153P EVIDENCE AGAINST MDMA-INDUCED HYPERTHERMIA BEING MEDIATED BY 5-HT RELEASE IN THE BRAIN

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A major clinical problem that can occur following recreational ingestion of 3,4-methylenedioxymethamphatamine (MDMA; "ecstasy") is hyperthermia. This induces other clinical problems and can lead to death (see Green *et al.*, 1995). Administration of MDMA to rats also produces acute hyperthermia, but the specific pharmacology of the response has been little studied. It has been assumed that the hyperthermia results from the MDMA-induced acute release of 5-HT from nerve endings (Schmidt *et al.*, 1987), since Grahame-Smith (1971) observed hyperthermia in rats when cerebral 5-HT release was increased by injection of tranylcypromine and L-tryptophan and Nash *et al.* (1988) reported that 5-HT_2 antagonists blocked MDMA-induced hyperthermia. We have now investigated the pharmacology of MDMA-induced hyperthermia in rats.

Male Dark Agouti rats (160-220 g) were used in all studies. In all experiments except the microdialysis study (see below), rats were injected i.p. with the investigative drug 10 min before MDMA (12.5 mg kg^{-1} i.p.). Temperature was measured by use of a rectal probe and digital readout thermocouple. In the microdialysis experiments, a dialysis probe was implanted in the right hippocampus as described previously (Colado *et al.*, 1997). The concentration of 5-HT in the perfusate was measured by h.p.l.c. (Colado *et al.*, 1997).

The non-selective 5-$HT_{1/2}$ antagonist methysergide (5 or 10 mg kg^{-1}) 10 min prior to MDMA had no effect on MDMA-induced hyperthermia. The 5-HT uptake inhibitor zimeldine (10 or 20 mg kg^{-1}) was also without effect. The 5-HT uptake inhibitor fluoxetine (10 mg kg^{-1}) 5 min prior to and 55 min post MDMA (15 mg kg^{-1}) administration almost totally abolished the acute release of 5-HT as measured by the hippocampal microdialysis probe (Fig 1a). However, the hyperthermia was unaltered (Fig 1b). Injection of the dopamine D1 antagonist SCH23390 (0.3-2.0 mg kg^{-1}) 10 min before MDMA produced a dose dependent inhibition of hyperthermia (at 2.0 mg kg^{-1} no MDMA-induced hyperthermia was seen: $F(1, 27) = 33.80$, $p < 0.0001$). Pretreatment with the D2 selective antagonist remoxipride (10 mg kg^{-1}) was without effect.

These data suggest that acute release of 5-HT in the hippocampus and therefore by implication other regions is not responsible for MDMA-induced hyperthermia. They further suggest that the acute dopamine release which also occurs after MDMA (Colado *et al.*, 1999) may play a role in inducing hyperthermia by an action at dopamine D1 receptors.

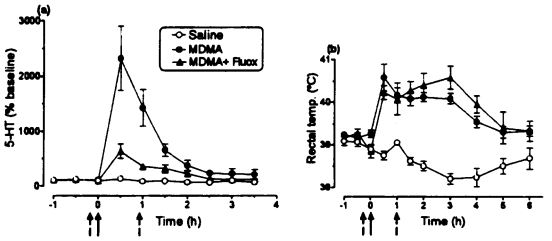

Fig. 1. (a) Increase in extracellular 5-HT in hippocampus and (b) rectal temp. in rats following saline, MDMA (injected at solid arrow) and fluoxetine (at dashed arrows). (a) MDMA different from MDMA +fluoxetine [$F(1,13)=4.8$, $p<0.05$], (b) MDMA different from saline [$F(1,9)=74,27$, $p<0.001$] but not MDMA+fluoxetine.

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154P CO-ADMINISTRATION OF D-AMPHETAMINE ALTERS THE ACUTE AND LONG-TERM EFFECTS OF 3,4-METHYLENE-DIOXYMETHAMPHETAMINE IN RATS

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The amphetamine derivative 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') is an illicit drug. In rodents, MDMA produces acute behavioural changes, including hyperlocomotion and hyperthermia, and long-term decreases in markers of forebrain serotonin (5-HT) function (White et al., 1996). This study aimed to assess the effects of co-administration of MDMA (20 mg/kg) with D-amphetamine (AMPH, 1.0 mg/kg) on the acute hyperlocomotor responses and long-term reductions in forebrain 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA).

Male Wistar rats (225 –250g) were used, and each treatment group (n = 10) received two i.p. injections of saline or AMPH, followed by saline or MDMA. The hyperlocomotor effects of treatment were assessed using an automated homecage activity (HCA) monitor. At 14 days post-treatment, rat brains were dissected and forebrain amine levels were measured using h.p.l.c.

MDMA produced a very significant increase in rat HCA counts ($p < 0.001$, repeated measures ANOVA, Bonferroni's multiple comparison test), while AMPH alone did not cause significant changes in HCA. Co-treatment with MDMA and AMPH increased rat HCA ($p < 0.001$), but this effect was significantly lower than MDMA alone ($p < 0.001$) (Fig. 1). MDMA caused significant decreases in frontal cortex levels of 5-HT and 5-HIAA at 14 days post-treatment (Table 1). AMPH alone did not alter levels of 5-HT and 5-HIAA, but co-administration of AMPH with MDMA produced significant reductions in both frontal cortex and striatal levels of these indoleamines. None of the treatments produced any changes in concentrations of dopamine or noradrenaline.

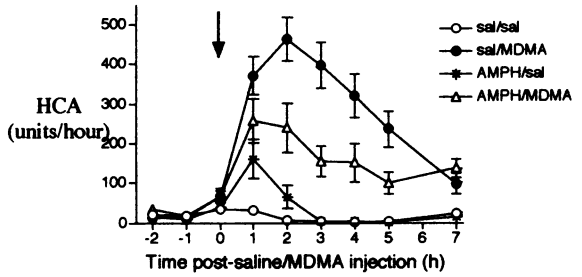

Fig.1: The effect of 1.0 mg/kg amphetamine on 20 mg/kg MDMA-induced acute increases in rat homecage activity (HCA).

Table 1

Treatment	Frontal cortex		Striatum	
	5-HT	5-HIAA	5-HT	5-HIAA
sal/sal	518 ± 16	191 ± 9	974 ± 70	793 ± 52
sal/MDMA	423 ± 9**	159 ± 8**	815 ± 65	699 ± 55
AMPH/sal	498 ± 16	175 ± 5	883 ± 78	760 ± 67
AMPH/MDMA	392 ± 18**	148 ± 9**	705 ± 77*	618 ± 60*

Mean ± SEM (n = 10), *p < 0.05, **p < 0.01 Vs. sal/sal group (2-way ANOVA, Fisher's LSD test).

These results suggest that the neurotoxic actions of MDMA is enhanced by AMPH, and may have implications for the risk of serotonergic neurotoxicity in human Ecstasy users who are co-using MDMA with amphetamine.

White S.R., Obradovic T., Imel K. M. et al. (1996) Prog. Neurobiol. 49: 455 –479.
This research was supported by Enterprise Ireland.

155P MODULATION OF GLYCINE RECEPTORS BY DIHYDROPYRIDINES IN RAT SPINAL NEURONES

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Glycine receptors are the main inhibitory receptors of the adult spinal cord, while dihydropyridines (DHPs) are widely used (both in clinic and research) for their L-type Ca^{2+} channel blocking properties. In the commonly used micromolar range, DHPs are not quite selective. We wondered whether they might affect glycine receptors.

Glycine responses were recorded from embryonic rat spinal cord cultures, using whole-cell voltage-clamp (at -30 mV, E_{Cl} = -57 mV) and fast perfusion (Chesnoy-Marchais, 1999). Usually, the DHP was added between and during glycine applications (of 2 s duration). All solutions contained the same amount of solvent (usually 14 mM DMSO, ≤ 17 mM ethanol when using BayK 8644). The extracellular solution was Ca^{2+}-free to eliminate possible effects *via* Ca^{2+} channel modulation.

At a concentration as low as 1 μM, nifedipine reduced the responses induced by 100 μM glycine. The blockade increased with time within each glycine application and reached 49.9 ± 1.4 % (3) (mean ± SEM (n)) at the end of the applications. The percentages of inhibition induced by 5 and 25 μM nifedipine were 69.5 ± 5.7 % (6) and 95.6 ± 0.9 % (3), respectively. This blocking effect was smaller for lower glycine concentrations, of only 29.2 ± 5.4 % (7) for 5 μM nifedipine and 15 μM glycine. A similar effect was observed with other DHPs, including a Ca^{2+} channel agonist, (*S*)-BayK 8644. With 100 μM glycine, the percentages of inhibition produced by (*S*)-BayK 8644, nitrendipine and nicardipine (5 μM) were 79.6 ± 1.8 % (3), 83.6 ± 3.7 % (3), and 96.6 ± 2.6 (4), respectively. In the same neurones, nicardipine (5 μM) also reduced the $GABA_A$ responses induced by 5 and 20 μM GABA, by 28.1 ± 5.3 % (4) and 50.3 ± 3.1 % (4), respectively. The time- and agonist concentration-dependence of the inhibition of glycine responses by DHPs, along with the similar modulation of $GABA_A$ responses by nicardipine, suggest that DHPs act as channel blockers.

