1P TRICYCLIC ANTIDEPRESSANTS AND 2nd GENERATION ANTIHISTAMINES INHIBIT VOLUME-SENSITIVE ANION CHANNELS IN HELA CELLS

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Tricyclic antidepressants (TCAs) and second generation non-sedating antihistamines (NSAs) exert several adverse cardiac effects, possibly associated with ion channel blockade. Both classes of compound block K^+, Na^+ and Ca^{2+} channels (Ming & Nordin, 1995). One of the effects of cardiac cation channel blockade is Toursades de Pointes (Coupland *et al.*, 1997), a condition caused by prolongation of the QT interval and associated with inhibition of the rapid component of the delayed rectifier I_K (Teschemacher *et al.*, 1999). However, it is plausible that some of the adverse effects of NSAs and TCAs may be associated with effects on anion transport pathways. We therefore examined the effects of the TCAs astemizole and terfenadine on volume-sensitive organic osmolyte/anion channels (VSOACs), anion exchange and lactate transport pathways, since blockers of VSOACs are known to be promiscuous (Mulvaney *et al.*, 2000).

In many cell types, the activation of VSOAC mediates the efflux of structurally dissimilar organic osmolytes such as the β-sulphonated amino acid taurine. Efflux measurements using ^{14}C-taurine were carried out as previously described (Hall, *et al.*, 1996). ^{14}C-taurine efflux was measured in either an isotonic medium (150 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM HEPES, pH 7.4) or a hypotonic medium (150 mM KCl substituted for 95 mM KCl). Anion exchange was measured by the uptake of $^{35}SO_4^{2-}$ by human erythrocytes and lactate transport as the uptake of ^{14}C-lactate in the presence of 0.1 μM DIDS to inhibit any uptake via anion exchange also in human erythrocytes (Mulvaney *et al.*, 2000). The NSAs were slightly more potent inhibitors of volume-sensitive ^{14}C-taurine uptake than the TCAs. IC_{50}'s for terfenadine and astemizole were 19.3 ± 3.6 μM (mean±standard error of the mean; throughout, n=3) and 21.0 ± 2.6 μM (n=3). In comparison those of amitriptyline and imipramine were found to be 45.7 ± 4.0 μM (n=3) and 36.7 ± 23.0 μM (n=3), respectively. Terfenadine (100 μM) was found to inhibit both anion exchange (80.9 ± 5.9%; n=3) and lactate transport (48.6 ± 11.8 %; n=3), whilst astemizole (100 μM) had a small effect on anion exchange (38.2 ± 7.1%; n=3) but no significant effect on lactate transport. Imipramine and amitriptyline had no significant effect on either anion exchange or lactate transport.

These results highlight the pharmacological promiscuity of anion transport pathways and may serve in the future to assist design of more specific modulators of such pathways. In addition, such effects may play a part in mediating some of the adverse cardiac effects of TCAs and NSAs.

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2P [^{3}H]DOFETILIDE BINDING TO HERG TRANSFECTED MEMBRANES; A POTENTIAL PRECLINICAL SCREEN?

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The K^+ channel encoded by the human ether-a-gogo-related gene (HERG; Warmke & Ganetzky, 1994) has received a considerable amount of attention in the last few years, as this channel may be responsible for the deleterious cardiotoxic side effects associated with the second generation antihistamines and other drugs (Netzer *et al.*, 2001). The aim of this study was to establish and characterise the binding of the classIII antiarrythmic, [^{3}H]dofetilide, to membranes from a cell line stably expressing the HERG channel. This assay could provide a useful preclinical screen for compounds that may show a propensity to induce long QT syndrome.

[^{3}H]dofetilide binding to membranes prepared from a cell line stably expressing the HERG channel (Zhou *et al.*, 1998) were performed essentially as described before (Finlayson *et al.*, 2001). On attaining confluency, cells were rinsed twice in pre-warned HBSS, scraped from flasks in ice cold 5 mM Tris-HCl (pH 7.4), left on ice for 60 min and spun at 10500 g for 15 min (4°C); membranes were stored in 1 ml of assay buffer/flask at –20°C. Assay buffer (mM; HEPES, 10; NaCl, 130; KCl, 5, $MgCl_2$, 0.8; NaEGTA, 1, glucose, 10; pH 7.4) or test drug (30 μl) was incubated with 20 μl of [^{3}H]dofetilide (final [10 nM]) and 150 μl of membranes at 37°C for 60 min. Binding was terminated by filtration onto glass filters (GF/C, pre-soaked in 0.25% polyethylenimine), followed by three washes (2 ml) with Tris-HCl wash buffer (mM: Tris-HCl, 25; NaCl, 130; KCl, 5.5; $MgCl_2$, 0.8; glucose, 5; $CaCl_2$, 0.05; pH 7.4).

A range of antiarrythmic compounds and other unrelated compounds known to cause QT prolongation were examined for their ability to inhibit [^{3}H]dofetilide binding. The antiarrythmics dofetilide, E4031 and d-sotalol all inhibited [^{3}H]dofetilide binding in a concentration dependent manner with a rank order of potency similar to that described previously (Table 1; Finlayson *et al.*, 2001). Similarly, the antipsychotic pimozide and the antihistamine terfenadine also inhibited [^{3}H]dofetilide binding (Table 1).

Table 1: Inhibition of [^{3}H]dofetilide binding to HERG transfected cell membranes. Data shown as mean ± s.e.m.

dofetilide	pimozide	E4031	terfenadine	d-sotalol
58.6±13.5 nM, n=5	82.5±14.1 nM, n=4	278±34 nM, n=4	444±70 nM, n=4	154±25 μM, n=5

In conclusion, we have established and characterised [^{3}H]dofetilide binding to membranes from a cell line stably expressing the HERG channel. As the list of compounds which prolong the QT interval continues to grow (Vandenberg *et al.*, 2001) it has been questioned whether drugs from different chemical classes would be detected in a non-functional assay (Netzer *et al.*, 2001). However, in preliminary studies, we have shown that as well as antiarrythmic compounds, unrelated molecules such as pimozide and terfenadine also inhibit [^{3}H]dofetilide binding to HERG transfected membranes. This assay might therefore provide a useful preclinical screen for compounds that cause QT prolongation.

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We would like to thank Pfizer for supplying [^{3}H]dofetilide

3P Kv2.1 CHANNELS ARE THE MAJOR CONTRIBUTORS TO HYPOXIA-SENSITIVE POTASSIUM CURRENTS IN PULMONARY ARTERY SMOOTH MUSCLE CELLS

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Small, resistance pulmonary arteries (PAs) constrict as a consequence of reduced oxygen tension. This mechanism is termed hypoxic pulmonary vasoconstriction (HPV). Inhibition of K channels, leading to membrane depolarisation, Ca^{2+}-influx and smooth muscle contraction has been proposed to underlie HPV. However, the molecular identities of the K channels involved remain unknown. Kv2.1 has been suggested to contribute to HPV. The aim of this study was to demonstrate the presence of this subunit in rat PAs and determine the contribution it makes to the O_2-sensitive K current.

RNA was extracted from the large (1st branch) and small (3rd and 4th branches) PA of male Wistar rats (200-250g). RT-PCR with primers based on the rat cDNA sequence (Genbank accession number X16476) produced the expected 514bp product, which was identical to the published sequence, obtained from small and large PA RNA, confirming the presence of Kv2.1 mRNA in these vessels. Immunohistochemical studies on rat lung sections using anti-Kv2.1 antibodies revealed Kv2.1 subunit protein was specifically localised at the plasmolemma of the smooth muscle cells of pulmonary arteries.

Whole cell, patch-clamp experiments were carried out on isolated PA smooth muscle cells, using a nominally Ca^{2+}-free bath solution and a low intracellular Ca^{2+} solution. Single cells were voltage-clamped at –50mV and membrane currents were measured in response to 1000ms voltage steps from –100 mV to +60mV at 10mV intervals. Statistical significance was assured using an unpaired Student's *t*-test.

Addition of anti-Kv2.1 antibody (1:100 dilution) to the intracellular solution attenuated outward currents following 15 minutes incubation. Normalised current at 50mV (I_{50}) was reduced to 0.31±0.03 (mean±standard error of the mean; throughout, n=3) from 0.94±0.17 (n=3; P<0.05) in control cells. The anti-Kv2.1-sensitive current had a similar profile to the current of cloned Kv2.1 channel expressed in *Xenopus* oocytes (Elinder *et al.*, 1996). Hypoxia (~27mmHg; throughout) reversibly attenuated outward currents when compared to normoxia with the mean normalised I_{50} being reduced from 0.94±0.17 (n=3) to 0.31±0.06 (n=6; P<0.005). The profile of the hypoxia-sensitive current was also similar to that attributed to Kv2.1. Combination of incubation with anti-Kv2.1 antibody and hypoxic challenge reduced the mean normalised I_{50} from 0.94±0.17 (n=3) to 0.30±0.04 (n=5; P<0.005). However, there was no significant difference in I_{50} following this combined treatment compared with anti-Kv2.1 or hypoxia treatments individually.

In conclusion, we have demonstrated the presence of Kv2.1 in the PA at the mRNA and protein level. Furthermore, we have shown that channels containing Kv2.1 conduct a significant component of I_{Kv} in PA smooth muscle cells. The similarity of antibody-sensitive and hypoxia-sensitive currents, combined with the non-additive effects of the treatments implies that Kv2.1 channels are the major contributors to hypoxia-sensitive K-currents in these cells.

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4P EXPRESSION OF CALCIUM-SENSITIVE POTASSIUM CHANNELS IN HUMAN ENDOTHELIAL CELLS

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The existence of a novel endothelium-derived hyperpolarizing factor (EDHF) was first proposed by Taylor & Weston (1988) and this has been confirmed by many subsequent studies (see Garland *et al.*, 1995). More recently studies in rat hepatic and mesenteric arteries have identified EDHF as K^+ ions liberated from endothelial cells (Edwards *et al.* 1998). As the EDHF response can be inhibited by a combination of charybdotoxin and apamin, it has been proposed that the release of K^+ ions is via Ca^{2+}-sensitive K^+ (K_{Ca}) channels located on the vascular endothelium (Edwards *et al.* 1998; 1999).

RT-PCR and immunocytochemistry have been used to investigate K_{Ca} channel expression in human umbilical vein endothelial cells (HUVECs). Additionally, an immunohistochemical study of K_{Ca} channels in small arterioles from human buttock biopsies was carried out.

For RT-PCR, total RNA was isolated from third passage HUVECs, using the RNeasy mini kit (Qiagen). Following reverse transcription, gene-specific PCR was carried out using primers designed against subunits of the large-, intermediate-, and three subtypes of small-conductance K_{Ca} channels (BK_{Ca}, IK_{Ca}, SK1, SK2, SK3 respectively). Cloning and sequencing were used to confirm PCR product identities.

For immunocytochemical studies, third passage HUVECs were trypsinized, washed in PBS and allowed to adhere to silanated glass slides. The cells were fixed and blocked. Antibodies raised against BK_{Ca}, SK2 and SK3 (Alomone) were applied. Negative controls using antibody pre-incubated with the appropriate control antigen were also included. Cells were then stained with DAPI and a goat anti-rabbit secondary antibody conjugated to cy3.

Using these same antibodies immunohistochemical studies were carried out on 8μm sections of human arterioles (lumenal diameter of 150-250μm) that had been fixed and embedded in OCT. Ethical approval was obtained.

RT-PCR showed the presence of mRNA for BK_{Ca}, IK_{Ca} and SK3 subunits in HUVECs, whereas mRNA for SK1 and SK2 was not detected. Positive staining for BK_{Ca}, SK2 and SK3 was seen in the immunocytochemical studies. In the endothelium of arterioles from three healthy volunteers, staining for BK_{Ca} and SK2 was seen, while SK3 staining was weak or absent. Uncertainty about the expression of BK_{Ca} in the vascular endothelium remains (for example see Jow *et al.* 1999), so its presence in these cells is of special interest.

