the expression of CD11b on the neutrophil cell surface: both parameters were significantly increased at the end of the ischemic period, and much more markedly augmented after 2 h reperfusion, however, no difference among the experimental groups was observed (data not shown). Finally, the protective action afforded by DEX on the myocardial infarct was attenuated in rats immunised with the anti-LC1 serum but not in those treated with the non-immune rabbit serum. An IS/AR and IS/LV ratios of 55 ± 3% and 45 ± 6% were measured in control animals, down to 41 ± 2% and 30 ± 4% in DEX + RbS group (n=4, $P<0.05$), whereas no significance was reached in the DEX + LCS4 group (52 ± 2% for IS/AR and 54 ± 6% in IS/LV, n=4).

In conclusion, we propose that LC1 and its mimetics may have beneficial effects in the treatment of pathologies associated to ischemia/reperfusion, including those of the myocardium. It remains to be assessed if tissue protection will also be observed following a prophylactic administration.

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98P NCX-1015, A NOVEL DERIVATIVE OF PREDNISOLONE WITH ENHANCED ANTI-INFLAMMATORY ACTIVITY

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The synthesis of nitroso-butyl esters of anti-inflammatory molecules is an innovative strategy to design novel molecules, which has been successfully employed for non-steroidal anti-inflammatory drugs, including nitro-fluribuprofen and nitro-aspirin. These compounds slowly release nitric oxide (NO) in a biological microenvironment and this confers biological activities not shared by the non-nitrosylated molecules (Wallace *et al.*, 1995). Here we report the initial characterisation of a novel glucocorticoid hormone with a potent anti-inflammatory profile, which is prednisolone 21-[(4'-nitrooxy-methyl)benzoate] (NCX- 1015).

Human platelet rich plasma (PRP, 1 ml) was incubated with 100 μM NCX-1015, prednisolone or sodium nitroprusside (SNP) at 37°C. Aliquots (0.2 ml) were collected at different times, and nitrite levels measured with the nitrite reductase assay, as described (Verdon *et al.*, 1995). In a second set of experiments, NCX-1015 or prednisolone (5-500 μM) were incubated with PRP for 45 min, and then nitrite was measured in cell-free plasma whereas cGMP accumulation was measured in platelets using a specific immunoassay (Amersham International, UK). *In vivo*, Swiss Albino male mice (28-32g) were injected i.p. with peanut oil (vehicle alone), or prednisolone or NCX-1015 (0.5, 5, 10 mg/kg prednisolone equivalent dose) 1 h prior to induction of experimental peritonitis with zymosan A (1 mg in 0.5 ml PBS). Peritoneal cavities were washed 4 h later with 3 ml of PBS containing 3 mM EDTA and 25 Uml^{-1} heparin. Polymorphonuclear cells (PMN) were counted in a Neubauer haematocytometer and the levels of soluble mediators, namely the CXC chemokine KC, prostaglandin E_2 (PGE_2), nitrite and interleukin-1β (IL-1β), were measured in cell-free lavage fluids. The expression of inducible NO synthase (iNOS) in inflammatory cells was monitored by Western blotting using a rabbit anti-mouse iNOS (Santa Cruz Biotechnology, CA). In the intravital microscopy study, mice (~15g) received peanut oil, prednisolone (5 mg/kg) or NCX-1015 (0.5 mg/kg) 1h prior to induction of inflammation with IL-1β (5 ng i.p.). The extent of leucocyte rolling, adhesion and emigration in the mesenteric microvasculature was determined 2 h later, using a Zeiss Axioskop microscope equipped with a x40 water immersion objective lens, and a video camera (Hitachi) connected to a colour monitor (Sony). At least 1 to 3 randomly selected post-capillary venules (> 100μm in length) were analysed for each mouse. Data were analysed by ANOVA followed by the Bonferroni test taking a P value <0.05 as significant.