In addition to their blocking effect on glycine responses, nitrendipine and nicardipine also potentiated peak responses to low glycine concentrations. For 15 μM glycine, the increases induced by 1 and 5 μM nitrendipine were of 68 ± 24 % (4) and 212 ± 34 % (5), respectively. In contrast, for 100 μM glycine, the effect of nitrendipine was already inhibitory at the peak of glycine responses (inhibition of 34.7 ± 3.5 % (3) by 5 μM nitrendipine, getting stronger in the continued presence of glycine). The potentiation of peak responses to low glycine concentrations by nitrendipine persisted in presence of external Zn^{2+}, at a concentration that is saturating for its own potentiating effect (10 μM). Thus, the potentiation induced by the DHP is not due to Zn^{2+} contamination. Both effects of extracellular nitrendipine persisted when the drug was included in the recording pipette, eliminating the involvement of intracellular effects.

In conclusion, DHPs modulate glycine responses independently of Ca^{2+} channels modulations, in a time- and glycine concentration-dependent manner. These modulations might contribute to some of the clinical or experimental effects of DHPs, such as their effects on glycine receptors clustering at developing glycinergic synapses (Kirsch & Betz, 1998) and their neuroprotective and anticonvulsant effects.

Chesnoy-Marchais, D (1999) *Br J Pharmacol* 126, 801-9
Kirsch, J & Betz, H (1998) *Nature* 392, 717-720.

156P GLUTAMATE TRANSPORTERS AND MODULATION OF PRIMARY AFFERENT-MEDIATED SYNAPTIC TRANSMISSION IN SPINAL DORSAL HORN NEURONES FROM NORMAL AND ARTHRITIC RATS *IN VITRO*

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Glutamate transporters are critical for maintenance of a low extracellular level of glutamate at central synapses to avoid neurotoxic damage. In hippocampus, novel metabotropic glutamate receptors (mGluRs) are recruited following elevations in synaptic glutamate concentration (Scanziani *et al.*, 1997). In this study, we have compared the effects of L-trans-pyrrolidine-2,4-dicarboxylic acid (L-PDC), a glutamate uptake inhibitor, on primary afferent-mediated synaptic transmission in dorsal horn neurones *in vitro* from normal rats and those with a Freunds Complete Adjuvant (FCA)-induced hind paw monoarthritis. We also tested in normal and arthritic rats whether the selective group II/III mGluR antagonist RS-α-cyclopropyl-4-phosphonophenylglycine (CPPG) could affect the response to L-PDC. The actions of the selective group III agonist, L-2-amino-4-phosphonobutyrate (L-AP4) were examined in both rat groups.

Monoarthritis was induced in Wistar rats (5-6 day old) by injecting FCA (1 $mg.kg^{-1}$, 30 μl) into the plantar surface of one hind paw under halothane anaesthesia. Mechanical withdrawal thresholds and paw oedema were measured. Normal or arthritic rats (12-15 day old) were anaesthetised (urethane, 2 $g.kg^{-1}$) and spinal cords removed. Ipsilateral hemicords were used from the FCA-injected animals. Intracellular recordings were made from deep dorsal horn neurones. A lumbar dorsal root was stimulated at A- and C-fibre intensity (>250 μA, >250 μV) to evoke a dorsal root-excitatory post-synaptic potential (DR-EPSP).

After FCA injection, there was a significant reduction in mechanical threshold (33 ± 2 g versus 8 ± 1 g; uninjected paw versus FCA-injected) and an increase in paw swelling (4 ± 0.1 mm versus 2.8 ± 0.1 mm) ($P < 0.01$, paired t-test). In normal and arthritic rats, L-PDC (1 mM, 20 min, n = 6-10) reduced (paired t-test, $P < 0.05$; data expressed as mean ± s.e.m. % inhibition) the DR-EPSP amplitude (normal: 59 ± 12 %; arthritic: 84 ± 4 %) and duration (normal: 61 ± 10 %; arthritic: 91 ± 3 %). The L-PDC-induced reductions in DR-EPSP were significantly greater in arthritic rats ($P < 0.01$, un-paired t-test). CPPG (100 μM, 20 min, n = 6-10) reduced the L-PDC-induced inhibition of DR-EPSP amplitude (7 ± 10 %, $P < 0.01$, un-paired t-test) and duration (23 ± 6 %, $P < 0.01$, un-paired t-test) in normal but not arthritic rats (amplitude: 68 ± 9 %; duration: 81 ± 9; $P > 0.05$, un-paired t-test). L-AP4 (30 μM, 20 min, n = 6-10) significantly reduced (paired t-test, $P < 0.05$) DR-EPSP amplitude (normal: 23 ± 6 %; arthritic: 31 ± 3 %) and duration (normal: 35 ± 4 %; arthritic: 29 ± 3 %) with no significant difference between normal and arthritic rats ($P > 0.05$, un-paired t-test).

These data suggest that modulation of glutamate transporter activity impacts differentially on primary afferent-mediated spinal neurotransmission in normal versus FCA rats. The role of specific mGluR subtypes in mediating these actions requires further clarification. The data have implications for the mechanisms underlying chronic pain pathology.

(Supported by the MRC. Technical assistance by J. Daniel.)
Scanziani, M. et al. (1997) *Nature;* **385**: 630-634.

157P PROTEIN KINASE A INVOLVEMENT IN NEUROKININ- AND NOCICEPTIVE AFFERENT- INDUCED SLOW DEPOLARISATIONS AND FOS EXPRESSION IN RAT SPINAL CORD DORSAL HORN *IN VITRO*

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Synaptic activity has the capacity to alter gene expression in postsynaptic target neurones. In the spinal dorsal horn after nociceptive stimuli, the most rapid event is an eruptive expression of the immediate early gene (IEG) c-*fos*. The underlying stimulation-transcription pathway for IEG expression is unknown. In this *in vitro* study, we have examined the role of Protein Kinase A (PKA)-dependent signalling pathways in neurokinin-1 receptor (NK1R)- and nociceptive afferent-induced slow depolarizations and Fos expression in dorsal horn neurones.

Intracellular recordings were made from dorsal horn neurones in spinal cords taken from young Wistar rats after fatal anaesthesia (urethane, 2 g.kg $^{-1}$, i.p.). Slow potentials were elicited by either suprathreshold A- and C- afferent dorsal root single shock stimuli (> 250 μs, > 250 μA) or by superfusion of the selective NK1R agonist, [Sar^9,Met(O$_2$)^{11}]-Substance P (2 μM). Fos expression was induced by prolonged NK1R agonist superfusion (10 μM, 1 hr) or nociceptive afferent stimulation (> 250 μs, > 250 μA, 2 Hz for 1 hr). In some protocols, spinal cords were pre-treated with the PKA inhibitor Rp-cAMP (100 μM, 30-60 min). Fos was visualized using a selective antisera (kindly supplied by Prof. S. Hunt, University College London) and a standard avidin-biotin-peroxidase complex technique.

Stimulation of dorsal roots typically generated a polysynaptic potential characterized by a slowly decaying 'tail' EPSP with a mean duration of 13.71 ±. 2.73 s and a peak amplitude of 12.16 ± 2.18 mV (n=5). After treatment of the spinal cord with Rp-cAMP (100 μM, 30 min), the duration was reduced to 12.39 ± 2.58 s and the amplitude to 7.37 ± 0.71 mV ($P < 0.05$, Students paired t-test). In control, [Sar^9,Met(O$_2$)^{11}]-Substance P (2 μM, 5 min) induced a slow depolarization of amplitude 6.6 ± 0.66 mV which was reduced to 3.5 ± 0.5 mV (n = 6, $P <$ 0.05, Students paired t-test) after Rp-cAMP (100 μM, 30 min).

After synaptic stimulation, the number of Fos immuno-positive neurones was significantly increased from basal levels to 1126 ± 55 in superficial laminae and 569 ± 122 in deep laminae ($P <$ 0.005, Students unpaired t-test). Under conditions of c-AMP inhibition (n=5), stimulus-evoked Fos expression was attenuated to values of 457 ± 49 (59 % reduction) and 124 ± 14 (78 % reduction) ($P < 0.005$) within superficial versus deep laminae respectively. After Rp-cAMP, [Sar^9,Met(O$_2$)^{11}]-Substance P (10 μM) evoked significantly less Fos expression in both superficial and deep laminae (Fig. 5). The number of agonist-induced Fos immuno-positive cells was reduced by 17 % and 70 % ($P < 0.05$) in these laminae respectively.

These data support a role for PKA/c-AMP in the stimulation-transcription coupling pathways that underlie IEG Fos expression in nociceptive dorsal horn neurones.

This work was supported by The Wellcome Trust. M. Worsley is in receipt of a University of Leeds Ph.D. studentship. Technical support from Mrs J. Daniel.