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5P ADENOSINE, THROUGH A_1 RECEPTORS, FACILITATES RECOVERY FROM HYPOXIA BY REDUCING SYNAPTIC NMDA RECEPTOR ACTIVATION

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Adenosine and glutamate are released during hypoxia. Increased levels of glutamate and activation of NMDA receptors are responsible for neuronal damage that occurs upon hypoxia. In contrast, adenosine through A_1 receptor activation may protect neurones by inhibiting excessive excitatory input (de Mendonça *et al.*, 2000). To investigate whether the ability of adenosine to inhibit synaptic transmission could be related to its neuroprotective action we evaluated the influence of adenosine A_1 receptor antagonism, of pre- and postsynaptic blockade of synaptic transmission, as well as of NMDA receptor blockade, on the recovery of synaptic transmission in hippocampal slices exposed to prolonged hypoxia.

Field excitatory postsynaptic potentials (fEPSPs) or excitatory postsynaptic currents (EPSCs; whole cell patch clamp) were recorded from the CA1 area of rat hippocampal slices upon stimulation of the Schaffer collaterals. The NMDA component of the EPSCs was evaluated by subtracting the whole EPSC from the EPSC obtained in the presence of DL-2-amino-5-phoshovaleric acid (AP5, 100μM); the holding potential was set at -40 mV. pO_2 levels were measured in the slice through a O_2-sensitive microelectrode. Hypoxia was applied to each slice only once by switching the $95\%O_2+5\%CO_2$ saturated perfusion solution (pO_2 in the slices 461±63 mmHg, n=5) into a $95\%N_2+5\%CO_2$ saturated solution for 90 min ($pO_2 \cong$ zero), followed by reoxygenation (pO_2 416±55 mmHg at 30 min).

During hypoxia there was a marked, but fully reversible, inhibition of fEPSPs (Table 1). When adenosine A_1 receptors were antagonised by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), recovery of synaptic transmission from hypoxia was attenuated. This impairment could be overcome by preventing synaptic transmission during hypoxia with tetrodotoxin (TTX), by switching off afferent stimulation (stim. off), or by blockade of NMDA receptors with AP5, but not by postsynaptic blockade of transmission with 6-cyano-7-nitroquinoline-2,3-dione (CNQX) (Table 1). A NMDA receptor-mediated component of pyramidal neurones EPSCs was observed during hypoxia in the presence, but not in the absence, of DPCPX (50 nM).

Table 1 Slope of the fEPSPs (as percentage of their pre-hypoxia values) after 90 min hypoxia (N_2) and at maximum recovery from hypoxia (O_2, recovery).

	N2	*O2, (recovery)*
Control (no drug, n=12)	8.8 ± 2.1%	111.1 ± 2.8%
50 nM DPCPX (n=12)	43.8 ± 4.9%[a]	75.4 ± 3.5%[a]
50 nM DPCPX + 0.1μM TTX (n=5)	-	95.0 ± 4.8%[b]
50 nM DPCPX (stim off, n=5)	-	98.6 ± 2.3%[b]
50 nM DPCPX + 50μM AP5 (n=6)	52.5 ± 7.9%[a]	99.4 ± 3.9%[b]
50 nM DPCPX + 4μM CNQX (n=5)	-	38.5 ± 10.2%[a,b]

[a] $P<0.05$ compared with control; [b] $P<0.05$ compared with DPCPX alone (ANOVA followed by Tukey's multiple comparison test).

It is concluded that impairment of synaptic transmission recovery after a hypoxic insult results from activation of synaptic NMDA receptors via synaptically released glutamate, and that adenosine by preventing this activation efficiently facilitates recovery. *Supported by FCT, Portugal.*

de Mendonça, A., Sebastião, A.M. & Ribeiro, J.A. (2000). *Brain Res. Rev.*, 33, 258-274.

6P POSITIVE ALLOSTERIC MODULATORS OF METABOTROPIC GLUTAMATE RECEPTOR 1: CHARACTERISATION AND PUTATIVE BINDING SITE.

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The group I metabotropic glutamate (mGlu) receptors, mGlu1 and mGlu5 are G-protein-coupled receptors which couple to phosphoinositide hydrolysis. Group I mGlu receptors are activated by glutamate, quisqualate and the selective agonist, S-DHPG to modulate K^+ and non-specific cation channels and inhibit voltage-gated Ca^{2+} channels.

We have identified two chemical series of compounds acting as selective positive allosteric modulators of recombinant and native metabotropic glutamate receptor 1 exemplified by: (1) Ro 67-7476 ((S)-2-(4-fluoro-phenyl)-1-(toluene-4-sulfonyl)-pyrrolidine) and (2) Ro 01-6128 (diphenyl-acetyl-carbamic acid ethyl ester) or Ro 67-4853 ((9H-xanthene-9-carbonyl)-carbamic acid butyl ester). As assayed by single cell Fura-2 imaging, application of Ro 67-7476 alone (1μM) did not elicit a calcium response in HEK293 cells transiently transfected with rat mGlu1 receptors. However, co-application of 1μM Ro 67-7476 with 1 μM glutamate, a concentration that elicited little or no response when applied alone, resulted in large glutamate response (90±12% of control maximum; n=15). Similar enhancing effects were observed for Ro 01-6128 and Ro 67-4853.

As assayed by the whole-cell patch clamp technique, the compounds also enhanced a 3μM glutamate-activated K^+ current in CHO cells stably expressing a G-protein-coupled, inwardly rectifying K^+ (GIRK) channel, transiently transfected with the rat mGlu1 receptor (pEC_{50}s: Ro 01-6128, 6.68±0.12; Ro 67-7476, 6.76±0.08; Ro 67-4853, 7.16±0.20; n=5-8). The compounds were devoid of enhancing effect at recombinant mGlu2, mGlu4 and mGlu8 receptors. In binding experiments, Ro 67-7476 and Ro 01-6128 concentration-dependently increased [^{3}H]-quisqualate affinity for the mGlu1 but not mGlu5 receptor (control: K_D=29±3; +10μM Ro 01-6128: K_D=18.5±5; +10μM Ro 67-7476: K_D=12±4) and reduced [^{3}H]-quisqualate K_{off} in dissociation kinetic experiments by 1.5- and 1.7-fold, respectively.

To localise molecular determinants that mediate the enhancing effect of the compounds, we constructed chimeric and point-mutated rat and human mGlu1/5 receptors. Ro 67-7476 (1μM) enhanced the glutamate-induced GIRK current in cells expressing rmGlu1 (397±79% of control; n=5) but not in cells expressing hmGlu1 or rmGlu5 receptors. Ro 67-7476 was effective on chimeric receptors containing the TM region of rmGlu1 but not on those containing the TM region of rmGlu5. Sequence alignment of the TM regions of rmGlu1, rmGlu5 and hmGlu1 revealed that a valine at position 757 in rmGlu1 is not conserved. Generation of point-mutated rmGlu1 (R1-V757L) and hmGlu1 (H1-L757V) resulted in loss and gain of function mutants, respectively. However, Ro 67-7476 had only a very weak effect in the rmGlu5 mutant (R5-L743V). Double mutation of 2 residues critical for the effect of the mGlu1 receptor antagonist CPCCOet in rmGlu1 (R1-T815M,A818S) did not alter the enhancing effect of Ro 67-7476. However, a triple mutant of rmGlu5 carrying mutated residues critical for MPEP binding (P654S,S657C), together with the L743V mutation, conferred a strong enhancing effect of Ro 67-7476.

These compounds are the first selective positive allosteric enhancers of mGlu receptors and represent the prototype of a novel class of mGlu receptor modulators. Furthermore, residues on the transmembrane domain of rmGlu1 are critical for the positive allosteric modulating action of these compounds.

7P POSITIVE ALLOSTERIC MODULATION OF NATIVE METABOTROPIC GLUTAMATE 1 RECEPTORS

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The major CNS excitatory neurotransmitter, glutamate, acts via both ionotropic and G-protein–coupled metabotropic (mGlu) receptors. Eight mGlu receptors have now been cloned, which can be divided into three groups according to their amino-acid sequence identity: group I, mGlu1 and 5; group II, mGlu2 and 3; and group III, mGlu 4, 6, 7 and 8. A number of agonists and antagonists selective for the mGlu subtypes have been described.

We have identified selective compounds acting as positive allosteric modulators at recombinant mGlu1 receptors (see Kemp *et al.*, this meeting). In this study, we have characterised the effects of these compounds at native mGlu receptors. Activation of neuronal mGlu receptors results in a G-protein-mediated inhibition of voltage-gated calcium channels (VGCCs) (e.g. Knoflach *et al.*, 2001). VGCCs were recorded from rat acutely dissociated CA3 pyramidal neurones and Golgi cells using the whole-cell patch clamp technique as previously described (Knoflach *et al.*, 1996;2001). Application of the group I selective mGlu agonist S-DHPG resulted in a concentration-dependent inhibition of VGCCs elicited in CA3 pyramidal neurones. The VGCC inhibition mediated by an EC_{30} concentration of S-DHPG (5μM) was potentiated concentration-dependently by co-applied Ro 01-6128 (pEC_{50} = 5.97±0.11, maximum potentiation = 333±34% of control, n=5). Ro 01-6128 was devoid of effect when applied alone at up 10μM. In the continual presence of Ro 01-6128 (3μM) the S-DHPG EC_{50} for VGCC inhibition was decreased and the maximum inhibition increased (control: pEC_{50} = 5.05±0.07, maximum inhibition = 18.3±3.1%; +Ro 01-6128: pEC_{50} = 5.32±0.05, maximum inhibition = 35.0±3.1%, n=5, $p<0.05$, t-test). Thus, Ro 01-6128 binding increased agonist potency and maximum efficacy. Similar enhancing effects were observed with Ro 67-4853 and Ro 67-7476. However, inhibition of VGCCs in cerebellar Golgi cells by the group II mGlu receptor agonist LY354740 (1μM) was unaffected by Ro 01-6128 (control: 31.2±4.5%; +3μM Ro 01-6128: 31.5±4.0%; n=4).

mGlu1 receptors are expressed at high levels in cerebellar Purkinje cells. Application of S-DHPG (100μM) to rat cerebellar slices in a greased-gap preparation induced population depolarisations (35±5μV, n=22) which were significantly potentiated (88±10μV, $p<0.001$, paired t-test) in the presence of Ro 67-7476 (3μM), whilst AMPA responses were unaffected. To investigate the effects of Ro 67-7476 on synaptically activated mGlu1 receptors, we studied the MCPG-sensitive Purkinje cell postsynaptic current evoked by repetitive parallel fibre stimulation in rat cerebellar slices using the whole-cell patch clamp technique. Application of Ro 67-7476 (3μM) produced no effect alone, but resulted in a marked potentiation of an mGlu1 receptor-mediated EPSC evoked by a 15 pulse, 100Hz burst stimulus (247±65% of control, n=5, $p<0.05$, paired t-test), whilst AMPA EPSCs were unaffected. These observations indicate that these compounds are positive allosteric modulators of native mGlu1 receptors that modulate physiological mGlu1 activity in the brain.

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Knoflach, F., Woltering, T., Adam, G. et al. (2001) *Neuropharmacology* 40, 163-169.

8P BENEFICIAL EFFECTS OF GW274150, A NOVEL, POTENT AND SELECTIVE INHIBITOR OF INOS ACTIVITY, IN A RODENT MODEL OF COLLAGEN-INDUCED ARTHRITIS

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The aim of this study was to investigate the role of inducible nitric oxide synthase (iNOS) on the modulation of the inflammatory response in mice subjected to collagen-induced arthritis (CIA). CIA was induced in wild-type mice (iNOS-WT) treated with GW274150, a novel, potent and selective inhibitor of iNOS activity, and in mice lacking the gene for iNOS (iNOS 'knock-out', iNOS-KO), by an intradermal injection of 100 μl of emulsion containing 100 μg of bovine type II collagen (CII) and complete Freund's adjuvant (CFA) at the base of the tail. After 21 days, a second injection of CII in CFA was administered. iNOS-WT mice developed erosive hind paw arthritis when immunised with CII in CFA.