Incubation of NCX-1015 (100 μM) with PRP resulted in a time- and concentration dependent accumulation of nitrite, peaking at 60 min (30.6 μM), and with values significant above prednisolone at > 50μM (21.6 ± 0.5 vs. 11.4 ± 0.96 μM nitrite, n=3). At an equimolar concentration, SNP produced nitrite accumulation in a more rapid manner (peak at 10 min). This profile was mirrored by cGMP accumulation in the platelets: 8.31 ± 1.4, 15.6 ± 5.8 and 29.7 ± 6.7 nM cGMP for vehicle, prednisolone and NCX-1015, respectively ($P<0.05$ for NCX-1015 vs. vehicle). In experimental peritonitis, NCX-1015 was more potent than prednisolone in reducing PMN accumulation with approxiamte ED_{50} of 2 and 9.5 mg/kg, respectively. NCX-1015, but not prednisolone, reduced KC levels in the exudate with an ED_{50} of 2 mg/kg. Nitrite levels in the lavage fluids were reduced below the detection limit of the assay (1 μM) by all doses of NCX-1015, whereas only the highest dose of prednisolone had an inhibitory effect when compared to the vehicle group: 3.4 ± 0.2 and 10 ± 0.7 μM, respectively (n=6, $P<0.05$). Accordingly, NXC-1015 produced a dramatic inhibition of iNOS expression in inflammatory cell pellets. PGE_2 and IL-1β levels were significantly reduced by NCX-1015 and prednisolone to the same extent. In the intravital microscopy experiments, NCX-1015 (0.5 mg/kg) and prednisolone (5 mg/kg) did not affect IL-1β-induced cell rolling on the post-capillary endothelium, but NCX-1015 significantly ($P<0.05$) reduced cell adhesion and emigration by ~50% when compared to control values (4.26 ± 0.58 adherent cells and 5.24 ± 0.7 emigrated cells, n=5).

In conclusion, NCX-1015 was more potent than prednisolone in models of acute inflammation with particular efficacy on the process of cell extravasation. This was due to an inhibition of the initial interaction between circulating white blood cells and the post-capillary endothelium, as seen in the mesenteric microcirculation, likely related, at least in part, to a pronounced inhibition on the formation of pro-inflammatory cytokines such as KC. Future studies will confirm if NCX-1015 will be effective in the clinic at lower doses than those used for the parent molecule.

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99P CYCLOOXYGENASE SELECTIVITY OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs) IN HUMANS: *EX VIVO* EVALUATION

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We have recently described an assay capable of assessing *ex vivo* the activity and selectivity on cyclooxygenase (COX) -1 and -2 of NSAIDs administered *in vivo* to rats (Giuliano & Warner, 1999). Here we have extended our experiments to use plasma from humans.

Healthy male volunteers aged 18-40 were given orally one of the following drugs (mg) for four days: etodolac (200 b.i.d.), meloxicam (7.5 q.d.), nimesulide (100 b.i.d), nabumetone (500 b.i.d.) and naproxen (500 b.i.d.). All subjects also received lansoprazole 30 mg q.d. in order to minimize gastric disturbances. Blood samples were collected on day 0 prior to administration of the drug (basal), on day 4 immediately before the last dose (time 0) and then at 0.5, 1, 2, 4, 6, 8, and 24 h after. The samples were centrifuged and the plasma snap frozen. To assay NSAID activity in the plasma, 50 µl samples were added to 50 µl of culture medium bathing either, a) IL-1ß-treated A549 cells (COX-2 system) or, b) human washed platelets (COX-1 system). After 30 min calcium ionophore A23187 (50 µM) was added; 15 min later medium was removed and TXB_2 and PGE_2 levels determined by RIA as measures of, respectively, COX-1 and COX-2 activity.

Plasma from etodolac-treated subjects demonstrated slight selectivity towards inhibition of COX-2; an effect that was more prominent in plasma taken from subjects receiving meloxicam or nimesulide (Table 1). Plasma from nabumetone-treated subjects showed relatively less selectivity, while plasma taken from subjects receiving naproxen was markedly more active at inhibiting COX-1 than COX-2. Etodolac showed a peak of activity between 1 and 2 h after administration that quickly regressed back to control levels. Conversely the plasma from meloxicam-treated volunteers produced a more stable inhibition during the sampling period. The activity of nabumetone in the plasma samples was similarly stable, but weaker than the comparators.

The data we describe here are consistent with our previous *in vitro* observations (Warner *et al.*, 1999). Etodolac, meloxicam and nimesulide showed preferential inhibition of COX-2, while naproxen was selective towards COX-1. These relative activities are associated with assessments of the relative potential of NSAIDs to produce deleterious GI side effects (see Mitchell and Warner, 1999).