158P LOCAL NITRIC OXIDE INHIBITION ALTERS THE PATTERN OF CORTICAL SPREADING DEPRESSION (CSD) INDUCED BY HIGH EXTRACELLULAR K$^+$ IN ANAESTHETISED RATS

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Cortical spreading depression (CSD) is a transient suppression of electrical activity, resulting from a temporary disruption of local brain ionic homeostasis, which propagates slowly across the cortex. CSD may contribute to lesion progression in brain ischaemia and trauma, and underlie the aura in classical migraine. As direct amperometric measurements of nitric oxide (NO) showed a multiphasic release of NO associated with CSD (Read *et al.*, 1997), the purpose of this study was to examine whether local inhibition of NO synthesis (NOS) with Nω-nitro-L-arginine methyl ester (L-NAME) alters K$^+$-induced CSD.

Male Sprague-Dawley rats (321± 5g, mean ± s.e.mean, n = 22) were anaesthetised throughout with halothane (concentration reduced to 1.5 % after surgery) in N_2O:O_2 (2:1), with spontaneous breathing. Microdialysis probes, incorporating an electrode for the recording of CSD as negative shift of the extracellular direct current (d.c.) potential, were implanted in the frontoparietal cortex (Obrenovitch & Zilkha, 1996) and used for the following procedures: (i) elicitation of 5 episodes of repetitive CSD by perfusion of 160 mM K$^+$ through the probe for 20 min, with 40 min of recovery after each CSD episode; and (ii) perfusion of 0.3, 1 and 3 mM L- or D-NAME (inactive isomer of NAME)for the 2nd, 3rd and 4th CSD episode (in respective order) for 60 min, starting 20-min before the K$^+$-application.

NOS inhibition with L-NAME had no effect on the 1st CSD latency. The number of CSD elicited during each K$^+$ stimulus was dose-dependently reduced (e.g. 3.1 ± 0.2 under 1 mM L-NAME versus 5.0 ± 0.4 in controls; mean ± s.e.mean; n = 6-8, $p <$ 0.01, Student's t-test), but this change was associated with a marked increase in the cumulative CSD peak area (e.g. 23.5 ± 1.6 mV.min under 1 mM L-NAME versus 16.7 ± 1.7 in controls, $p < 0.05$), suggesting that under NOS inhibition CSD waves are fewer but larger. Indeed, L-NAME markedly broadened CSD peaks (53.2 ± 2.5 s under 1 mM L-NAME versus 25.0 ± 2.5 in controls; $p < 0.001$) primarily by delaying the repolarisation phase (Fig. 1). In comparison to L-NAME, D-NAME only slightly reduced both the number and magnitude of CSD at the highest dose tested (3mM).

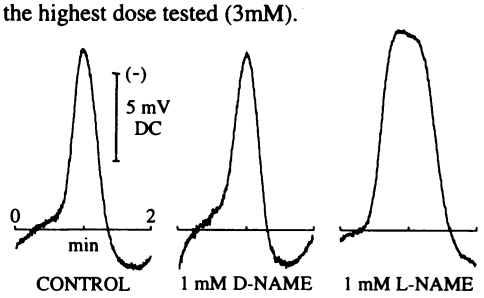

Figure 1. Effect of NOS inhibition (L-NAME) on the pattern of the 1st individual CSD produced by high extracellular K$^+$.

This study demonstrates that NOS inhibition does not inhibit the initiation of CSD by high extracellular K$^+$, but markedly alters its pattern by delaying repolarisation. This effect is likely to be detrimental to cerebroprotection in stroke and head trauma models.

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Obrenovitch, T.P. & Zilkha, E. (1996) *Br. J. Pharmacol.* 117, 931-937.

159P *IN VITRO* CHARACTERISATION OF J-113397 AT THE NATIVE AND RECOMBINANT RAT ORL_1 RECEPTOR

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Recently a non peptide ORL_1 receptor antagonist, J-113397 (1-[(3*R*,4*R*)-1-Cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one) was discovered by Banyu Pharmaceuticals (Kawamoto *et al.*, 1999). J-113397 has been shown to selectively bind to human ORL_1 receptors expressed in chinese hamster ovary (CHO) cells (Ozaki *et al.*, 2000) whilst exhibiting no efficacy at human recombinant or rodent peripheral sites (mouse/rat vas deferens, guinea pig ilium and mouse colon Bigoni *et al.*, 2000). Here we report on the effects of J-113397 at the rat ORL_1 receptor expressed in CHO cells and native receptors of the rat frontal cortex using [^{3}H]nociceptin and [^{35}S]GTPγS binding. We have also characterised its effect at peripheral ORL_1 receptors present on the terminals of sympathetic motor nerves serving the anococcygeus muscle of the rat, a preparation devoid of μ, δ and κ opioid receptors.

Radioligand binding: Membranes prepared from either CHO cells expressing the rat ORL_1 receptor or male Lister hooded rat frontal cortex (250-300g) were incubated with 50pM [^{3}H]Nociceptin, competing drug and Tris-HCl buffer (pH 7.4) for 1 hour at 25°C. Nonspecific binding (NSB) was defined by 1μM Ac-RYYRWK-NH_2. For the [^{35}S]GTPγS binding assay, membranes were incubated at 30°C for 90min with or without agonist/antagonist and 50pM [^{35}S]GTPγS in buffer containing (mM) Tris-HCl (50); $MgCl_2$ (3); EGTA (0.2); NaCl (100); GDP (0.05) and peptidase inhibitors (amastatin, bestatin, captopril and phosphoramidon, 0.01), pH 7.4. 10μM unlabelled GTPγS was used to define NSB. All incubations (0.5ml) were conducted in the presence of 0.01% BSA and were terminated by rapid filtration through GF/B filters (pre-soaked in 1% PEI for the ^{3}H assay only).

Organ bath: Anococcygeus muscles were set up for electrical-field stimulation (5x0.5ms biphasic pulses at 10Hz, every 30s) in 3ml silanised glass organ baths containing modified Krebs' solution at 37°C. To examine pure adrenergic contractions, 100μM Nω-nitro-L-arginine was included in the Krebs buffer. Ligands tested for agonist activity were added cumulatively; for experiments involving measurement of antagonist affinity, a preincubation time of 20-30min was used following a 60-minute period of washout/recovery, before the next cycle of agonist addition.

In CHO cells expressing the rat ORL_1 receptor, J-113397 fully inhibited [^{3}H]nociceptin binding with an affinity some 123 fold lower than nociceptin (pK_i 7.57±0.09 and 9.66±0.04 respectively, n=4). A similar affinity profile for J-113397 and nociceptin was observed for the native ORL_1 receptor expressed in the rat frontal cortex (pK_i 8.24±0.03 and 10.3±0.04 respectively, n=4). For all curves, Hill slopes did not significantly differ from unity.

Using [^{35}S]GTPγS binding as a measure of receptor function, J-113397 displayed no measurable efficacy at concentrations up to 10μM for the recombinant/native receptor. The lack of efficacy allowed the measurement of affinity for J-113397 using Schild regression. J-113397 produced a surmountable, concentration-dependent parallel rightward shift of the concentration response curve to nociceptin. The functional affinity at the native receptor was of the same order as the binding affinity (pA_2 7.19±0.67, slope 0.84±0.08, pK_B 8.39±0.14, n=3). A slightly higher affinity was observed for the recombinant site (pA_2 8.78±0.12, slope 1.01±0.10, pK_B 8.78±0.03, n=3).

In the rat anococcygeus, nociceptin fully inhibited electrically evoked contractions (pIC_{50} 8.13±0.07, slope 1.2±0.06, n=12) but J-113397 was completely devoid of efficacy. Interestingly, in contrast to the purported ORL_1 antagonist, [Phe^{1}ψ(CH_2-NH)Gly^2]Nociceptin(1-13)NH_2, J-113397 did not enhance contractions above baseline levels (Ho *et al.*, In press). However, J-113397 produced a surmountable concentration-dependent rightward shift of the agonist curve with Schild regression defining an antagonist affinity in line with binding values (pK_B 7.35 (7.17-7.53), slope 1.05)

These results generated using the rat receptor support previous findings that J-113397 is a competitive pure antagonist at the ORL_1 receptor. In addition this study has shown that the binding and functional pharmacological profile for J-113397 is comparable at both native central and peripheral sites.

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160P EFFECT OF PREGABALIN ON THE DEVELOPMENT OF CONDITIONED PLACE PREFERENCE (CPP) TO MORPHINE AND THE EFFECT OF MORPHINE TO INCREASE ACCUMBAL DOPAMINE IN THE RAT

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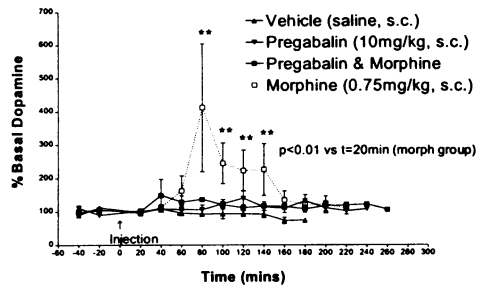
There is evidence for a synergistic benefit of combining a gabapentinoid with an opioid to achieve greater pain relief (Shimoyama et al., 1997). As such combination therapy would be used on a long term basis by patients suffering from chronic pain states we have assessed the rewarding properties of pregabalin, alone and in combination with morphine. The effect of pregabalin on the ability of morphine to increase dopamine levels in the nucleus accumbens is also reported.