Over a 35-day period, macroscopic clinical evidence of CIA first appeared as peri-articular erythema and oedema in the hind paws. By day 28, the incidence of CIA was 100% in CII-challenged iNOS-WT mice and the severity of CIA progressed with radiographic evaluation revealing resorption of bone. Histopathology of CIA mice demonstrated erosion of the cartilage at the joint margins. iNOS-WT mice treated with GW274150 (5 mg/kg i.p. daily) starting at the onset of arthritis (day 23), and iNOS-KO mice showed a delay of the development of the clinical signs at days 24-35 and an improvement of the histological status in the knee and paw (see histological score Table 1).

Levels of nitrite/nitrate were significantly increased in plasma obtained from CIA-iNOS-WT mice (Table 1). In contrast, nitrite/nitrate levels were significantly lower in the plasma of GW274150-treated iNOS-WT mice subjected to CIA (Table 1). No increases in plasma nitrite/nitrate levels in CIA iNOS-KO mice (Table 1). Immunohistochemical analysis for nitro-tyrosine and for poly (ADP-ribose) polymerase (PARP) revealed positive staining in inflamed joints from CII-treated iNOS-WT mice. The degree of staining for nitrotyrosine and PARP were markedly reduced in tissue sections obtained from CII-treated iNOS-WT mice, which had received GW274150 and from iNOS-KO mice. Furthermore, radiographic signs of protection against bone resorption were present in the joints of iNOS-WT mice treated with GW274150 as well as in the joint from iNOS-KO mice.

This study provides the first evidence that GW274150, a novel, potent and selective inhibitor of iNOS activity, attenuates the degree of chronic inflammation and tissue damage associated with CIA in mice. Furthermore, these results suggest that the induction of iNOS and NO production are essential for the up-regulation of the inflammatory response during experimental CIA.

	Plasma NOx (μm)	**Histological Score**
°Sham-iNOSWT	16 ± 1.6	0.1±0.8
Sham-iNOSKO	19 ± 2.5	0.08±0.05
CIA-iNOSWT	66 ± 4.3*	3±1*
CIA-iNOSKO	15 ± 2.6°	1.5±0.7°
CIA-iNOSWT + GW274150	32 ± 3°	1.3±0.9°

Table 1. Data are mean ± SEM. *$p < 0.01$ vs. normal (Sham) animals, °$p < 0.01$ vs. wild type mice subjected to CIA only.

9P EXPRESSION AND FUNCTION OF SODIUM TRANSPORTERS IN TWO OPOSSUM KIDNEY CELL CLONAL SUBLINES

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An interesting aspect in OK cells is that this is the only renal epithelial cell line possessing high affinity parathyroid hormone (PTH) receptors (Cole et al., 1988). Though certain properties of opossum kidney (OK) cells are consistent with a proximal tubular site of origin, this cell line was derived from the whole kidney (Koyama *et al.*, 1978), and different cell clones have been described (Cole *et al.*, 1989). The present study describes characteristic features of two clonal subpopulation of OK cells (OK_{LC} and OK_{HC}) that are morphologically identical but functionally different.

OK_{LC} and OK_{HC} cells derive from the same original batch obtained from the American Type Culture Collection (ATCC 1840-HTB, F-12476 at passage 36) and their functional differences were identified after 12 to 26 passages. OK cells (OK_{LC}, passages 52-65; OK_{HC} cells, passages 53-74) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Minimum Essential Medium supplemented with 10% foetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B and 100 µg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 µg of cell protein. 24 h before the experiments the cell culture medium was changed to a serum free medium. Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=5.

Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

The PTH-stimulated accumulation of cAMP in OK_{HC} cells (E_{max}= 1402±84 fmol $well^{-1}$) was markedly higher than that in OK_{LC} cells (E_{max}= 301±11 fmol $well^{-1}$). However, the forskolin-stimulated increase in cAMP accumulation was of similar magnitude in both types of cells. The EC_{50} values for stimulation of cAMP accumulation by PTH in OK_{HC} cells (10.2 [3.1, 33.7] nM) and OK_{LC} cells (5.4 [1.3, 23.4] nM) were similar to that described in OK parental cells (Cole et al., 1988). Transport systems for organic anions, organic cations, α-methyl-D-glucoside, L-type amino acids and activities of enzymes, such as aromatic L-amino acid decarboxylase and catechol-O-methyltransferase were also identical in OK_{LC} and OK_{HC} cells. The most impressive differences between OK_{HC} and OK_{LC} cells are that the former over-expresses Na^+-K^+ ATPase and type 3 Na^+/H^+ exchanger, this being accompanied by increased Na^+-K^+ ATPase activity (30.0±0.1 vs 57.6±5.6 nmol Pi mg $protein^{-1}$ min), increased ability to translocate sodium from the apical to the basolateral cell side, and enhanced sodium-dependent pH_i recovery (0.094±0.011 vs 0.254±0.016 pH units s^{-1}). It is suggested that OK_{LC} and OK_{HC} cells constitute an interesting cell model for the study of renal epithelial physiology and eventually in renal pathophysiology.

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10P EFFECTS OF L-NIL AND AE-ITU ON THE RENAL DYSFUNCTION MEDIATED BY ISCHAEMIA-REPERFUSION OF RAT KIDNEYS *IN VIVO*

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Nitric oxide (NO) is involved in ischaemia/reperfusion (I/R) and hypoxic tubular injury *in vivo* (Ling *et al.*, 1999). NO may react with superoxide to generate peroxynitrite, which is capable of nitrating protein tyrosine residues. Inducible nitric oxide synthase (iNOS)-generated NO and peroxynitrite formation may play a role in the pathogenesis of ischaemic acute renal failure (ARF) (Ling *et al.*, 1999). Here we investigate the effects of L-N^6-(L-iminoethyl)-lysine (L-NIL) and 2-aminoethylisothiouronium (AE-ITU) on the renal dysfunction mediated by I/R of rat kidneys *in vivo*.

Forty-seven male Wistar rats (215-330 g) were anaesthetised with sodium thiopentone (120 mg kg^{-1} i.p.). After performing a midline laparotomy, rats were divided into 6 groups; (i) **'Shams'**, in which rats were maintained under anaesthesia for the duration of the experiment, (ii) **'I/R only'**, in which rats underwent bilateral clamping of the renal pedicles for 45 min followed by reperfusion for 6 h and (iii) **'I/R + L-NIL'**, in which rats underwent I/R, but were administered an i.v. bolus of L-NIL (3 mg kg^{-1} in saline) 15 minutes prior to I/R followed by an infusion of L-NIL (1 mg kg^{-1} h^{-1} in saline) throughout I/R, (iv) **'S + L-NIL'**, which was similar to the sham group except for the administration of L-NIL as described for the I/R + L-NIL group, (v) **'I/R + AE-ITU'**, in which rats also underwent I/R, but were administered an i.v. bolus of AE-ITU (1 mg kg^{-1} in saline) 15 minutes prior to I/R followed by an infusion of AE-ITU (1 mg kg^{-1} h^{-1} in saline) throughout I/R, (vi) **'S + AE-ITU'**, which was similar to the sham group except for the administration of AE-ITU as described for the I/R + AE-ITU group.

Subsequent to I/R, serum urea (Ur) and creatinine (Cr) levels were measured and used as indicators of glomerular function. Plasma nitrite/nitrate concentrations (NO_2/NO_3) were measured using the Griess assay and used as an indicator of NO production. Serum aspartate aminotransferase (AST) levels were measured and used as an indicator of renal I/R injury.

Renal I/R produced significant increases in Ur, Cr, NO_2/NO_3 and AST concentrations, which were significantly reduced by both L-NIL and AE-ITU (**Table 1**).

Data are expressed as mean±s.e.mean for *N* rats, **P*<0.05 vs. I/R only, analysed using one-way ANOVA / Bonferroni's test.

Table 1	*N*	Ur (µmol L^{-1})	Cr (µmol L^{-1})	NO_2/NO_3 (µmol L^{-1})	AST (iu L^{-1})
Shams	12	6±0.4*	43±4*	11±2*	144±7*
I/R only	10	24±1	234±13	69±17	2403±239
I/R + L-NIL	9	19±2*	161±8*	4±2*	703±145*
S + L-NIL	4	7±2*	47±7*	10±2*	218±13*
I/R + AE-ITU	8	21±1*	169±9*	23±2*	790±120*
S + AE-ITU	4	6±0.4*	47±2*	10±3*	137±15*

These results suggest that L-NIL and AE-ITU reduce the glomerular and I/R injury caused by renal I/R *in vivo*. We propose that selective and specific inhibitors of iNOS may be useful against NO-mediated renal dysfunction and injury.

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11P THE PPAR-γ LIGAND 15d-PGJ$_2$ REDUCES RENAL DYSFUNCTION AND INJURY MEDIATED BY ISCHAEMIA/REPERFUSION OF THE RAT KIDNEY

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The peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors related to retinoid, steroid and thyroid hormone receptors (Evans, 1988). The endogenous prostaglandin (PG)D$_2$ metabolite, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$) is a PPAR-γ ligand, modulating the transcription of target genes, e.g. inducible nitric oxide synthase (iNOS) (Ricote *et al.*, 1998). However, not all the effects of 15d-PGJ$_2$ can be attributed to PPAR-γ activation; e.g. inhibition of iNOS expression by 15d-PGJ$_2$ is independent of PPAR-γ activation in activated microglia (Petrova *et al.*, 1999). This aim of this study was to investigate the effect of 15d-PGJ$_2$ on the renal dysfunction and injury caused by ischaemia/reperfusion (I/R) of the rat kidney.

Thirty-eight male Wistar rats (220-320 g) were anaesthetised with sodium thiopentone (120 mg kg^{-1} i.p.). After performing a midline laparotomy, rats were divided into 4 groups; (i) **'Sham'**, in which rats were maintained under anaesthesia for the duration of the experiment, (ii) **'I/R only'**, in which rats underwent bilateral clamping of the renal pedicles for 45 min followed by reperfusion for 6 h, (iii) **'I/R + 15d-PDJ$_2$'**, in which rats underwent I/R, but were administered an i.v. bolus of 15d-PGJ$_2$ (1 mg kg^{-1} in 10 % v v^{-1} dimethyl sulphoxide, 90 % w v^{-1} saline) 5 min before reperfusion and at 3 h after commencement of reperfusion and (iv) **'Sham – 15d-PGJ$_2$'**, which were similar to the Sham group except for the administration of 15d-PGJ$_2$ as described for the I/R + 15d-PGJ$_2$ group. On completion of experiments, renal function was assessed by measurement of serum levels of urea (sUr) and creatinine (sCr), and estimation of creatinine clearance (C_{CL}) and fractional excretion of Na^+ (FE_{Na}). Renal I/R injury was determined by measurement of serum levels of aspartate aminotransferase (AST) and γ-glutamyl transferase (γ-GT).

Compared to the Sham group, renal I/R produced significant increases in sUr, sCr, FE_{Na}, AST and γ-GT and significantly reduced C_{CL} (**Table 1**), indicating renal dysfunction and injury. Administration of 15d-PGJ$_2$ significantly reduced I/R-mediated increases in sUr, sCr, FE_{Na}, AST and γ-GT and reduction in C_{CL} (**Table 1**), suggesting improvement of renal function and attenuation of renal I/R injury.