In conclusion, we have demonstrated that this assay can be used to assess *ex vivo* the activity of NSAIDs administered *in vivo* to humans. This assay may, therefore, represent a useful complement for existing systems as it allows simple and direct measure of the relative activity against COX-1 and COX-2 of NSAIDs consumed by human volunteers. It is to be hoped that data from such systems will aid in our understanding of the relationships between NSAIDs differential inhibition of COX-1 and COX-2 and their reported gastrointestinal toxicities.

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Table 1. Relative activities of COX-1 and COX-2 exposed to plasma from subjects consuming test compounds. Activity in basal plasma = 100%. Results are expressed as mean ± s.e.m.

Drug / Time	etodolac (n = 4)		meloxicam (n = 4)		nabumetone (n =4)		naproxen (n = 9)		nimesulide (n = 3)	
	COX-1	COX-2	COX-1	COX-2	COX-1	COX-2	COX-1	COX-2	COX-1	COX-2
0	90±12	87±10	94±7	87±13	74±10	79±13	22±8	55±6	118±11	44±4
4	102±12	76±13	76±7	42±8	77±9	59±6	5±2	41±6	102±10	23±1
24	95±5	93±3	86±4	59±10	92±7	71±11	28±12	52±5	108±10	82±6

100P CONDITIONED MEDIUM FROM HUMAN STIMULATED VENOUS SMOOTH MUSCLE CELLS INHIBITS NEUTROPHIL APOPTOSIS: RELEVANCE OF ENDOGENOUSLY PRODUCED GM-CSF AND G-CSF

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Neutrophil apoptosis is inhibited by cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF). We have recently shown that human venous smooth muscle cells (SMCs) can be induced to release GM-CSF and G-CSF which are influenced both negatively (GM-CSF) and positively (G-CSF) by the co-expression of cyclo-oxygenase-2 (COX-2) (Stanford *et al.* 1999). In this study we compared the relative abilities of GM-CSF and G-CSF to inhibit neutrophil apoptosis. Furthermore we investigated the effects of conditioned medium taken from venous SMCs, treated with IL-1β and/or NSAIDs, on neutrophil apoptosis.

Samples of saphenous vein (SV) were cultured as described previously (Bishop-Bailey *et al.*, 1998). Following explantation, cultured human venous SMCs were plated onto 96 well plates. Cells were then treated accordingly for 24 hours and medium removed. GM-CSF and G-CSF levels were measured by ELISA and effects on neutrophil apoptosis determined. Neutrophils, isolated from healthy adults were treated with culture medium directly, or with conditioned medium removed from venous SMCs subjected to various treatments, for 6 hours. In separate experiment neutrophils were treated with rhGM-CSF or rhG-CSF (0.01-10ng/ml). Neutrophils were then lysed and apoptosis assessed using a commercial ELISA (Boehringer Mannheim) to determine levels of histone-associated mono- and oligonucleosomes as described previously (Fortenberry *et al.*, 1998).

GM-CSF was a more potent inhibitor of neutrophil apoptosis than G-CSF (1a). Basal apoptosis was not significantly affected (student t-test) by conditioned culture medium from unstimulated SMCs (OD at 405nm: 1.11±0.07, n=16 vs. 1.38±0.12, n=6). Furthermore neither IL-1β, indomethacin nor the selective COX-2 inhibitor, DFU (Riendeau *et al.*, 1997), had any direct effect on neutrophil apoptosis (n=6). Conditioned medium taken from venous cells treated with DFU or indomethacin alone contained undetectable levels of GM/G-CSF but had a small effect on neutrophil apoptosis (1b). Conditioned medium taken from venous SMCs treated with IL-1β contained elevated levels of GM-CSF (0.08±0.01ng/ml) and G-CSF (4.7±1.2ng/ml) and inhibited neutrophil apoptosis. In the presence of IL-1β both factors were effected by treatment with DFU (GM-CSF: 0.4±0.04ng/ml; G-CSF: 1.7±0.4ng/ml) or indomethacin (GM-CSF: 0.4±0.03ng/ml; G-CSF: 1.4±0.2ng/ml), however no appreciable difference in neutrophil survival was seen by medium from cells co-treated with these drugs.