Male Hooded Lister rats (250-350g) were used for all studies. For behavioural studies, the place preference chambers were balanced for tactile and visual cues (unbiased) and training took place over 4 days. Briefly, each rat was injected and placed immediately into one side or other of the box for 45 min (with no access to the other side). On Day 5 each rat was placed (uninjected) into the box and allowed free access to both sides of the box for 15 min. Time spent in each side was recorded and preference expressed as time in drug side minus time in saline side (Students paired t-tests were used to analyse for differences between time spent in the drug and saline-paired sides). In Experiment 1 rats (n=9/group) were trained to associate saline with one side and pregabalin (3-30mg/kg p.o. or 10mg/kg s.c.) with the other side of the two-compartment box. In Experiment 2, rats (n=12-15) were trained to associate morphine (0.75mg/kg s.c.) or morphine plus pregabalin (1-30 mg/kg p.o. or 3-100mg/kg s.c.) with one side of the box and saline with the other side of the box. In Experiment 3, rats (n=7-10) were implanted (under isoflurane anaesthesia) with dialysis probes (BAS, 2mm membrane, ~8000 mwt cut-off) in the nucleus accumbens 18h prior to the experiment and perfused with aCSF (mM:NaCl 140, $CaCl_2$ 1.2, KCl 4.0, glucose 11) at 1μl min^{-1}. Samples were collected every 20 min and analysed by HPLC-ECD for their content of dopamine. Drugs were administered following the collection of 3 samples that did not differ in their content of dopamine by more than 10%. Data were analysed by Wilcoxon signed rank tests for matched pairs.

Briefly, Experiment 1 showed pregabalin (p.o. and s.c.) to induce no significant CPP (veh-40.9±58.7; 3mg/kg -35.8±55.5; 10mg/kg 21.8±41.0; 30mg/kg -31.6±60.9; 10mg/kg s.c. 132.0±105.0). In Experiment 2 morphine alone significantly increased the time spent on the drug-paired side of the box (151.8±46.0, $p<0.01$) and this effect was significantly reduced when the morphine was co-administered with pregabalin (M.E.D. 10mg/kg p.o. 63.8±35.3, and 10mg/kg s.c. 72.5±67.5, both $p>0.05$). In Experiment 3 morphine (0.75mg/kg s.c.) significantly increased levels of dopamine in the nucleus accumbens, but this action was blocked following a 40-minute pretreatment with pregabalin (10mg/kg s.c.) at a dose that had no effect by itself (see Figure below).



In conclusion these results show that pregabalin is able to block morphine-induced CPP and morphine-induced increases in accumbal dopamine levels, while inducing no place preference or increases in dopamine levels itself. Furthermore, these results indicate that if given in combination with morphine, pregabalin may actually reduce unwanted side-effects of the opioid e.g. dependence.

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161P MRP1-MEDIATED TRANSPORT OF 2,4-DINITROPHENYL-S-GLUTATHIONE (DNP-SG) IN MEMBRANE VESICLES PREPARED FROM HUMAN ERYTHROCYTES AND COR-L23/R LUNG TUMOUR CELLS

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Inside-out membrane vesicles from human red blood cells (rbcs) or L23/R cells, both cell types of which express MRP1, transport DNP-SG. The apparent kinetic parameters in rbc vesicles, K_m = 6.3±1.2 μM, and V_{max}= 62±10 pmol mg^{-1} protein min^{-1} (mean±sem, n = 6), are similar to those reported previously for rbc (7 μM) (Pulaski *et al.*, 1996) and other MRP1-overexpressing cells (8.1 μM)(O'Brien *et al.*, 1999).

DNP-SG transport was competitively inhibited by oxidised glutathione (GSSG), an established substrate of MRP1, with K_i values of 63±6 μM (n = 6) for rbc-derived and 75 ± 12 (n= 6) for L23/R-derived vesicles. Lithocholic acid 3-sulphate but not taurocholic acid also inhibited DNP-SG uptake with K_i values of 7.2±2 μM (n = 4) for rbc vesicles and 8.7±2.9 (n= 4) for L23/R vesicles. In addition, the MRP1-specific mAb QCRL-3, which recognises a conformation-dependent epitope of MRP1 (Hipfner *et al.*, 1999), inhibited at least 75% of the transport activity in rbc vesicles when applied at 19 μg ml^{-1}. The concentration to inhibit 50% of activity was 8.6±0.7 μg ml^{-1}.

GSH has been found to be required for transport of vincristine (VCR) and daunorubicin (DNR) by MRP1 in tumour cell vesicles (Loe *et al.*, 1996; Renes *et al.*, 1999). With L23/R vesicles, uptake of DNP-SG was not affected by GSH (1-5 mM) in agreement with previous reports (Loe *et al*, 1996) but with rbc vesicles, it was enhanced to 177±16% (n=6, p<0.05) at 3 mM GSH. DNR and doxorubicin (DOX) at 100μM either alone or in the presence of GSH (1-5 mM) had no significant effect on DNP-SG uptake in L23/R vesicles. 100 μM VCR alone also showed little inhibition (<13% of activity lost, n=6) in L23/R vesicles but significant inhibition was seen in the presence of 1 mM GSH (74±2%, n=6, p<0.001) and 5 mM GSH (83±4%, n=6, p<0.001). With rbc vesicles, 100 μM VCR and DOX alone did not cause any inhibition but in the presence of GSH produced significant lowering of DNP-SG uptake: values for percentage activity lost due to VCR and DOX in the presence of 1 mM GSH (58±5%, n=3, p<0.01; and 36±10%, n=6, p<0.05, respectively) or 5 mM GSH (75±2%, n=6, p<0.01, and 38±5%, n=6, p<0.05, respectively). By contrast 100 μM DNR inhibited uptake irrespective of the absence (33±10%, n=6, p<0.05) or presence of GSH (1 mM, 32±3%, n=6, p<0.01; 5 mM, 35±6%, n=6, p<0.05).

The evidence in this report, especially the inhibition of DNP-SG transport by QCRL-3, supports the previous suggestion that MRP1 mediates the high affinity component of DNP-SG transport in human rbc vesicles (Pulaski *et al.*, 1996). The different effects of GSH in vesicles prepared from rbc and L23/R cells indicate that MRP1 function differs in important respects between these two systems, and for the first time suggests that GSH enhances MRP1-mediated transport of the organic anion, DNP-SG in rbcs.

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162P THE EFFECT OF S6c ON PLATELET AGGREGATION AND LEUKOCYTE FREE RADICAL GENERATION IN RAT BLOOD *IN VITRO* AND *EX VIVO*

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We have previously shown that Sarafotoxin-6c (S6c; an ET_B receptor agonist), given in a preconditioning protocol, was protective against myocardial ischaemia-induced arrhythmias in the rat heart *in vivo* and *in vitro* (Crockett *et al.*, 2000). This effect was more marked *in vivo*, suggesting that the antiarrhythmic effect could be mediated, at least in part, by indirect effects of S6c. Platelets have been shown to increase arrhythmias (Flores, 1996), while some leukocyte-generated free radicals (FR's) such as peroxynitrite decrease arrhythmias (Altug *et al.*, 1999). The aim of this study was to investigate the actions of S6c on platelet aggregation and leukocyte FR generation, both *in vitro* and *ex vivo*, as a possible mechanism of its antiarrhythmic effects.

Male Sprague-Dawley rats (300-450g) were anaesthetized using Hypnovel/Hypnorm®. The right carotid artery was cannulated for measurement of mean arterial blood pressure (MABP) and withdrawal of blood and the left jugular vein for administration of drug/vehicle. The extent of platelet aggregation (Ω) in response to collagen (3 $\mu g ml^{-1}$) was determined in whole blood by impedence aggregometry. FR generation in whole blood in response to zymosan (10^6 particles) was determined using luminol-enhanced chemiluminescence (CL). For the *in vitro* studies, S6c (10^{-9}–$3x10^{-8}$M) was added to the blood sample prior to stimulation. For the *ex vivo* studies, blood (0.6ml) was withdrawn 20 min before and 1, 20 and 35 min after injection of vehicle (0.9% saline; n=12) or S6c (0.24, or 0.8nmolkg^{-1}, n=6 and 8, respectively) and subjected immediately to assessment of aggregation and CL measurement. Data (mean ± s.e.m.) was expressed as a % of saline control (*in vitro*) or –20 min (*ex vivo*) samples, and compared using one-way ANOVA with Dunnett's post-hoc test. Statistical significance was taken if P≤0.05.