Thus, administration of 15d-PGJ$_2$ significantly reduced the renal dysfunction and injury associated with I/R of the kidney of the anaesthetised rat. The mechanism of the renoprotective effect of 15d-PGJ$_2$ and the role of the PPAR-γ warrants further investigation.

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Table 1	*N*	sUr (mmol L^{-1})	sCr (μmol L^{-1})	C_{CL} (ml min^{-1})	FE_{Na} (%)	AST (iu L^{-1})	γ-GT (iu L^{-1})
Sham	12	6 ± 0.4$^+$	43 ± 9$^+$	1 ± 0.2$^+$	1 ± 0.2$^+$	144 ± 7$^+$	0.3 ± 0.1$^+$
I/R only	10	24 ± 1*	234 ± 13*	0.02 ± 0.01*	48 ± 11*	2403 ± 240*	6 ± 1*
I/R + 15d-PGJ$_2$	12	21 ± 1*$^+$	187 ± 10*$^+$	0.06 ± 0.01*$^+$	23 ± 4*$^+$	787 ± 69*$^+$	3 ± 0*$^+$
Sham - 15d-PGJ$_2$	4	6 ± 1$^+$	46 ± 7$^+$	1 ± 0.3$^+$	1 ± 0.3$^+$	239 ± 53$^+$	0.5 ± 0.3$^+$

Table 1: Effect of I/R and 15d-PGJ$_2$ on biochemical indicators of renal dysfunction and I/R injury. *P<0.05 vs. Sham, ^+P <0.05 vs. I/R only. Data are expressed as mean ± s.e. mean of N rats, analysed using one-way ANOVA followed by the Bonferroni's post significance test. A P value of less than 0.05 was considered to indicate significance.

12P EFFECTS OF HUMAN HIGH-DENSITY LIPOPROTEINS (HDLs) ON THE MULTIPLE ORGAN INJURY IN HAEMORRHAGIC SHOCK IN THE ANAESTHETISED RAT

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Increased plasma levels of HDL have been shown to be associated with reduced mortality in rodent models of endotoxaemia (Levine *et al.*, 1993). It has been proposed that the beneficial effects of HDL are due to the ability of HDL to bind to and inactivate lipopolysaccharide. However, HDL also directly inhibits the TNF-α induced expression of E-selectin, VCAM-1 and ICAM-1 in endothelial cells (Cockerill *et al.*, 1995 & 1999). The multiple organ injury and failure resulting from trauma and or acute blood loss is secondary to the adhesion and transmigration of neutrophils into the perivascular space of the affected organs (Vedder *et al.*, 1988). The aim of this study was to investigate the effects of HDL on the multiple organ injury and/or dysfunction associated with severe haemorrhage and resuscitation in the anaesthetised rat.

Thirty-eight male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1} i.p.). The carotid artery was cannulated to measure mean arterial blood pressure (MAP) and heart rate (HR), the jugular vein for the intravenous administration of drugs, and the femoral artery for blood withdrawal. After a 30 min stabilisation period, rats were subjected to haemorrhage to lower MAP to 50 mmHg for 90 min. The animals were then treated with either human reconstituted HDL (rHDL, 80 mg kg^{-1}), native HDL isolated from the blood of healthy human volunteers (nHDL, 80 mg kg^{-1}) or saline (1 ml kg^{-1}) and subsequently resuscitated (shed blood plus an equivalent volume of Ringers Lactate solution). Four hours after the onset of resuscitation, blood samples were taken for the measurement of biochemical markers of organ injury. In animals treated with saline, haemorrhagic shock (HS) caused a delayed fall in MAP (p<0.05).

In addition, HS caused significant rises in the serum levels of (i) urea and creatinine (renal dysfunction), (ii) aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (liver injury) (Table 1). HDL significantly attenuated the renal dysfunction and the liver injury caused by HS (n=9, p<0.05). (see Table 1).

Group	n	Urea (mmol L^{-1})	Creatinine (μmol L^{-1})	AST (iu L^{-1})	ALT iu L^{-1})
Sham	9	7±1*	41±5*	244±41*	149±20*
S-HDL	4	7±0*	53±7*	245±18*	134±15*
HS	9	19±1	106±8	1194±197	540±69
HS-rHDL	9	14±2*	46±5*	424±75*	248±56*
HS-nHDL	7	14±1*	66±14*	431±112*	207±56*

Table. 1 Data are expressed as mean±sem, *p<0.05 vs. HS, analysed using one-way ANOVA followed by Dunnet's post hoc test.

Thus, both rHDL and nHDL attenuate the renal dysfunction and the hepatocellular injury caused by severe haemorrhage and resuscitation.

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13P GENDER-DEPENDENT EXPRESSION OF SULFONYLUREA RECEPTORS IN GUINEA-PIG HEART

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Cardiac K_{ATP} channels are composed of, at least, two structurally distinct subunits, i.e. Kir6.2, primarily responsible for K^+ permeance, and the regulatory subunit, also known as the sulfonylurea receptor, or SUR2A, implicated in ligand-dependent channel gating. Recently, some evidence has been provided that gender difference in cardiac response to a metabolic stress may be due to gender difference(s) in efficiency of endogenous cardioprotective mechanism(s), such as ATP-sensitive K^+ (K_{ATP}) channels. Therefore, the main objective of the present study was to establish whether gender regulates expression of subunits forming cardiac K_{ATP} channel.

RT-PCR using primers specific for Kir6.2 and SUR2A subunits was performed on total RNA from guinea-pig ventricular tissue. Western blotting using anti-Kir6.2 and anti-SUR2A antibodies was performed on cardiac membrane fraction. Whole cell, single channel electrophysiology and digital epifluorescent Ca^{2+} imaging were performed on isolated guinea-pig ventricular cardiomyocytes. RT-PCR analysis of ventricular cardiac tissue demonstrated higher levels of SUR2A mRNA in females relative to males (PCR product band intensity was 20±2 arbitrary units (AU) for males and 36±6 for females, n=3, P<0.05). In contrast, the difference between Kir6.2 mRNA levels was not statistically significant (17±2 AU in males compared to 13±1 AU in females, n=3; P>0.05). Western blotting with the specific anti-Kir6.2 and anti-SUR2A antibodies uncovered signals at sizes expected for Kir6.2 and SUR2A, i.e. ~35 kDa and ~150 kDa, and both signals were more intense in females than males (n=3). From the holding potential of –40 mV, 400 ms voltage pulses between –80 to +80 mV were applied to the cell in a 20-mV increment every 1 s and the whole cell K^+ current was recorded.

Initially, there was no statistical significant difference in current density between male and female myocytes (males: 12±1 pA/pF; females: 14±1 pA/pF, n=6, P>0.05). However, in the presence of the K_{ATP} channel opener, pinacidil, current density rose from 12±1 pA/pF to 14±1 pA/pF in males (P<0.01, n=6), and from 14±1 pA/pF to 19±2 pA/pF in females (P<0.01, n=6). The average current density of pinacidil-sensitive component was 2.0±0.3 pA/pF in males and 5.0±0.5 pA/pF in females (n=6 for each). This difference was statistically significant (P<0.05). To test whether single channel properties differs between genders, we have employed single channel patch clamp electrophysiology. There were no statistically significant differences in single channel amplitude (males: 4.14±0.08 pA; females: 4.29±0.20 pA at HP=-60 mV; n=3, P=0.35), mean open time (males: 1.72±0.11 ms; females: 1.98±0.12 ms at HP=-60 mV; n=3, P=0.19) and mean closed time (males: 0.28±0.03 ms; females: 0.23±0.02 ms at HP=-60 mV; n=3, P=0.27) between cells harvested from male or female guinea-pigs. On the other hand, in single cardiomyocytes from males, but not females, ischemia-reperfusion induced significant Ca^{2+} loading (males: from 40±3 to 173±50 nM, P=0.02, n=8; females: from 54±10 to 62±10 nM, P=0.59, n=10). There was a statistically significant interaction between gender and cellular response to the ischemia-reperfusion (P<0.05).

Thus, this study has demonstrated that females express higher levels of sulfonylurea receptors than males forming more sarcolemmal cardiac K_{ATP} channels, which may influence cardiac response to metabolic challenge.

14P PHARMACOLOGICAL CHARACTERISATION OF THE LONGITUDINAL FEMALE RAT URETHRA *IN VITRO*

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Genuine Stress Incontinence is a common condition thought to be due to urethral dysfunction. However the mechanisms controlling urethral function still remain unclear. We investigated the nerve-mediated response of the female rat urethra to electrical field stimulation (EFS) and the responses to exogenous agonists *in vitro*.

Female Sprague Dawley (CD) rats (250-300g) were killed by cervical dislocation, the urethra removed and mounted longitudinally in a tissue bath and equilibrated under 1g tension. Noradrenaline (NA) and isoprenaline concentration-effect curves (CEC) were constructed in the presence and absence of prazosin and propranalol. The change in baseline (Area Under Curve (AUC)) in response to EFS (0.8ms pulse width, 20 Volts, 10s train, every 5 minutes, 1-64 Hz) was measured in the presence of various pharmacological agents. Amplitude data are expressed as mean ± s.e.m. g tension. All other data are expressed as mean ± s.e.m. percentage change in AUC, relative to the control response at 64Hz.

NA (1nM - 3μM) produced a concentration-dependent contraction of the urethra yielding an EC_{50} value of 115.0 ± 25.2nM (n=10). Prazosin (1,3,10nM) shifted the NA CEC to the right in a competitive manner. Schild regression analysis gave a pA_2 of 8.22 ± 0.22 (n=10). Thus post-junctional α1 receptors are present. Isoprenaline (1nM-3μM) relaxed the urethra with an EC_{50} value of 28.8 ± 5.4nM (n=8). Propranalol (10,30,100nM) shifted the isoprenaline CEC to the right in a competitive manner. Schild regression analysis gave a pA_2 value of 8.2 ± 0.2 (n=8) indicating that the post-junctional β-receptors are not β_3.

Nerve stimulation (1-64Hz) produced a relaxation of the urethra (406.9 ± 37.2mg amplitude at 16 Hz, n=16). The magnitude of this relaxation was reduced with increased frequency. A contraction subsequent to the relaxation was evident at frequencies greater than 16Hz. The response was nerve-mediated as it was abolished by 100nM Tetrodotoxin. 300μM N_ω-nitro-L-arginine methyl ester (L-NO-Arg) caused an increase in resting tension, suggesting endogenous nitric oxide (NO) release mediates relaxation. L-NO-Arg completely removed the EFS-induced relaxation to leave a phasic frequency-dependent contraction (585.7 ± 50.8mg amplitude at 16 Hz, n=16) followed by a non-frequency dependent relaxation (154.6 ± 16.9 mg amplitude at 16 Hz, n=16). Thus NO appears to be the major transmitter involved in the EFS-induced relaxation of the rat urethra, as previously demonstrated in the rabbit (Persson & Andersson, 1994).

Further studies were carried out in the presence of L-NO-Arg to characterise the EFS-induced contraction. 1μM atropine reduced the EFS-induced contraction by 56.3 ± 8.8% at 16Hz (n=5). 3μM guanethidine inhibited the EFS-mediated contraction by 79.2 ± 4.5% at 16Hz (n=6). In the presence of guanethidine and atropine the EFS-induced contraction was abolished (97.6 ± 0.7% at 16Hz, n=6). However, the secondary EFS-induced relaxation was unchanged. 1μM prazosin reduced the magnitude of the EFS-mediated contraction by 48.5 ± 8.8% at 16Hz (n= 8) suggesting that the noradrenergic response is mediated via α_1 adrenoreceptors (AR). Yohimbine (1μM) and clonidine (100nM) had no significant effect on the noradrenergic or cholinergic nerve-mediated response. Clonidine produced a small contraction (303.6 ± 29.7mg amplitude, n=8), however at this concentration it has affinity for α_1-AR.