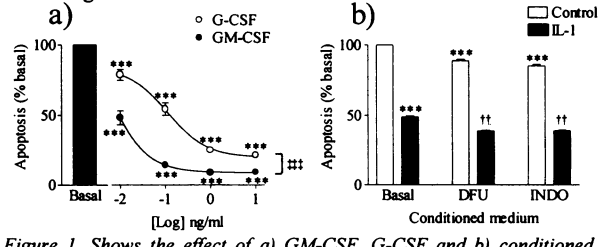

*Figure 1. Shows the effect of a) GM-CSF, G-CSF and b) conditioned medium (diluted 1:100) taken from SMCs treated with indomethacin (INDO: 10µM), DFU (10µM) ± IL-1β (1ng/ml). ***P<0.001 One-sample t-test vs. basal: ‡‡‡P<0.001 two-way ANOVA: ††P<0.01 one-way ANOVA. vs. basal+IL-1β. Molecular wts, GM-CSF 14-35kDa; G-CSF 21kDa.*

Here we show that GM-CSF is a more potent inhibitor of neutrophil apoptosis than G-CSF. Conditioned medium taken from IL-1β-stimulated venous SMCs, containing elevated levels of GM-CSF and G-CSF, also inhibited neutrophil apoptosis. When IL-1β-stimulated cells were pre-treated with NSAIDs, despite alterations in CSFs levels, the observed effect was unaltered from that seen with conditioned medium from SMCs treated with NSAIDs alone. We conclude that the the increase in GM-CSF, and decrease in G-CSF, levels seen in conditioned medium from IL-1β-stimulated venous SMCs pretreated with NSAIDs counterbalance each other with respect to their effect on neutrophil apoptosis.

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101P TOXINS ASSOCIATED WITH GRAM NEGATIVE AND GRAM POSITIVE BACTERIA STIMULATE IL-8 RELEASE FROM HUMAN BLOOD: IMPLICATIONS FOR ARDS

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Neutrophil recruitment and activation play crucial roles in many disease processes affecting the lung such as the acute respiratory distress syndrome (ARDS). Interleukin (IL)-8 is a powerful chemotactic agent for neutrophils and the level in bronchalveolar lavage fluid has been shown to predict the development or ARDS in 'at risk' patients (Donnelly *et al.* 1993). Recently we have shown that vascular endothelial and smooth muscle cells are capable of releasing IL-8 when stimulated with the cytokines IL-1β and TNFα (Jordan *et al.* 1999) and other investigators have shown that the gram negative toxin lipopolysaccharide (LPS) stimulates whole blood to release IL-8 (DeForge *et al.* 1993). Thus, the purpose of this study was to investigate the effects of these stimuli as well as the gram positive toxin lipoteichoic acid (LTA) on IL-8 release by human whole blood both in healthy volunteers and patients with ARDS.

Whole blood was obtained from patients with ARDS on the intensive care unit at the Royal Brompton Hospital and healthy volunteers, immediately placed in heparinised tubes and transferred into a 96 well plate within 30 minutes, with 100µl of blood per well. Inflammatory mediators made up in phosphate buffered saline were then added to the wells and the plates were incubated at 37°C. After 24 hours the plates were centrifuged 200xg for 5 minutes and the plasma was removed and frozen at -80°C. After thawing IL-8 levels were measured by ELISA (R&D Systems). All data are n=9 from 3 experiments.

When stimulated with LPS or LTA (0.01 to 100µg ml^{-1}) IL-8 was released by whole blood in a concentration dependant manner. Treatment with IL-1β or TNFα (0.001-100ng ml^{-1}), however, failed to significantly increase the release of IL-8.

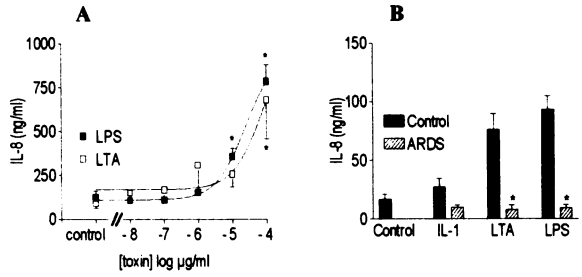

Figure 1: (A) The effect of LPS and LTA (0.01 to 100µg ml^{-1}) on the release of IL-8 by whole blood from healthy volunteers. *P<0.05 vs control. (B) The effect of ARDS on the release of IL-8 by whole blood treated with IL-1β (10ng ml^{-1}), LTA and LPS (10µg ml^{-1}). *P<0.05 vs blood from healthy volunteers. (one-way ANOVA followed by Dunn's test for multiple comparisons).