In vitro, S6c had no significant effect on platelet aggregation, although at higher concentrations (10^{-8}M) there was a tendancy to decrease the extent of aggregation (76±21% of control, n=3; P=NS). In contrast, there was a concentration-dependent inhibition of CL generated from leukocytes (89±13 and 37±11% of control with 10^{-8} and $3x10^{-8}$M S6c; n=5). *In vivo* administration of S6c (0.24nmol.kg^{-1}) caused a transient decrease in MABP (baseline 80±2mmHg), followed by an increase to 106 ± 11 and 108 ± 13mmHg at 5 and 20 min post administration, respectively. This dose of S6c significantly increased leukocyte CL to 215±60% of control (from a baseline of 1098±449 units) and induced a significant fall in platelet aggregation (65±13% of baseline; P<0.05 vs the same time point in saline-treated rats) 20 min after administration. The higher dose of S6c (0.8nmol.kg^{-1}) caused a significant decrease in MABP 1 min post administration (63±4mmHg), with no subsequent increase in MABP, and cardiovascular collapse occurred in 5 of the rats between 20 and 30 min post-administration. This dose of S6c also caused a significant increase in leukocyte CL at both 20 min (702±292% of baseline) and 35 min (605±308% of baseline) post-administration but had no effect on platelet aggregation at any time point. These results demonstrate that S6c has opposing effects on leukocyte FR production *in vitro* and *in vivo*, with minimal effects on platelets. Since certain FR's have been shown to be antiarrhythmic, any link between the effects of S6c on arrhythmias and the blood components is likely to be related to the effect on leukocyte FR generation.

TRC is supported by the British Heart Foundation.

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163P THE EFFECT OF OXIDATIVE STRESS ON ENDOTHELIUM-DEPENDENT AND NITRIC OXIDE DONOR-INDUCED RELAXATION IN RAT AORTIC RINGS: IMPLICATIONS FOR NITRATE TOLERANCE

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Organic nitrates such as glyceryl trinitrate (GTN) are nitric oxide (NO) donor drugs that have been used for many years to relieve the symptoms of angina and heart failure. A major limitation of organic nitrates is the development of tolerance, with a decline in haemodynamic and antianginal effects within 24 h when given without interruption (Zimrin *et al.*, 1988). Recently, several clinical studies have implicated increased inactivation of NO by superoxide as the primary cause of tolerance to GTN (Munzel *et al.*, 1995). The aim of this study was to determine whether superoxide can inhibit GTN-induced vasodilatation in rat aorta, using an established model of oxidative stress. The effect of superoxide on GTN was compared to that on acetylcholine (ACh)-stimulated endothelium-dependent relaxation, and on another NO donor drug, S-nitrosoglutathione (GSNO). GSNO does not induce tolerance (Megson *et al*, 1999) and is known to generate NO via mechanisms different from GTN. The actions of both drugs are guanylate cyclase-dependent.

Vasodilator responses to ACh, GSNO and GTN (10 nM – 30 μM) were investigated in thoracic aortic rings from adult male Wistar rats (300 – 500 g). Following precontraction with phenylephrine (PE, 0.1 μM), rings were treated with either the superoxide generator pyrogallol (300 μM) alone or combined with the Cu/Zn superoxide dismutase (SOD) inhibitor diethyldithiocarbamate (DETCA 0.1 mM). Catalase (3000 U/ml) was present in all experiments to prevent hydrogen peroxide accumulation. Vasodilator responses to ACh were abolished in the presence of pyrogallol, irrespective of DETCA-induced inhibition of SOD (Figure 1a). Responses to GTN, however were partially inhibited by pyrogallol in DETCA-treated rings only, suggesting the vasodilator responses to GTN are relatively resistant to inhibition by pyrogallol-derived superoxide (Figure 1b). However, responses to tolerance-resistant GSNO were significantly inhibited by pyrogallol ($P<0.05$, 2-way ANOVA; n=8), and the effect was exacerbated in DETCA-treated rings ($P<0.05$; n=12).

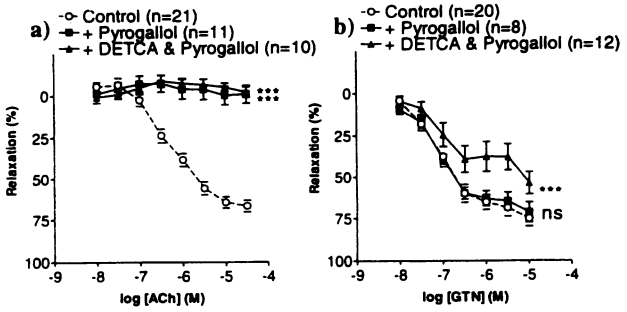

Figure 1. Effect of superoxide on responses to (a) ACh and (b) GTN. *** - $P<0.001$; ns - $P>0.05$ (two-way, repeated measures ANOVA).

Taken together, these results suggest that superoxide might not be a major factor in tolerance. In order to test this hypothesis further, endothelium-intact PE-precontracted rings were treated with a supramaximal concentration of GTN (10 μM) for 2 h. The initial vasodilatation (96±5%) recovered to around pre-treatment levels within the 2 h period, indicating nitrate tolerance. Responses to ACh (0.1 - 10 μM), however, were not inhibited in GTN-tolerant rings ($P>0.05$, 2-way ANOVA n=6). Given the apparent susceptibility of endothelium-derived NO to superoxide, our results suggest that oxidative stress is not an important factor in this model of vascular tolerance.

ISH was the recipient of a BPS Intercalated Award, 1998-99.

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164P NO-ASPIRIN, BUT NOT NATIVE ASPIRIN, REDUCES MYOCARDIAL INFARCT SIZE IN ANAESTHETISED PIGS SUBJECTED TO ISCHAEMIA/REPERFUSION

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While the use of aspirin as a means of reducing mortality from AMI is well documented, its effects are most likely due to prevention of re-infarction rather than any direct cardioprotective effect (Verheught et al., 1990). Thus, should coronary occlusion occur then aspirin would not provide any protection against either arrhythmias or myocardial injury. NO donors have been shown to reduce myocardial infarct size (Lefer et al., 1993) and to have moderate antiarrhythmic activity (Wainwright & Martorana, 1993). The recent development of NSAID's with a NO moiety (NO-NSAID's) may provide additional benefit in patients at risk from AMI. The aim of this study was to compare the effects of NO-aspirin with native aspirin on ventricular arrhythmias and myocardial infarct size in anaesthetised pigs.

Large White/Landrace cross breed male pigs (20-25kg) were used for the study. Aspirin ($10mgkg^{-1}$, n=6), NO-aspirin (NCX4016; 18.4 $mgkg^{-1}$, n=6 or $60mgkg^{-1}$, n=7) or placebo (n=9) were given orally either in the food or by gavage daily for 7 days prior to the procedure. 1 hour after the last dose the pigs were sedated with azaperone ($7mgkg^{-1}$ i.m.), and anaesthetised with chloralose ($100mgkg^{-1}$). The animals were intubated for artificial ventilation and prepared for occlusion of the left anterior descending coronary artery following a mid-sternal thoracotomy. Following stabilisation, the LAD was occluded for 1 hour, followed by 3 hours reperfusion. A Lead II ECG (to detect ventricular arrhythmias) and all standard haemodynamic (HR, ABP, $LVdP/dt_{max}$) variables were recorded continuously on a computerised data acquisition system. VF was converted to sinus rhythm by direct cardioversion. At the end of 3 hours reperfusion the heart was removed and myocardial infarct size (IS), expressed as a % of area at risk (AAR) measured using a dual staining technique. Platelet aggregation responses to collagen were measured in blood samples taken prior to surgery, using whole blood impedence aggregometry. Adhesion of ^{51}Cr-labelled isolated autologous leukocytes to strips of coronary artery from the normal and infarct zones was measured to assess the effects of drug intervention on leukocyte adhesion. All data is given as mean±s.e.m. and analysed by either one-way or two-way ANOVA and Dunnet's post-hoc test.

The higher dose of NO-aspirin significantly reduced the total number of ventricular premature beats (VPB's) during ischaemia (61±16 vs 273±40 in controls: $P<0.05$), without affecting VF (100% in all groups), and significantly decreased IS (22.6±3.7% of AAR) compared to control pigs (53.0±2.8%; $P<0.01$). Neither aspirin nor low dose NO-aspirin had any antiarrhythmic (349±130 and 281±68 VPB's, respectively) or cardioprotective (IS 53.9±4.59% and 51.0±0.6% of AAR, respectively) effects. Aspirin almost completely prevented platelet aggregation in whole blood (0.94±0.22Ω in response to $10\mu gml^{-1}$ collagen *vs* 8.21 ±2.16Ω in controls; $P<0.05$ n=3). Both doses of NO-aspirin significantly inhibited aggregation to the same extent (2.1±0.13Ω and 2.25±0.43Ω with 18.4 and 60 $mgkg^{-1}$, respectively; $P<0.05$), but less effectively than native aspirin. All three interventions also significantly inhibited TxA_2 release from platelets. Finally, high dose NO-aspirin did not modify the ischaemia/reperfusion induced increase in leukocyte adhesion to coronary endothelium (19.0±0.7% and 25.5±% in normal and infarcted zones; $P<0.05$, respectively, *vs* 19.7±0.4 and 23.9±1.8% in control pigs). None of the drugs had any significant haemodynamic effects. These results suggest that NO-aspirin reduces infarct size by a mechanism independent of its antiplatelet effects and effects on leukocyte adhesion.