In conclusion we have found evidence for cholinergic, adrenergic and nitrergic drive to the female rat longitudinal urethra. However, there is a neurotransmitter whose identity remains to be elucidated.

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15P THE EFFECTS OF 12,14-DICHLORODEHYDROABIETIC ACID ON HUMAN $GABA_A$ CURRENTS IN OOCYTES AND NATIVE RAT $GABA_A$ RECEPTORS IN PRIMARY CORTICAL CULTURES

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12, 14-dichlorodehydroabietic acid (Cl_2DHA) is a product of the bleaching of wood pulp. It has recently been found to block GABA gated chloride channels in rat cortical neuronal cultures (Nicholson *et al.*, 1999). To further probe underlying mechanisms, human $GABA_A$ ($\alpha_1\beta_2\gamma_{2l}$) receptors expressed in *Xenopus Laevis* oocytes were exposed to Cl_2DHA and compared to results in cultured rat pyramidal cells. Picrotoxin was used as a positive control.

Recording salines, cell-culture protocols and oocyte isolation procedures have been published (Nicholson *et al.*, 1999; Lees & Edwards, 1998). Human receptors were expressed in *Xenopus laevis* oocytes by blind nuclear injection of cDNAs for $GABA_A$ $\alpha_1\beta_2\gamma_{2L}$ subunits (kindly provided by Dr P Whiting, MSD, UK). Two-electrode voltage clamp, 1-5 days later, was used to measure GABA currents at 22-24°c (Lees & Edwards, 1998).

Cl_2DHA was dissolved in dimethylsulphoxide (0.033 – 0.1%, in salines throughout). GABA (EC_{50}) was used at a concentration of 1μM in oocytes and 10μM in neurones. Data, presented as mean ± s.e.mean, were analysed by paired or unpaired, two-tailed Students t-test as appropriate (Spike 2 from CED Ltd., Prism 3 from Graphpad and Uni of Strathclyde software). P values of <0.05 were significant.

In oocytes, at a holding potential of –60mV, 50 μM Cl_2DHA had no significant effect on the amplitude of the GABA induced current after 8 minutes, (Pre-treatment = 1129 ± 442 nA; Cl_2DHA: 1161 ± 439 nA; P = 0.58, n = 4) nor did it alter the holding current. In contrast, 1 μM ionomycin (a Ca^{2+} ionophore) evoked inward Cl^- currents in all cells: 262.5 ± 83.8nA (n = 4). 1 μM picrotoxin produced a profound depression in the amplitude of GABA induced oocyte currents (Pre: 104.5 ± 3.4% of 0 time response; picrotoxin: 9.4 ± 9%; P = 0.015, n = 3). In standard physiological saline using a standard K-gluconate intracellular saline, at a holding potential of –45mV, the amplitude of GABA induced currents in cultures was significantly decreased by 10μM Cl_2DHA (Pre-treatment: 850 ± 247 pA; Cl_2DHA: 37.5 ± 23.9pA; P = 0.038, n = 4) and 1 μM picrotoxin after 8 minutes (Pre-treatment: 102.7 ± 6.0%; Picrotoxin: 30.9 ± 11.7%; P = 0.013, n = 4).

In synaptosomes, Cl_2DHA activates transmitter release, which is concurrent with an increase in cytosolic free [Ca^{2+}] (Zheng & Nicholson, 1998). In our study, the most common human $GABA_A$ receptor isoform was not modulated by the resin acid, nor could Cl_2DHA evoke a significant Ca^{2+}-activated Cl^- current in the oocytes. Although we cannot totally exclude a species difference, an indirect antagonistic mechanism at the $GABA_A$ receptor seems more likely. This may require the involvement of an, as yet, undefined neural receptor or second messenger pathway.

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16P BLOCK OF SUSTAINED REPETITIVE FIRING IN RAT CULTURED CORTICAL NEURONES BY *CIS-9,10-OCTADECENOAMIDE* AND THE STATUS OF ENDOGENOUS FATTY ACID AMIDE HYDROLASE

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The sleep hormone cis-9,10-octadecenoamide (cOA or oleamide) modulates $GABA_A$ receptors and inhibits transmitter release in cultured neurones or synaptoneurosomes. Radioligand binding assays ([^{3}H]batrachotoxin A 20-α-benzoate) on nerve terminals have also suggested a modulatory effect on site 2 of the neuronal voltage gated sodium channel (VGSC) (Nicholson *et al.*, 2001). This is supported by the ability of cOA to block isolated murine Na^+ currents (Nicholson *et al*, 2001) and sustained repetitive firing in cultured rat neurones, suggesting a state-dependent block of the VGSC (Verdon *et al.*, 2000). The aim of this work was to determine whether the potency of cOA in suppressing burst firing is limited by cleavage by endogenous fatty acid amide hydrolase enzymes (FAAH).

Sprague-Dawley rat cultured cortical pyramidal cells were used after 14-21 days *in vitro*. Cells were current clamped at 22-24°C and per-fused with cobalt containing saline (Verdon *et al*, 2000). Lipids and drugs were dissolved in dimethylsulphoxide (DMSO: 0.1% in saline throughout). Only cells with resting membrane potentials < –50mV were selected. Stimuli sufficient to produce sustained repetitive firing (SRF) of over-shooting action potentials were applied for 750ms at a frequency of 0.1Hz. Results are expressed as mean ± s.e.mean. Statistical analysis (compared to time-matched blanks) was by unpaired t-test or one way ANOVA (Tukey post test) where appropriate. $P < 0.05$ was significant: n = 4 throughout, unless stated. Oleamide concentrations were determined using a GCMS assay (Basile *et al*, 1999). Oleamide produced a concentration dependent inhibition of SRF, EC_{50} = 3.8 μM (log EC_{50} 0.58 ± 0.09 μM; Hillslope = 2.0 ± 0.9, 21 degrees of freedom). The high affinity FAAH inhibitor 1,1,1-trifluoro-10(Z)-nonadecen-2-one (TFNO) at 10nM produced no significant block of SRF expressed as % age residual spikes [DMSO blank : 96.4 ± 6.7, n = 5 ; TFNO : 90.9 ± 3.6] . TFNO at 10nM [84.8 ± 4.2, n = 7] and 320nM [99.0 ± 7.8, n = 5] did not significantly alter the response to a low supra-threshold concentration of 2 μM cOA. Similarly, phenylmethylsulphonyl fluoride (PMSF) produced no significant effect at 100 μM alone [90.5 ± 11.0, n=6] and did not potentiate exogenous cOA (2μM) [96.2 ± 6.6]. 100 μM PMSF significantly enhanced extracellular cOA titres in cell cultures maintained at 37°C ($P < 0.01$) but not at room temperature. The release of oleamide was enhanced *circa* 3 fold by depolarisation by 50mM K^+ ($P < 0.005$) in a Ca^{2+} dependent manner.

These results suggest that at ambient temperatures FAAH is not physiologically active nor does it limit the intrinsic efficacy of cOA (2-10 μM). GCMS experiments suggest that these concentrations are locally achievable adjacent to release sites in perisynaptic regions.

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17P A NATURALLY OCCURRING HUMAN NORADRENALINE TRANSPORTER MUTATION IN TRANSMEMBRANE DOMAIN 9 CAUSES DIFFERENTIAL CHANGES IN NORADRENALINE AND COCAINE AFFINITIES

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A naturally occurring human noradrenaline (NA) transporter (hNAT) variant, which showed very low NA uptake, caused orthostatic intolerance due to increased plasma NA levels (Shannon *et al.*, 2000). An alanine (A) residue at position 457 in transmembrane domain (TMD) 9 of the hNAT is a proline (P) residue in the variant. The aim of this study was to further characterise the properties of the A457P variant of the hNAT.

The hNAT cDNA was mutated to produce cDNA encoding the A457P mutant (Stratagene QuikChange site-directed mutagenesis kit), and automated sequencing was used to confirm the mutation. COS-7 cells were transiently transfected with hNAT or A457P cDNA by lipofection and experiments were carried out two days later. In uptake experiments, cells were incubated in Krebs-HEPES buffer ± 10 μM nisoxetine (Nisox) (to determine non-specific uptake) for 15 min at 37°C, and then 10 nM [^{3}H]NA was added for 2 min. In binding experiments, cells were incubated in Krebs-HEPES buffer containing 0.5-60 nM [^{3}H]Nisox and 20-6000 nM NA, 2-3000 nM dopamine (DA), 3-2000 nM 1-methyl-4-phenylpyridinium (MPP^+), 0.6-60 nM desipramine (DMI) or 20-3000 nM cocaine ± 200 μM DA (to determine non-specific binding) at 0°C for 60 min. [^{3}H] and protein contents were used to calculate specific [^{3}H]NA uptake or [^{3}H]Nisox binding. Data (n=3-4) were analysed by non-linear regression analysis and kinetic parameters were compared by paired *t*-test on log K_D and K_i values or absolute values for other data.

Specific [^{3}H]NA uptake by the A457P mutant was 2% of that by hNAT [mean±s.e.m. (fmol/mg protein): A457P 21 ± 6.8, hNAT 1067 ± 57, $P<0.001$], as previously shown (Shannon *et al.*, 2000). Specific [^{3}H]Nisox binding (0.5 nM) by A457P was 33% of that by hNAT [mean±s.e.m. (fmol/mg protein): A457P 23 ± 2.7, hNAT 70 ± 1.1, $P<0.001$]. In [^{3}H]Nisox binding kinetics experiments, A457P showed a small decrease in both Nisox affinity and maximal binding compared with hNAT [K_D (nM; geometric mean and 95% CI): A457P 8.9 (7.2, 11), hNAT 4.6 (3.2, 6.7), $P<0.05$; B_{max} (pmol/mg protein; mean ± s.e.m.): A457P 1.36 ± 0.04, hNAT 3.43 ± 0.44, $P<0.05$]. Substrate affinities for inhibition of [^{3}H]Nisox binding were markedly lower for A457P than hNAT [K_i (nM; geometric mean and 95% CI): NA A457P 1356 (1227, 1499), hNAT 86 (52, 142), $P<0.01$; DA A457P 90 (68, 119), hNAT 9.3 (6.3, 14), $P<0.001$; MPP^+ A457P 430 (365, 507), hNAT 77 (52, 113), $P<0.01$]. Inhibitor affinities for inhibition of [^{3}H]Nisox binding showed differential effects, with an increase in affinity of cocaine for A457P compared with hNAT [K_i (nM): A457P 139 (116, 166), hNAT 652 (580, 734), $P<0.001$], but no difference in DMI affinity for A457P and hNAT.

The study shows that the A457P mutant of hNAT exhibits very limited functional uptake, with markedly reduced affinities of, not only NA, but also other substrates. However, the affinity of cocaine is increased by the mutation. In conclusion, the study provides (i) the first evidence for a critical role of TMD 9 of hNAT for substrate binding and (ii) further evidence for different hNAT binding sites for cocaine and antidepressants.

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18P TOPOGRAPHICAL ASSESSMENT OF DOPAMINE RECEPTOR-MEDIATED MOTOR BEHAVIOURAL PHENOTYPE FOLLOWING DARPP-32 KNOCKOUT

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Dopamine [DA] and cAMP-regulated phosphoprotein MW 32 kDa [DARPP-32] is, when phosphorylated, a cytosolic inhibitor of protein phosphatase 1; DA exerts bidirectional control of the phosphorylation state of DARPP-32 via D_{1A} and D_2 receptors. DARPP-32 knockout (ko; Fienberg et al., 1998) allows examination of its role in DAergic behavioural regulation, here using ethologically-based techniques which resolve all topographies within the mouse repertoire.