The findings here that both LPS and LTA effectively stimulate whole blood to release IL-8, implicates this chemokine in the pathology of both gram positive and gram negative infection. Furthermore, the paradoxical finding that blood from ARDS patients releases less IL-8 than blood from healthy volunteers, suggests differences in the functioning of circulating inflammatory cells with those recruited/resident to the lung.

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102P INVOLVEMENT OF p38 MITOGEN-ACTIVATED PROTEIN KINASE IN INTERLEUKIN-8 RELEASE FROM HUMAN NEUTROPHILS

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Generation of specific neutrophil chemokines, in particular interleukin-8 (IL-8) plays a key role in neutrophil recruitment to sites of inflammation. Neutrophils are an important source of IL-8, which is released in response to inflammatory mediators such as bacterial products and cytokines. However, little is known about the mechanisms by which these mediators cause the release of IL-8 from neutrophils. In this study we compared the effects of lipopolysaccharide (LPS) and the cytokines, tumour necrosis factor α (TNFα) and interleukin-1β (IL-1) on IL-8 release from human neutrophils. We also used a selective p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1H-imidazole, HCL] (Lee *et al*, 1994), to investigate the involvement of p38 MAPK in IL-8 release.

Human neutrophils were isolated from peripheral blood of healthy adult volunteers by discontinuous plasma-Percoll gradient centrifugation (Burke-Gaffney & Hellewell, 1996). Neutrophils were resuspended at 10^7 cells per ml in RPMI culture medium containing foetal calf serum (10%), penicillin (200U ml^{-1}) and streptomycin (200µg ml^{-1}) and dispensed into flat-bottomed 96-well plates, at 100µl per well. Neutrophils were incubated for 24h (37°C, 5% CO_2) with 0.01 to 10µg ml^{-1} LPS (*Escherichia coli*, 055:B5) and 0.01 to 10ng ml^{-1} of TNFα or IL-1. In experiments with SB203580, neutrophils were preincubated with 0.1 to 3µM of SB203580 for 1h at 37°C before addition of inflammatory mediators. Plates were centrifuged at 200g for 8 min and supernatants were collected and assayed for IL-8 using a specific ELISA (R & D Systems, Oxford). Results show mean data from n separate donors and are expressed as mean ± s.e mean of IL-8 release in pg ml^{-1}. Statistical analysis was carried out using one-way analysis of variance and Dunnett's multiple comparison post test, which compares all values with a control.

Incubation of neutrophils with LPS or TNFα, but not IL-1, caused a concentration-dependent increase in IL-8 release, therefore, we investigated the effects of SB203580 on LPS and TNFα-stimulated release. IL-8 release from neutrophils stimulated with LPS (10µg ml^{-1}) was significantly increased from basal levels of 82 ± 16 pg ml^{-1} to 6223 ± 927 pg ml^{-1} (n=6, P<0.01) and TNFα (10ng ml^{-1}) significantly increased release from 90 ± 10 pg ml^{-1} to 1157 ± 115pg ml^{-1} (n=3, P<0.01). SB203580 caused a concentration-dependent inhibition of IL-8 release (Figure 1) and a concentration of 3µM reduced LPS-induced IL-8 release to 32 ± 7% and TNFα-induced release to 13 ± 3%, of respective stimulated levels.

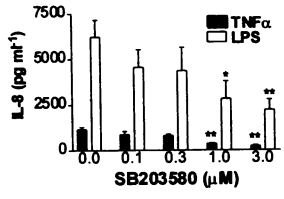

Figure 1 IL-8 release from LPS- and TNFα-stimulated neutrophils in the presence of SB203580. *P<0.05 and **P<0.01, represent significant differences compared to effects without SB203580.

These results suggest an important role for p38 MAPK in IL-8 release from human neutrophils.

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