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165P STUDIES ON THE ROLE OF LEUKOCYTES AND TNFα IN NEOINTIMA FORMATION AND LEUKOCYTE ADHESION IN A RABBIT MODEL OF BALLOON INJURY

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Previous work in our laboratory has demonstrated that leukocyte adhesion to balloon injured rabbit subclavian arteries is enhanced 24 and 48 hours after injury and that this enhanced adhesion may be related to increased expression of leukocyte adhesion molecules on the injured vessel (Kennedy *et al*, 2000). Vascular injury generates a range of cytokines (Ross, 1993) which may result in the upregulation of the adhesion molecules. Leukocytes could then adhere and migrate into the vessel wall where they may contribute to neointima formation. The aim of the current study was to assess the effect of leukocyte depletion on neointima formation in balloon injured rabbit left subclavian artery and to determine the effect of the cytokine TNFα on leukocyte adhesion *in vitro* and then *in vivo* after balloon angioplasty. To evaluate the importance of leukocytes in neointima formation, angioplasty of the left subclavian artery was performed under halothane/nitrous oxide anaesthesia (Hadoke *et al*, 1995) in leukopenic male New Zealand White rabbits (2-2.5kg). Leukopenia (n = 9) was induced by administering an antibody to leukocyte common antigen (1mg/kg i.v.) 2 days before angioplasty. Control animals (n = 9) received pre-immune serum. Vessel areas (mm^2) were assessed by planimetry 28 days after angioplasty. Groups were compared using one-way ANOVA followed by a Tukey Test. To assess the effect of TNFα on adhesion of leukocytes to normal arteries, isolated subclavian artery rings were incubated with TNFα (0.01-10ng/ml) for 4 hours. After washing, each artery was opened longitudinally and incubated with 5μl of ^{51}Cr-labelled leukocytes for 30 minutes at 37°C, washed and gamma counted. Leukocyte adherence was calculated as a % of total leukocytes added. Groups were compared using one-way ANOVA followed by Dunnett's Multiple Range Test. To assess the effect of TNFα administered to injured arteries *in vivo*, another group of rabbits then underwent balloon angioplasty of the left subclavian artery followed by local catheter delivery of 0.9% saline (n = 5) or TNFα (10ng/ml, n = 6) over a 15 minute infusion period at a constant rate of 200μl min^{-1} (total perfused volume 3ml). The effect on leukocyte adhesion was assessed *ex vivo* 24 hours later. Groups were compared using Students Unpaired T-tests.

Induction of leukopenia (~90% decrease in leukocyte count) decreased neointimal size in injured arteries from 0.224±0.038mm^2 in controls to 0.072±0.005mm^2 ($p<0.001$). No difference was seen in the areas of adventitia or media between antibody- and serum-treated arteries. Pre-treatment of isolated rings of rabbit subclavian artery *in vitro* with TNFα 10ng/ml for 4 hours significantly enhanced leukocyte adhesion from 21±5% in control arteries to 47±4% (n=6; $p<0.01$). Local delivery of TNFα to the left subclavian artery after balloon angioplasty *in vivo* significantly enhanced leukocyte adhesion from 34±1% in the injured artery of control rabbits to 44±2% in the TNFα treated rabbits ($p<0.05$). In conclusion, the reduction in neointima in leukopenic rabbits suggests a role for leukocytes in restenosis. TNFα is capable of enhancing leukocyte adhesion to normal isolated blood vessels *in vitro* and *in vivo* after balloon angioplasty. This effect may be important in the events that occur after vascular injury.

A.M.M. is the recipient of an A.J. Clark Scholarship.

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166P LIPOPOLYSACCHARIDE INCREASES ENDOTHELIN-1 SYNTHESIS IN ENDOTHELIAL CELLS WITHOUT INCREASING PREPROENDOTHELIN-1 GENE TRANSCRIPTION RATE

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Preproendothelin-1 (ppET-1) mRNA levels and ET-1 release are increased in cultured endothelial cells by LPS (Marsden & Brenner, 1992; Nakamura *et al.*, 1991) and TGF-β (Kurihara *et al.*, 1989). These stimuli may be important in pathologies involving endothelial dysfunction that lead to ET-1 dependent changes in vessel tone and remodelling. To investigate the regulation of the human ET-1 gene we have developed a reporter gene assay. Here we have investigated the effects of LPS and TGF-β on ET-1 reporter gene expression and ET-1 release in cultured bovine aortic endothelial cells (BAEC).

The promoter region of the human ET-1 gene was obtained by PCR of the 5kb region immediately upstream from the ppET-1 coding sequence using clone PAC 451B15 (from the human DNA library RPCI3, Genbank Z98050, The Sanger Centre) as template and the proofreading Platinum *Pfx* DNA polymerase. The reporter gene construct pET1881-luc, comprising the firefly luciferase gene under the control of the human ppET-1 promoter (positions −1881 to +167), was generated by insertion of a 2kb fragment of the 5kb PCR product into the promoterless vector pGL3 basic. BAEC were transiently co-transfected with pET1881-luc and the pSVβ-galactosidase control vector using TransFast reagent. 48 h after transfection, cells were subjected to 6 h incubation with TGF-β (1 ng/ml, serum-free) or LPS (10 μg/ml, 0.5% FCS). Cell culture and measurement of ET-1 were performed by described methods (Corder & Barker, 1999). Luciferase and β-galactosidase activities in cell extracts were measured by commercial assays. To correct for variation in transfection efficiency, ppET-1 reporter gene activity was expressed as the ratio of luciferase to β-galactosidase activity (relative light units, RLU).

	ET-1 release (% of control)	RLU (% of control)
Control	100 ± 3.8	100 ± 4.5
TGF-β (1 ng/ml)	149 ± 9.2 *	139 ± 14.0 *
LPS (10 μg/ml)	130 ± 6.2 *	93 ± 6.6

Table 1 ET-1 release and ppET-1 promoter activity in pET1881-luc transfected BAEC stimulated for 6 h with TGF-β or LPS. Data are mean ± s.e.mean, n=13, * $p<0.001$ vs control (ANOVA with Fisher's LSD test).

TGF-β and LPS significantly increased ET-1 release in pET1881-luc transfected BAEC (Table 1). Reporter gene activity was significantly increased in response to TGF-β but remained unchanged in LPS stimulated cells. These data suggest that induction of ET-1 by TGF-β is, at least in part, mediated by an increase in ppET-1 gene transcription. In contrast, increased ET-1 synthesis in response to LPS occurs independently of the rate of gene transcription.

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167P CHARACTERISATION OF P2 RECEPTORS MEDIATING CONTRACTION OF THE RAT ISOLATED PULMONARY VASCULATURE

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In the pulmonary circulation, purines and pyrimidines such as adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) produce complex vasomotor responses. ATP was a potent vasoconstrictor in perfused rat lung (Rubino & Burnstock, 1996), but a weak agonist in rat isolated pulmonary artery (Rubino *et al.*, 1999). This may reflect multiple P2 receptor subtypes localised differentially along the pulmonary arterial tree. Therefore, the aim here was to characterise the P2 receptors present in rat small (SPA, 300-500 μm i.d.) and large (LPA, 1-1.5 mm i.d.) intrapulmonary arteries.

Isolated artery rings from male Sprague Dawley rats were bathed in a HEPES-buffered solution at 37 °C. Changes in isometric force were measured with a MacLab data acquisition system. Cumulative contractile concentration-response curves were constructed for ATP, UTP, α,β-methyleneATP (α,β-metATP), 2-methylthioATP (2-MeSATP) and adenosine 5′-diphosphate (ADP). As these did not reach a maximum, agonist potency was compared at the level equivalent to 40% of the contraction to 40 mM K^+ (EC_{40}). The data was analysed statistically by one-way ANOVA with Tukey's comparison.

The P2 receptor agonists evoked concentration-dependent contraction of both tissues (Table 1). The EC_{40} values for α,β-metATP, 2-MeSATP, ATP and ADP were greater in the LPA. α,β-metATP, 2-MeSATP and ADP were inhibited by pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and suramin (100-300 μM), consistent with an action at P2X receptors. Interestingly, α,β-metATP caused a transient contraction in SPA, but a sustained response in LPA. Thus, non-desensitising P2X receptors may be present in the LPA.

Table 1 EC40 (M) values for agonist induced contraction

Agonist	Rat SPA	Rat LPA
α,β-metATP	1.1×10^{-6}* (n=9)	3.2×10^{-5} (n=5)
2-MeSATP	6.3×10^{-5} (n=5)	$>3 \times 10^{-4}$ (n=5)
ATP	5.1×10^{-5} (n=5)	$>3 \times 10^{-4}$ (n=7)
UTP	4.7×10^{-5} (n=7)	7.3×10^{-5} (n=8)
ADP	$>3 \times 10^{-4}$ (n=6)	Inactive (n=4)

*P<0.01 for α,β-metATP of rat SPA versus 2-MeSATP, ATP, UTP of rat SPA and α,β-MetATP of rat LPA

The EC_{40} values for UTP in rat SPA and LPA were similar and in both tissues the contractions were partially blocked by suramin, but unaffected by PPADS. This is consistent with the expression of $P2Y_2$ and $P2Y_6$ receptors in these vessels.

These results show that both P2X and P2Y receptors mediate contraction of rat large and small intrapulmonary arteries. The potency of P2X receptor agonists, but not of P2Y receptor agonists, appears to vary along the pulmonary arterial tree.