Congenic wildtype [wt] and homozygous DARPP-32 kos [10 back-crosses into C57BL/6; Rockefeller University, NY] were bred in Dublin; progeny [20 male, 20 female of each genotype] were assessed for spontaneous behaviour using previously described procedures (Clifford et al., 1998, 2000), over an initial exploratory period of 60 min. followed by habituation over a total period of 370 min. Data were analysed using analysis of variance [ANOVA] followed by Student's t-test or Mann-Whitney 'U' test.

Female but not male DARPP-32 kos evidenced a 33% increase in locomotion over the initial 60 min. [means±SEM, females: wt 13.0±1.1, ko 17.3±0.8, P<0.05; males: wt 12.6±0.9, ko 13.0±1.4, NS] and a 44% decrease in total grooming [females: wt 5.5±0.8, ko 3.1±0.4, P<0.01; males: wt 3.9±0.6, ko 4.4±0.8, NS]; there was an overall decrease in rearing seated in DARPP-32 kos of both genders [P<0.05]. Sniffing, total rearing, rearing free, rearing to wall, intense grooming, sifting, chewing and stillness were unaltered over either the initial 60 min. of exploration or subsequent habituation.

The *ethogram* of DARPP-32 ko was characterised by an overall decrease in non-exploratory rearing, and in females by an increase in exploratory locomotion and a decrease in total grooming; these findings indicate a shift in behaviour from non-exploratory to exploratory topographies, with greater conservation of behaviour in males than in females. While increased locomotion and grooming have been reported in D_{1A} kos (Clifford et al., 1998), with decreased locomotion in D_2 kos (Clifford et al., 2000), no gender specificity was apparent. In the regulation of behaviour, DARPP-32 may not be influenced solely by either D_{1A} or D_2 receptors, and may exert gender-specific effects.

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19P A CCK_B ANTAGONIST DECREASED EFFECTS OF SOCIAL DEFEAT ON ALCOHOL CONSUMPTION

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The experience of social defeat causes behavioural changes in rodents, including increased intravenous self-administration of cocaine (Tidey and Miczek, 1997) and increases in blood and brain corticosterone concentrations in defeated animals (Croft and Little, this meeting). We examined the effects of social defeat, using the resident/intruder paradigm, on alcohol consumption. We further investigated the actions of a CCK_B antagonist on these effects.

Male C57 strain mice, bred in house were housed singly, with ad lib access to food, maintained on reverse phase 12h/12h cycle (lights on 19.00h). Mice were first identified as low preference mice by prior screening for the ratio of 8% v/v ethanol to water consumed; those with ratios below 0.4 for alcohol to total fluid intake were used as 'intruder' mice in the subsequent experiments.

Resident animals were housed singly and screened previously for aggression. For each session, an 'intruder' mouse was placed in the cage of a resident for up to 5 min. When an unambiguous display of upright submissive posture was seen the intruder was immediately removed (confrontations between mice are ritualistic, actual fighting being rare).

Groups of mice (n=8 per group) were given 5 daily experiences of either defeat (Def) or a novel environment (Nov) for times corresponding to the defeat exposure duration. Immediately prior to each exposure they were either given intraperitoneal injections of saline (Sal) or the CCKB antagonist, CAM1028 (CAM) 1 mg/kg, or no treatment (no inj.). They were then given 24h access to a free choice between tap water and 8% v/v ethanol and the fluid consumption measured. Results were calculated as mean values for each week, analysed by one way analysis of variance followed by Bonferroni post hoc test.

Repeated defeat plus saline injection significantly increased alcohol preference in Weeks 1 and 2, compared with repeated novelty experience ($P < 0.01$) or with defeat plus CAM1028 ($P < 0.05$). Defeat with no treatment significantly increased preference compared with novelty plus no treatment in Week 2. No changes in total fluid consumption were found.

The results show that repeated experience of social defeat increased the alcohol preference of C57 low drinking mice and that this effect was decreased by a CCKB antagonist. This is in agreement with our previous work showing CAM 1028 prevented the increased alcohol consumption in C57 mice caused by three weeks of saline injections (Little et al., 1999).

Table 1. Alcohol preference ratio in first week after defeat or novelty experiences, mean ± s.e.m. (*significant changes)

Alcohol	Baseline	Defeat week	Week 1	Week 2
Sal+Def	0.15±0.06	0.19±0.04	0.54±0.05*	0.44±0.05*
Sal+Nov	0.16±0.03	0.17±0.02	0.31±0.04	0.24±0.05
CAM+ Def	0.15±0.04	0.18±0.03	0.37±0.04	0.31±0.05
CAM+ Nov	0.18±0.03	0.21±0.04	0.39±0.05	0.25±0.04
Def + no inj.	0.19±0.02	0.22±0.03	0.39±0.04	0.41±0.03*
Nov+no inj.	0.17±0.03	0.22±0.03	0.37±0.04	0.23±0.03

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20P EVIDENCE THAT ENDOGENOUS GRK6 CONTRIBUTES TO AGONIST MEDIATED PHOSPHORYLATION OF THE M_3 MUSCARINIC RECECPTOR AND SUBSEQUENT UNCOUPLING FROM $G\alpha_{q/11}$

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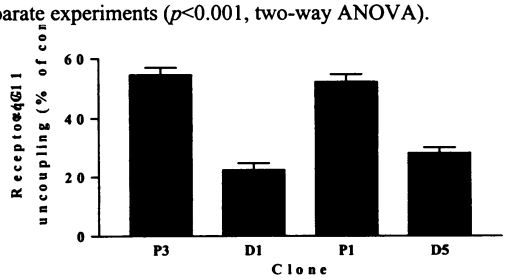
G protein-coupled receptor kinases (GRKs) play a crucial role in the regulation of a variety of G protein-coupled receptors (Ferguson, 2001). We have previously examined the role of GRK6 in the desensitization of the M_3 muscarinic acetylcholine (mACh) receptor endogenously expressed in human SH-SY5Y neuroblastoma cells. Stable over-expression of wild-type GRK6 enhanced M_3 mACh receptor phosphorylation, and led to increased receptor/$G\alpha_q$ uncoupling, at both maximal and sub-maximal agonist concentrations (Willets *et al* 2001). To determine whether endogenous GRK6 is involved in M_3 MCh receptor desensitization in SH-SY5Y cells, we have over-expressed a dominant-negative 'kinase dead' GRK6, created by the introduction of a single point mutation (^{K215R}GRK6).

SH-SY5Y cells, stably expressing either pcDNA3 (empty-vector) or pcDNA3-^{K215R}GRK6, were pre-treated with methacholine (MCh; 100 μM) for 3 min at 37°C. Cells were washed thoroughly, and membranes prepared. Membrane preparations from either vector or ^{K215R}GRK6-expressing cells (50 μg per tube) were incubated ±MCh (100 μM) for 2 min at 30°C, in the presence of GDP (1 μM) and [^{35}S]-GTPγS (1 nM). Non-specific binding was defined in the presence of 10 μM GTPγS. $G_{q/11}\alpha$-[^{35}S]-GTPγS was immunoprecipitated (Akam *et al.*, 2001) and quantified by scintillation counting. The degree of receptor uncoupling was determined as the percentage reduction of [^{35}S]-GTPγS binding after pre-treatment, compared to the maximal [^{35}S]-GTPγS binding in vehicle-treated controls.

MCh-stimulated M_3 receptor phosphorylation was studied in P3 (control) and D1 (^{K215R}GRK6-expressing) cell-lines. Densometric analysis of autoradiographs showed that over-expression of ^{K215R}GRK6 inhibited MCh (100 μM, 3 min) stimulated M3 mACh receptor phosphorylation by 50% (P3: basal, 2497 ± 1077, MCh, 14203 ± 1147; D1: basal, 2018 ± 1047, MCh, 7868 ± 880 optical density mm^{-2} ± s.e.mean; n = 4; $p<0.001$ for agonist-stimulated increases in P3 vs D1 cell membranes). MCh-stimulated M_3 mACh receptor/$G_{q/11}\alpha$ uncoupling was also inhibited by approx. 50% with over-expression of ^{K215R}GRK6 (clones D1 and D5), when compared to vector controls (P1 and P3, Table 1).

Figure 1 Effect of ^{K215R}GRK6 over-expression on MCh-induced M_3 mACh receptor/$G_{q/11}\alpha$ uncoupling in SH-SY5Y cells. Data are expressed as the % increase in uncoupling when compared to vehicle-treated controls. Values are shown as means ± s.e.mean for 3 separate experiments ($p<0.001$, two-way ANOVA).



The ability of the dominant-negative ^{K215R}GRK6 to inhibit M_3 mACh receptor phosphorylation and receptor/$G_{q/11}\alpha$ uncoupling strongly suggests that GRK6 regulates, at least in part, the desensitisation of the M_3 mACh receptor in human SH-SY5Y neuroblastoma cells.

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21P INTERNALIZATION OF mGluR1 SPLICE VARIANTS INDUCED BY MUSCARINIC RECEPTOR ACTIVATION IS PKC- AND CaM KINASE II-DEPENDENT

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Recently, we demonstrated that glutamate-induced internalization of metabotropic glutamate receptor 1a (mGluR1a) is an arrestin- and dynamin-dependent process (Mundell *et al.*, in press). In the present study, we investigated the internalization of the mGluR1 splice variants 1a, 1b, and 1c following heterologous activation of another G_q-coupled receptor (M1 muscarinic), and assessed the possible arrestin- and dynamin-dependency of this process.

Internalization of surface receptor was quantified by an immunosorbent assay (ELISA; Daunt *et al.*, 1997), making use of a haemagglutinnin (HA) epitope tag inserted into the extracellular domain of the rat mGluR1a, b and c splice variants (Mundell *et al.*, in press). HEK293 cells were transiently transfected with $pcDNA_3$ containing either HA-mGluR1a, b or c using Lipofectamine 2000.

Following activation of endogenously-expressed muscarinic receptors by carbachol (1 mM; 0-60 min; Mundell and Benovic, 2000), each splice variant internalized with a $t_{0.5}$ of 27.6 ± 4.8, 16.1 ± 4.6 and 12.5 ± 3.7 min for HA-mGluR1a, 1b and 1c respectively. The carbachol-induced internalization was partially blocked by pre-treatment (15 min) with inhibitors of either protein kinase C (PKC; GF 109203X; 2μM) or Ca^{2+} calmodulin-dependent kinase II (CaM kinase II; KN-93; 1μM). Co-addition of both kinase inhibitors almost completely abolished carbachol-dependent HA-mGlu1a, 1b and 1c internalization (e.g. surface HA-mGluR1a loss (%) - with carbachol (1mM; 30 min) alone, 42.3 ± 3; carbachol + GF 109203X, 14.5 ± 2.3; carbachol + KN-93, 15.5 ± 4.9; carbachol + both kinase inhibitors 1.0 ± 7.8). Similar results were obtained for HA-mGluR1b and 1c indicating that activation of both PKC and CaM kinase II can promote mGluR1a, 1b and 1c internalization. These findings were supported by experiments using phorbol 12-myristate 13-acetate (PMA; 1μM, 30 min), an activator of PKC. PMA promoted the internalization of HA-mGluR1a, 1b and 1c (surface receptor loss (%) was: HA-mGluR1a, 35.6 ± 4.0; 1b, 46.4 ± 3.2; and 1c, 39.3 ± 2.1). In these experiments pretreatment with the PKC inhibitor GF 109203X blocked PMA-induced receptor internalization whilst, as expected, KN-93 was ineffective.