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168P EXPRESSION OF MIP-2, KC AND MCR-1 AND THEIR RECEPTORS IN CHRONIC GRANULOMATOUS INFLAMMATION

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We have recently reported the time-dependent formation of two CC chemokines, macrophage inflammatory protein (MIP)-1α and monocyte chemoattractant protein (MCP)-1 in a mouse model of granulomatous air-pouch (Carollo *et al.*, 1999). Here we investigated the mRNA and protein expression of several CXC and CC chemokines, and receptors, in relation to the different phases of the inflammatory response.

Dorsal air-pouches were formed on female Swiss Albino mice (30 ± 2 g) at day -1, and injected 24 h later with 0.5 ml Complete Freund's Adjuvant (5 mg/ml *M. Tuberculosis*) emulsified in 0.1% of croton oil. At several days post-injection, mice were killed by CO_2 exposure and air-pouches dissected and divided into two parts: the upper part was fixed in paraformaldehyde 3% and glutaraldehyde 0.5% in sodium cacodylate buffer (pH 7.4) for 24 h and subsequently embedded in historesin (Technovit 7200, Unicryl™, TAAB Laboratories) or paraffin. The lower part was divided into two portions, snap-frozen in liquid nitrogen and used subsequently for i) determination of mRNA for selected CC and CXC chemokines and chemokine receptors; ii) determination of chemokines levels by ELISA. Quantitative analysis of mRNA for CXCR2, CCR2, MIP-2, KC, MCP-1 and MCP-3 was performed by TaqMan® RT-PCR: 2 μg of total RNA were converted into cDNA by addition of 1 μl of Superscript II reverse transcriptase (RTEnzyme, 10,000-50,000 units/ml) (GIBCO BRL). TaqMan® quantitative assay was performed in a 96 wells plate using for each well 1 μl of cDNA plus a mixture of forward and reverse primers, probes and TaqMan MASTER MIX (AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP) (Gibco BRL), in according to optimised ratio (not shown). Specific ELISA for mouse MCP-1, MIP-2 and KC were purchased from BioSource International (Camarillo, CA) and R&D Systems (Abingdon, UK). Semiquantitative cell counting was performed in 1.5 μm historesin sections, using May-Grumwald (1: 2) and Giemsa (1: 10) staining (TAAB Laboratories, showing results as number of cells per mm^2, in five distinct serial sections. Date (mean ± s.e.mean of *n* mice per group) were analysed using ANOVA plus Bonferroni test taking a P value < 0.05 as significant. Non-parametric Kruskal-Wallis analysis was used for TaqMan® data.

A low expression of CXCR2 mRNA was detected in non inflamed skin by TaqMan® analysis, followed by a sharp increase at day 3, decreasing thereafter in a time dependent manner. The mRNA of two ligands for CXCR2, KC and MIP-2, mirrored the profile of the receptor, with high levels at day 3. TaqMan® data were confirmed by ELISA: KC protein level peaked at day 7 (372 ± 71 pg/mg, n = 13, *vs.* a basal value of 109.6 ± 17.7 pg/mg, n = 5), decreasing after the first week of inflammation; MIP-2 was mainly measured within the first week of inflammation, reaching the highest value also by day 7 (807 ± 151 pg/mg, n = 12 vs. a basal of 21.8 ± 5.5 pg/mg, n=6). CCR2 mRNA expression was not basally detected; and a two-three fold increase was measured during the first week of inflammation, followed by a more significant increase at day 14. mRNA for CCR2 ligands (MCP-1 and MCP-3) mirrored the receptor profile, showing a first peak by day 3, then a second peak for MCP-3 (day 14) and MCP-1 (> day 21). The last finding was confirmed by ELISA with a peak for MCP-1 protein at day 21 (52 ± 14 pg/mg, n =20 vs a basal of 17.9 ± 4.1 pg/mg, n=8). This CC chemokine was mainly associated with mast cells and and monocytes as observed by immunohistochemistry using 1/1000 diluition of a polyclonal rabbit anti-mouse antibody against MCP-1 (gift of Dr. Cory Hogaboam, University of Michigan, Ann Arbor, MI). Semiquantitative analysis in historesin sections (1.5 μm) showed an increase in neutrophil influx during the first week of inflammation. At a later stage of the inflammatory response (day 21-28) a predominant influx of mono/macrophages was observed together with an increased number of fibroblasts. Mast cell degranulation (~70%) was also detected. These histological finding are in accordance with our previous study in which cell influx was monitored using enzymatic markers (Carollo *et al.*, 1999).

In conclusion, we propose that i) the CXC chemokines MIP-2 and KC, and their receptor CXCR2 play a pro-inflammatory role in this model, promoting neutrophils influx by day 7; ii) MCP-1, and CCR2 expression preceded the influx of mono/macrophage. This CC chemokine may be involved in resolving the inflammatory reaction by promoting collagen fiber deposition in the granuloma.

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This work was supported by a BBSRC-AstraZeneca PhD Studentship. MP is a fellow of the Arthritis Research Campaign

169P NERVE CELL-MEDIATED EOSINOPHIL DEGRANULATION

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Eosinophils localise to airway cholinergic neurones (Costello *et al.*, 1997) and may contribute to the pathogenesis of asthma via the release of their granule contents. In vitro, the secretion of these toxic mediators can be induced by a number of factors including N-formyl-methionyl-leucyl-phenylalanine (fMLP), immunoglobulins and platelet activating factor (Takafuji *et al.*, 1992, Bartemes *et al.*, 1999). However, the mechanisms underlying eosinophil degranulation in the presence of nerve cells are not well characterised.

We have examined the release of one of these granule proteins, eosinophil peroxidase (EPO), from human eosinophils cultured in the presence of neuroblastoma IMR32 cells. Differentiation of IMR32 cells for 7 days by reduction of serum and addition of 2 mM butyrate led to neurite outgrowth and increased cholinergic function. Eosinophils were isolated from venous blood of volunteers by negative immunomagnetic selection and either suspended in culture medium alone or added to IMR32 cells. After 2 h incubation, EPO activity was measured in the culture medium following conversion of homovanillic acid to a fluorescent product (Menegazzi *et al.*, 1991).

Eosinophils secreted significantly ($p<0.005$) more EPO when cultured in the presence of IMR32 cells. Eosinophils alone released 0.002 ± 0.003 U peroxidase activity and those in coculture released 0.021 ± 0.007 U (mean ± SD, n = 6). Eosinophils express very late antigen-4 (VLA-4) and the CD11/18 integrins, which act as counterligands to the cell adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, on IMR32 cells. The IMR32-stimulated EPO release was significantly ($p<0.02$, n = 6) reduced by 47.49 ± 24.20% after a 30 min pre-incubation of eosinophils with ZD7349 (10 μM), a specific peptide inhibitor of VLA-4 (Haworth *et al.*, 1999), and by 53.34 ± 12.27% after a 30 min pre-incubation with a specific antibody directed against CD11/18 (1:1000 dilution). ZD7349 and antibody together, inhibited release by 46.73 ± 20.50%. Following paraformaldehyde fixation to abolish nerve cell viability, EPO release was significantly inhibited by 99.03 ± 2.89% ($p<0.05$, n = 3). Coculture with IMR32 cells also significantly potentiated EPO release following stimulation of eosinophils with fMLP (10 nM) from 0.018 ± 0.008 U to 0.033 ± 0.014 U ($p<0.05$, n = 5).

These results suggest that interaction with nerve cells enhances both spontaneous and stimulated eosinophil degranulation. Part of this process depends on adhesion.

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This work was supported by the Wellcome Trust.

170P THE ADHESION OF EOSINOPHILS TO A HUMAN NEURONAL CHOLINERGIC CELL LINE IMR32

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Eosinophils have previously been shown to localise to airway parasympathetic nerves in antigen-challenged guinea pigs and to the airway nerves of humans who died during an asthma attack. Once localised to these nerves, eosinophils release Major Basic Protein onto muscarinic M_2 autoreceptors. The resulting reduced activity at the receptors may contribute to airway bronchoconstriction and hyperreactivity (Costello *et al*, 1997; Evans *et al*, 1997). In this study we have tested the hypothesis that eosinophils adhere to a cholinergic nerve cell line through specific adhesion molecules on the nerves and that the adhesion may by inhibited by the anti-inflammatory compounds heparin and dexamethasone.

Human cholinergic IMR32 cells were induced to differentiate for 7 days by exposure to dibutyryl cyclic AMP (1 mM). Immunohistochemical analysis showed the presence of cell adhesion molecules ICAM-1 and VCAM-1. Eosinophils were prepared from blood of normal human volunteers by negative immunoselection. Cell adhesion assays were performed by co-incubating ^{51}chromium-radiolabelled eosinophils with IMR32 cells for 30 min at 37°C. After three washes to remove non-adherent eosinophils, the cells were lysed with Triton-X-100 and radioactivity was measured. Statistical analysis was performed by one-way ANOVA with Dunnett's post-hoc test.

Pre-incubation of eosinophils for 30 min with monoclonal antibodies to CD11 (1:1000 dilution) or CD18 (25 pg.ml^{-1}), counterligands for ICAM-1, reduced the adhesion of eosinophils to IMR32 cells to 25.4 ± 32.7% (mean ± SD, $p<0.005$, n=6) and 8.4 ± 7.1% ($p<0.01$, n=3) of control, respectively. ZD7349 (10^{-5} M), an inhibitor of VLA-4 (Haworth *et al*, 1999), a counterligand for VCAM-1, inhibited adhesion to 21.3 ± 34% (n=7, $p<0.005$). Adhesion was similarly inhibited in the presence of a non-basic equivalent VLA-4 peptide inhibitor, whereas an inactive equivalent peptide had no inhibitory effect. Dexamethasone (10^{-6} M) and heparin sulphate (20 U/ml) significantly reduced eosinophil adhesion to IMR32 cells to 8.5 ± 5.8 % and 10.8 ± 5.8 %, respectively ($p<0.01$, n=3).

These results show that IMR32 cholinergic nerves express cell adhesion molecules that participate in eosinophil adhesion by a mechanism that can be inhibited by both heparin and dexamethasone.

Supported by the British Lung Foundation and a Samuel Crossley-Barnes studentship to DAS.

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171P ANALYSIS OF OSTEOPROTEGERIN LIGAND EFFECTS ON THE FLG 29.1 PRE-OSTEOCLASTIC CELL LINE

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Osteoprotegerin (OPG) and OPG ligand (OPGL) regulate osteoclastogenesis, with OPGL promoting osteoclast differentiation, and OPG acting as a soluble decoy receptor neutralizing osteoclast formation and activity (Simonet *et al.*, 1997; Lacey *et al.*,, 1998). Since OPGL in combination with macrophage colony-stimulating factor (CSF)-1 can induce osteoclast–like cell formation from blood monocytes, spleenocytes and macrophages, we investigated the effects of these cytokines on the pre-osteoclastic cell line FLG 29.1 (Gattei *et al.*, 1992). Blood mononuclear cells (PBMC) were used as control.

FLG 29.1 cells were routinely grown in RPMI-1640 culture medium, supplemented with 10% foetal calf serum (FCS), and 100 μg/ml gentamicin, in an atmosphere of 5% CO_2/95% air at 37 °C. PBMC were isolated from human blood sample by separation on a gradient of Histopaque 1119/1077; The mononuclear cell population (~20% monocytes) was kept in RPMI-1640 culture medium as for the FLG 29.1 cells. Osteoclast differentiation was monitored by measuring tartrate resistant acid phosphatase (TRAP) activity. In both cases, cells were plated in 6 well plates ($5x10^4$/well) in 2ml RPMI-1640 medium with 1% FCS in the presence or in the absence of various concentrations of stimuli including OPGL (0.1-30 $ngml^{-1}$), CSF-1 (3 $ngml^{-1}$) and calcitonin (20 pM). Cell numbers were counted with a Neubauer hemocytometer after 3 and 6 days of culture. For TRAP activity, cell lysates were tested using a kit based upon hydrolysis of *p*-nitrophenyl phosphate (Sigma Chemical Co., Poole, UK). Briefly, 500μl of substrate solution, 500μl of tartrate acid buffer solution and 200μl of the sample were incubated at 37 °C for exactly 30 min. The samples were read using a spectrophotometer (Labsystem Multiskan Bicromatic, Basingstoke Hants, UK) with a measurement filter of 405 nm. Bone resorption, was determined using the bone-slice assay. Cells were incubated in 24 well plates coated with a film of calcium phosphate apatite (OAAS™, OCT Inc). After 24h cells were removed by bleaching, the number of excavation pits counted for each well, and the pit area measured with an Argus-10 image-processing system (Hamamatsu Photonics, Enfield, UK). The resorbed area was calculated as total areas of individual excavations and expressed as a percentage of control values. All experiments were carried out in triplicate and results (mean ± s.e.mean) were analysed by analysis of variance followed by the Dunnett's test.

FLG 29.1 cells responded to OPGL with a decrease in cell replication and an increase in TRAP activity. These effects were observed after 3 days of culture but were more pronounced after 6 days: OPGL (30 $ngml^{-1}$) led to a significant ($P<0.01$) decrease in cell number (-59±5.5%, n=3 experiments). This was associated with a marked increase in TRAP activity: +77±24.8%, n=3, $P<0.01$ vs. non-stimulated cells. Co-addition of CSF-1 (3 $ngml^{-1}$) did not modify OPGL effect on cell replication, while produced a shift to the left of the concentration-response curve to the cytokine with respect to FLG 29.1 differentiation as measured by TRAP activity: an approximate ED_{50} of 1 $ngml^{-1}$ (vs. a value between 10 and 30 $ngml^{-1}$ in the absence of CSF-1) could be calculated. Interestingly the addition of calcitonin (a potent inhibitor of osteoclast activity) completely abolished the increase in TRAP activity caused by OPGL 30 $ngml^{-1}$ while it had minor effects on cell replication (n=3, $P<0.01$ vs. OPGL alone). Treatment of PBMC with OPGL 10 $ngml^{-1}$ and CSF-1 3 $ngml^{-1}$ for 6 days increased TRAP activity by 55±7% above the values measured in untreated cells, however a similar effect was measured with CSF-1 alone. Treatment of FLG 29.1 cells with either combination of stimuli led only to some morphological changes (cells become bigger and multinucleated), without producing resorptive activity. PBMC differentiation (9 d) with OPGL 10 $ngml^{-1}$ and CSF-1 3 $ngml^{-1}$, but not with either stimuli alone, led to induction of bone resorptive activity: 2220 ± 250 μm^2 (n=3).

In conclusion, FLG 29.1 cells respond to OPGL and a synergism between this cytokine and CSF-1 was observed. FLG 29.1 cells are a useful model for studying osteoclast precursors, but not to extrapolate mature osteoclast behavior.

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This work was supported by the Joint Research Board of Bart's Hospital (grant XMJQ). MP is supported by the ARC

172P CARDIOVASCULAR EFFECTS OF ANANDAMIDE AND OF 5-HT IN THE ABSENCE AND PRESENCE OF THE $5HT_3$-RECEPTOR ANTAGONIST, AZASETRON IN CONSCIOUS RATS

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Intravenous administration of a high dose of anandamide in conscious rats causes initial, short-lived, pronounced bradycardia and hypotension (Lake *et al.*, 1997), reminiscent of the $5\text{-}HT_3$ receptor-mediated, chemosensitive (von Bezold-Jarisch) reflex (see Veelken *et al.*, 1993). This raises the possibility that some of the cardiovascular effects of anandamide may be secondary to 5-HT release. We have, therefore, measured the cardiovascular responses to anandamide and to 5-HT in conscious rats, in the absence and presence of the $5\text{-}HT_3$-receptor antagonist, azasetron (Fukuda *et al.*, 1991).

Under anaesthesia (fentanyl and meditomidine, 300μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1mg kg^{-1} of each s.c.), 9 male, Sprague-Dawley rats (350-400g) had pulsed Doppler flow probes and intravascular catheters implanted, in a 2 stage procedure, separated by at least 2 weeks. Measurements of heart rate (HR), mean arterial blood pressure (BP) and renal (R) mesenteric (M) and hindquarters (H) vascular conductances (VC) began 24h after catheterisation (Gardiner & Bennett, 1988). On Day 1, animals were given increasing i.v. doses of anandamide (75μg kg^{-1} to 2.5mg kg^{-1}), and a single dose of 5-HT (25μg kg^{-1}). On Day 2, the anandamide and 5-HT doses were repeated in the presence of azasetron (10μg kg^{-1}, 10μg kg^{-1} h^{-1}). Data are expressed as mean ± s.e.mean.

At the lower doses (up to 750μg kg^{-1}), anandamide caused initial (at 10s), dose-dependent, increases in BP and vasoconstriction (e.g., anandamide 750μg kg^{-1}, BP + 18±2 mmHg, RVC - 18±3%, MVC - 19±3%, HVC - 20±2% at 10s), which were not significantly affected by azasetron (e.g., anandamide 750μg kg^{-1} after azasetron, BP + 20±3 mmHg, RVC - 15±3%, MVC - 21±4%, HVC - 20±9% at 10s),. There was no significant change in HR at that time, in either the absence or the presence of azasetron.

At the highest dose (2.5mg kg^{-1}), anandamide caused an early (5±1s), pronounced, fall in HR (-245±35 beats min^{-1}), and BP (-43±8 mmHg), associated with falls in RVC (-32±5%), MVC (-61±6%) and HVC (-70±7%), which were not significantly affected by azasetron (after azasetron, HR -251±22 beats min^{-1}, BP -40±9 mmHg, RVC -39±9%, MVC -66±7%, HVC -83±4% at 6±1s).

In contrast, the bradycardic and hypotensive responses to 5-HT (-113±45 beats min^{-1}, -31±4 mmHg at 7±3s) were abolished by azasetron (-14±21 beats min^{-1}, -5±3 mmHg at 9±1s), although the associated changes in VC were not significantly affected (before *vs* after, RVC -10±9 *vs* -8±4%, MVC -29±7 *vs* -31±7%, HVC -27±9 *vs* -27±9%).

The present results, therefore, do not support the hypothesis that the initial cardiovascular effects of anandamide are secondary to 5-HT release.

This work was supported by the British Heart Foundation

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