To determine whether carbachol-induced mGluR1a, 1b and 1c internalization is an arrestin- and dynamin-dependent process, cells were cotransfected with HA-mGluR1a, 1b or 1c and dominant negative mutant arrestin (arrestin-2 (319-418); arr-DNM) or dominant negative mutant dynamin (dynamin-K44A; dyn-DNM). Interestingly, expression of either the arr-DNM or dyn-DNM construct with the HA-mGluR1a or 1c strongly inhibited carbachol-induced (1 mM; 30 min) internalization (inhibition (%): HA-mGluR1a / arr-DNM 65.0 ± 8.5; 1a / dyn-DNM, 51.0 ± 9.2 and HA-mGluR1c / arr-DNM, 66.5 ± 10.1; 1c / dyn-DNM 58.3 ± 6.9). Meanwhile, co-expression of the arr-DNM construct with the HA-mGluR1b was found to be less effective in reducing carbachol-induced receptor internalization whilst the dyn-DNM strongly inhibited receptor internalization (inhibition (%) was 19.1 ± 6.0 and 60.0 ± 9.2 for arr-DNM and dyn-DNM respectively).

To further characterize the arrestin-dependency of this process we investigated if arrestin-2-GFP or arrestin-3-GFP underwent carbachol-induced translocation from cytosol to membrane, in HEK293 cells coexpressing HA-mGluR1a, 1b or 1c. We found that carbachol-dependent arrestin translocation was only evident in cells co-expressing HA-mGlu1a or HA-mGlu1c, and not in cells co-expressing HA-mGlu1b. In addition, carbachol-induced arrestin-GFP translocation was absent in cells not transfected with HA-mGluR constructs.

These experiments demonstrate that the internalization of mGluR1a, 1b and 1c splice variants are all subject to PKC and CaM kinase II regulation, and consensus phosphorylation sites for these kinases exist in all three splice variants. In addition, regulation by these kinases confers differential arrestin dependency, with the mGluR1a and 1c undergoing arrestin-dependent internalization and the mGluR1b largely arrestin–independent internalization.

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22P A C-TERMINAL TAIL CAN TARGET GONADOTROPHIN-RELEASING HORMONE RECEPTORS (GnRH-R) FOR DYNAMIN-DEPENDENT INTERNALISATION

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Mammalian GnRH receptors (type I) are G-protein coupled receptors which, uniquely, lack C-terminal tails, do not rapidly desensitise and internalise only slowly (McArdle *et al.* 1999). All cloned non-mammalian GnRH-R have C-terminal tails and where studied do rapidly desensitise and internalise (McArdle *et al.* 1999). Using recombinant adenovirus to express human and Xenopus receptors (XGnRH-R and hGnRH-R) in Hela cells expressing a dominant negative GTPase deficient mutant of dynamin (K44A dynamin) under control of a tetracycline regulable promoter, we have found that internalisation of XGnRH-R is dynamin-dependent whereas internalisation of hGnRH-R is not (Hislop *et al.* 2000a).

In order to determine the importance of the C-terminal tail, adenovirus were engineered expressing a chimeric GnRH-R (H/XGnRH-R, full length hGnRH-R with C-terminal tail of the XGnRH-R). Here we have used confocal microscopy to visualise beta-arrestin 2-GFP (Mundell and Benovic, 2000) after co-transfection with GnRH-R. We found that activation XGnRH-R or H/XGnRH-R caused translocation of the beta-arrestin-GFP to the plasma membrane, indicating agonist-induced receptor phosphorylation and consequent beta-arrestin binding. Activation of the hGnRH-R caused no such translocation. Receptor internalisation was explored using whole cell binding assays in K44A Hela cells. The radioligands used were [^{125}I]chicken GnRH-II (selective for XGnRH-R) and [^{125}I]Buserelin (selective for hGnRH-R and H/XGnRH-R), and acid washing was used to distinguish cell surface from internalised ligand (Hislop *et al.* 2000b).

In K44A Hela cells, the XGnRH-R rapidly internalised (80% after 2 hours, t ½ 10-20 minutes) and internalisation was reduced to 25% of control by allowing the expression of the K44A dynamin. The hGnRH-R internalised only slowly (60% after 2 hours, t ½ 30 minutes) and this was not inhibited by K44A dynamin. Internalisation of the H/XGnRH-R was, surprisingly, slower than that of hGnRH-R, and was reduced to 40% of control by K44A dynamin. Receptor expression levels were also considerably higher with the tailed GnRH-R than with the hGnRH-R (e.g. 50,000, 200,000 and 2,000,000 sites/cell in K44A Hela cells infected at 100 p.f.u./cell with adenovirus expressing hGnRH-R, XGnRH-R and H/XGnRH-R, respectively). However, varying receptor expression levels (by varying viral titre from 3-100 p.f.u./cell) revealed that the functional differences between these receptors (in terms of internalisation rates and dynamin-dependence) of internalisation were not dependent upon receptor number.

Thus it appears that the C-terminal tail facilitates beta-arrestin binding and that this targets the tailed receptors for dynamin-dependent internalisation. However, this is clearly not the only structural determinant of GnRH-R internalisation rate, and the adapter and fission proteins mediating internalisation of the tail-less mammalian GnRH-R remain unknown.

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23P RELATIVE EFFICACIES OF NEUROPEPTIDE Y AND PEPTIDE YY IN Y_1 RECEPTOR-STIMULATED GTPγ[^{35}S] BINDING STUDIES

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The measurement of GTPγ[^{35}S] binding is a useful tool with which to examine agonist stimulation of G_i coupled receptors, since it directly assesses the generation of activated Gα-GTP subunits on guanine nucleotide exchange (e.g. Williams *et al.*, 1997). We have applied this technique to rat Y_1 receptors stably expressed in Chinese hamster ovary (CHO K1) cells, in order to compare the efficacies of two endogenous agonists with similar affinity for the Y_1 receptor, neuropeptide Y (NPY) and peptide YY (PYY).

CHO cells were stably transfected with Y_1 receptor cDNA by calcium phosphate co-precipitation and glycerol shock, from which the G418 resistant rY1-2 clone was selected for further study ([^{125}I]PYY total binding sites 1.2 ± 0.2 pmol mg^{-1}; PYY pIC_{50} 9.46 ± 0.02, NPY pIC_{50} 9.48 ± 0.04; n = 3 - 6). rY1-2 membranes were preincubated at 21°C in optimal buffer (10 mM HEPES, 50 or 100 mM NaCl, 10 μM GDP, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 % BSA and 0.1 mg ml^{-1} bacitracin) with or without agonist/antagonist for 90 min, to ensure equilibrium binding. GTPγ[^{35}S] (200 pM) was added for a further 20 min, over which basal and stimulated binding rates were linear. Incubations were terminated by rapid filtration over GF/B filters and the retained radioactivity was detemined by liquid scintillation counting. Non-specific binding was assessed with 10 μM GTPγS (< 5% of total counts). EC_{50} values with 95% confidence limits were generated from the fitted pooled data using GraphPad Prism; statistical comparisons were made by Student's unpaired *t*-test.

In a 100 mM NaCl buffer, PYY and NPY stimulated the rate of GTPγ[^{35}S] binding in rY1-2 membranes (Table 1; expressed as a percentage of basal levels of 65.7 ± 3.2 fmol mg^{-1}, n = 4 – 6); these responses were not observed in non-transfected CHO K1 cells (n = 3). Both peptides were equipotent, but the response elicited by 1 μM NPY was significantly greater than that for 1 μM PYY (Table 1; P = 0.011). In a less stringent assay buffer (50 mM NaCl), the lower percentage maximum responses (1 μM) mediated by PYY and NPY were equivalent (over basal binding of 81.6 ± 4.3 fmol mg^{-1}, each n=5) but NPY was 4.4-fold more potent than PYY (Table 1). Under these conditions, the potency of NPY was decreased 12.1-fold (EC_{50} 275 nM (143 – 532); n = 3) by co-incubation with the antagonist BIBO3304 (3 nM; Wieland *et al.*, 1998), yielding a pK_B value of 9.5 (compared to a pIC_{50} value in [^{125}I]PYY displacement studies of 9.36 ± 0.04, n = 3). We conclude that both PYY and NPY stimulate GTPγ[^{35}S] binding in rY1-2 membranes exclusively via transfected Y_1 receptors, but that NPY may exhibit a slightly greater efficacy relative to PYY.

Table 1 Agonist-stimulated GTPγ[^{35}S] binding in rY1-2 clone

	100 mM NaCl		*50 mM NaCl*	
Agonist	*EC_{50} (nM)*	*1 μM (%)*	*EC_{50} (nM)*	*1 μM (%)*
PYY	141 (123–163)	247.6±13.0	99.6 (30.4–326)	210.2±11.4
NPY	103 (39.0–274)	300.7±6.7	22.7 (14.3-36.0)	222.6±14.6

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24P TRYPSIN STIMULATES THE NFκB TRANSCRIPTIONAL ACTIVITY VIA AN IKK ISOFORM-INDEPENDENT PATHWAY IN NCTC 2544 TRANSFECTED WITH HUMAN PROTEINASE-ACTIVATED RECEPTOR-2

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Proteinase-activated receptor-2 (PAR-2) is a member of a four member family of G-protein coupled receptors activated by a novel 'tethered ligand' strategy (reviewed in Macfarlane *et al.*, 2001). It has become increasingly clear that PAR-2 may play an important function in inflammatory responses in various physiological systems, including the epidermis (Wakita *et. al.*, 1997). In addition it appears that PAR-2 may have wider reaching functions in the epidermis, including the regulation of pigmentation (Seiberg *et al.*, 2000). Recent evidence also implicates the inflammatory transcription factor NFκB as playing an important role in the control of epidermal structure and function (Hu *et al.* 1999; Seitz et al., 2000). Here we report that PAR-2 expressed in a model keratinocyte cell line activates the NFκB transcriptional activity via a novel pathway that is independent of the 'classical' upstream kinases IKKα and IKKβ.

NFκB-DNA-binding was assessed in nuclear extracts by electrophoretic mobility shift assay and the activation of the IKK isoforms by *in vitro* kinase assay. NFκB transcriptional activity was investigated by transient transfection of a κB reporter plasmid containing a LacZ construct.

30nM trypsin strongly activated NFκB-DNA-binding, the IKK isoforms and κB-mediated gene transcription. However, the addition of DN-IKKα and/or DN-IKKβ had little effect upon the ability of trypsin to activate κB dependent transcription, whereas the mutant IKK isoforms greatly abrogated the ability of TNFα (25ng/ml) to effect NFκB-driven gene expression. The addition of PKC inhibitors GF109203X (3μM) and Gö6983 (10μM) only partially reduced 30nM trypsin-activated NFκB-DNA-binding (67.2% ± 1.7% and and 53.2% ± 1.0%, respectively) and κB-dependent gene transcription 54.4% ± 17% and 78.2% ± 8.8%, respectively).

In the presence of Ca^{2+}-free extracellular buffer, trypsin-activated IKK activation and NFκB-DNA-binding were unaffected. However, this treatment did greatly reduced trypsin-stimulated NFκB-driven gene transcription by 83.3% ± 3.2%, indicating that Ca^{2+} may play an important role in the activation of NFκB gene transcription by PAR-2 agonists.

These findings indicate that PAR-2-mediated activation of NFκB transcriptional activity may occur via a novel pathway and whilst activation of the transcription factor by the 'classical' NFκB cascade may well play some part in PAR-2 signalling, the major signal is carried via an IKK-independent pathway. This novel route for the activation of NFκB by PAR-2 agonists is likely to involve typical and atypical PKC isoforms. In addition, it is likely that Ca^{2+}, the intracellular levels of which are known to be affected by PAR-2 activation, also plays a role in this IKK-independent pathway of NFκB activation.

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25P 1-ETHYL-2-BENZIMIDAZOLINONE ACTIVATES ENDOTHELIAL CELL IK_{Ca} AND SMOOTH MUSCLE HYPERPOLARIZATION IN RAT ISOLATED MESENTERIC ARTERY

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The putative IK_{Ca} channel activator, 1-ethyl-2-benzimidazolinone (1-EBIO) has been employed recently to investigate the role of IK_{Ca} channels in the arterial endothelium-dependent hyperpolarizing factor (EDHF) pathway (Edwards *et al.*, 1999). Little is known about the mechanism of action of 1-EBIO. However, 1-EBIO does activate K_{Ca} channels in epithelial and endothelial cells (Devor *et al.*, 1996; Edwards *et al.*, 1999) and cloned IK_{Ca} channels in heterologous expression systems (Jensen *et al.*, 1998). The aim of the present investigation was to determine if 1-EBIO selectively activates IK_{Ca} channels and cause smooth muscle relaxation as a consequence of the resulting hyperpolarization.

Small mesenteric arteries were dissected from male Wistar rats (200 - 250 g) and a segment of a third order branch was mounted in a Mulvany-Halpern myograph and superfused with oxygenated Krebs (37°C). Smooth muscle membrane potential (sharp electrodes filled with 2 M KCl, tip resistances approximately 100 MΩ) and tension were recorded simultaneously. Single cells were also isolated by enzymatic treatment of rat mesenteric arteries and used for whole-cell patch-clamp recordings. From a holding potential of -10 mV, voltage ramps from +50 mV to -150 mV were applied to both freshly isolated vascular smooth muscle and endothelial cells.

In rat small mesenteric arteries contracted with phenylephrine, 1-EBIO (3-300 μM) evoked concentration-dependent relaxation. However, smooth muscle hyperpolarization was only associated with concentrations of 100 μM and above. 1-EBIO-evoked hyperpolarization (maximum 22.1 ± 3.6 mV with 300 μM, n = 4) was endothelium-dependent and inhibited by charybdotoxin (ChTX 100 nM; n = 4) but not iberiotoxin (IbTX 100 nM; n = 4). In freshly isolated mesenteric endothelial cells, 1-EBIO (600 μM) evoked a ChTX-sensitive outward K-current with a mean amplitude of 42.8 ± 3.4 pA at +50 mV (n = 6). In contrast, 1-EBIO had no effect on smooth muscle cell conductance.

So in high concentrations, 1-EBIO can be used to activate an outward current in mesenteric endothelial but not smooth muscle cells, which appears to reflect the activation of IK_{Ca}. Transfer of current to the smooth muscle presumably then underlies the endothelium-dependent hyperpolarization and contributes to relaxation. However, 1-EBIO also relaxes the smooth muscle directly, by an undefined mechanism independent of any change in membrane potential.

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26P MODULATION OF RESPONSES TO EXOGENOUS POTASSIUM BY POTASSIUM CHANNEL ACTIVITY IN THE RAT ISOLATED MESENTERIC ARTERY

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In small mesenteric arteries, smooth muscle hyperpolarization and relaxation evoked with 10.8 mM potassium (K^+) is abolished if phenylephrine stimulates contraction and depolarisation above around 10 mN and –40 mV (Dora & Garland, 2001). As the effect of exogenous K^+ on the smooth muscle in this artery appears predominately to reflect stimulation of Na^+/K^+ ATPase, it may be that endogenous K^+ accumulation around the smooth muscle pre-stimulates the pump. Since K^+ efflux through K_{Ca} occurs during stimulation with phenylephrine (Dora *et al.*, 2000), we investigated whether inhibition of this efflux enhanced the ability of exogenous K^+ to evoke relaxation in arteries mounted in both wire and pressure myographs.

Male Wistar rats (200-250g) were stunned and killed by cervical dislocation. The mesenteric arteries were removed and cleaned of adherent tissue in Krebs buffer. Small segments (2-3mm in length; 200-400 μm diameter, third order branch) were dissected and mounted in either a wire or a pressure myograph (Danish Myotechnology). Arteries were normalized to 0.9 the diameter at 100mmHg or pressurized at 50 or 80 mmHg, incubated in Krebs containing L-NAME at 37°C and then stimulated with phenylephrine (0.2-3.0 μM). In the wire myograph, sharp microelectrodes were used to measure simultaneous changes in smooth muscle membrane potential and tension.

The K_{Ca} channel blockers apamin (50 nM) and charybdotoxin (100nM) in combination, did not modify the resting potential of –51.9 ± 2.3 mV (n=5). Phenylephrine (0.3 μM) contracted the arteries to 13.8 ± 1.6 mN, which was associated with a membrane potential of –32.2 ± 2.3mV (n=5). Raising $[K^+]_o$ from 4.8 to 10.8mM, which had no effect in the absence of the blockers, evoked repolarization and relaxation of 107.6 ± 8.6% and 98.8 ± 0.6%, respectively. In arteries pressurized to either 50 or 80 mmHg, the diameters were 305 ± 9 and 360 ± 7 μm (n=7 & 5), and reduced to 107 ± 19 and 136 ± 10 μm with 2-8 μM phenylephrine. In all vessels at 50 mmHg, and 3 at 80 mmHg, 10.8 mM $[K^+]_o$ reversed the contraction by 77 ± 11:90 ± 5 %. In the other arteries at 80mmHg, complete K^+ evoked relaxation was obtained in the presence of charybdotoxin.

Therefore, the efflux of K^+ through calcium-activated K channels can suppress the ability of 10.8 mM $[K^+]_o$ to hyperpolarize and relax smooth muscle cells in the mesenteric artery, possibly by pre-stimulation of the Na^+/K^+ ATPase. The influence of pre-stimulation is more marked in wire-mounted as opposed to pressurized arteries, and may explain the small and variable response to 10.8 mM $[K^+]_o$ reported in these arteries by others (Doughty *et al.*, 2000).

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27P EFFECTS OF PLASMA PROTEINS AND BLOOD ELEMENTS ON THE POTENCIES OF FLURBIPROFEN AND NO-FLURBIPROFEN AS INHIBITORS OF THROMBOXANE A_2 FORMATION BY HUMAN PLATELETS

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Non-steroid anti-inflammatory drugs (NSAIDs) are the most widely used drugs worldwide. However their therapeutic use is limited by their side effects, particularly their gastrointestinal toxicity. In recent years, new approaches to develop safer drugs have lead to a new set of drugs called NO-NSAIDs. The addition of a nitrobutylester to traditional NSAIDS, such as flurbiprofen, has reduced gastric toxicity in rats (Wallace *et al.*, 1994). The release of NO from these compounds is thought to have beneficial effects and one study has shown that nitrofenac (NO-diclofenac) actually improved the healing of gastric ulcers in rats (Elliot *et al.*, 1995). NSAIDs are often highly bound by plasma proteins and this effect can be a key determinant of their pharmacokinetic properties. Here we have compared the potencies of flurbiprofen and NO-flurbiprofen as inhibitors of platelet cyclooxygenase-1 (COX-1) in whole blood, and following platelet washing.

Blood from 6 healthy volunteers, who had not taken any NSAIDs for at least two weeks was withdrawn by venepuncture and collected in sodium citrate (3.15% w/v). The blood was divided and one half spun at 200 g for 7 min to obtain platelet rich plasma (PRP). Prostacyclin (300 ng ml^{-1}) was added to the PRP, which was then spun at 1000 g for a further 15 min. The supernatant was removed and replaced with an equal volume of Ca^{2+}-free modified Krebs-Ringer solution at 37 °C. The platelet pellet was re-suspended and prostacyclin was further added. The platelets were pelleted again and re-suspended in Krebs solution as above. 30 min later the platelets were diluted 1:5 v/v in DMEM for preparation of washed platelets (WPL). 90 µl of whole blood (WHB) or WPL was incubated in each well of a gelatine coated 96-well plate with flurbiprofen, NO-flurbiprofen (each drug at 10^{-10}-10^{-3} M), or vehicle (0.1 % DMSO) for 1 h. Calcium ionophore, A23187 (5 x 10^{-5} M) was then added to each well and incubation continued for a further 30 min. Diclofenac (10^{-3} M) was then added to stop the production of prostanoids. The plates were spun at 3000 rpm for 5 min and the concentration of thromboxane B_2 in samples measured by radioimmunoassay as an index of thromboxane A_2 production, and so of COX-1 activity (Warner *et al.*, 1999).

Drugs	WHB IC_{50}s (M)	WPL IC_{50}s (M)	IC_{50} ratio WHB/WPL
flurbiprofen	1.6×10^{-8}	1.4×10^{-8}	1
NO-flurbiprofen	4.0×10^{-6}	4.0×10^{-7}***	10

Table 1: *IC_{50}s (M) of flurbiprofen and NO-flurbiprofen as inhibitors of thromboxane B_2 formation in WHB and WPL. Unpaired students t-test for n=6 patients, WHB vs. WPL. *** $P < 0.001$.*

Comparison of the potencies of the test agents as inhibitors of COX-1 demonstrated that washing of platelets was associated with increased potency of NO-flurbiprofen but not flurbiprofen. Thus, blood factors such as plasma proteins and/or blood cells could limit to a greater extent the availability of NO-NSAIDS than NSAIDs. Hence these blood factors would influence the potencies of NO-NSAIDs more strongly than the potencies of NSAIDs as inhibitors of COX. In other compartments lacking such factors NO-NSAID potencies could well be less affected.

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28P EFFECTS OF N^G-MONOMETHYL-L-ARGININE ON THE VASORELAXANT RESPONSES TO NOVEL THIENYLCYANOGUANIDINE POTASSIUM CHANNEL OPENERS IN RAT ISOLATED AORTA.

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The chemical diversity of ATP-sensitive potassium channel openers (K_{ATP}COs) suggests the involvement of multiple sites of action on the target membrane (Lawson, 1996). L-arginine analogues have been shown to discriminate between the vaso-relaxant responses to the cyanoguanidine K_{ATP}CO, pinacidil, and the benzopyran K_{ATP}CO, cromakalim, in rat isolated aorta (Carr & Lawson, 1999). To gain a greater understanding of the mechanisms involved in the sensitivity of pinacidil responses to N^G-nitro-L-arginine methyl ester and N^G-monomethyl-L-arginine (L-MMA), a series of thienylcyanoguanidine K_{ATP}CO (Yoshiizumi *et al.*, 1997) have been studied in the rat isolated aorta.

Aortic rings, devoid of endothelium, from male Wistar rats (200-250g) were suspended under a resting tension of 2g in Kreb's bicarbonate solution (gassed with 95% O_2 / 5% CO_2 at 37° C). After 80 mins equilibration, cumulative concentration response curves (CRC) to pinacidil (0.05-25.6 µM), KB-R10101 (0.05-25.6 µM), KB-R6844 (0.05-25.6 µM), KB-R6907 (0.05-25.6 µM), KB-R10757 (0.05-25.6 µM) and KB-R10758 (0.001-0.256 µM) were constructed 30 mins after incubation with L-MMA (100 µM), L-N^5-(1-iminoethyl) ornithine (L-NIO 100 µM), methylene blue (10 µM) or vehicle (control) in phenylephrine (1.0 µM)-contracted tissues. EC_{50} (concentration of K_{ATP}CO to evoke 50% relaxation of phenyl-ephrine contraction) values are reported as mean ± s.e.m. (n = 4-7). Concentration ratios with 95% confidence limits CR(cl) were determined from EC_{50} values of paired preparations.

Concentration related relaxations were produced by pinacidil (EC_{50} 0.41 ± 0.06 µM), KB-R10101 (EC_{50} 0.16 ± 0.03 µM), KB-R6844 (EC_{50} 0.66 ± 0.05 µM), KB-R6907 (EC_{50} 2.28 ± 0.08 µM), KB-R10757 (EC_{50} 1.07 ± 0.30 µM) and KB-R10758 (EC_{50} 0.0024 ± 0.0003 µM). L-MMA (100 µM) significantly displaced the CRC to pinacidil (CR(cl) 1.65 (1.15-2.15)), KB-R6844 (CR(cl) 2.67 (1.50-3.85)), KB-R6907 (CR(cl) 3.22 (1.66-4.77)) and KB-R10757 (CR(cl) 2.12 (1.32-2.93)), but not to KB-R10101 (EC_{50} 0.19 ± 0.05 µM) or KB-R10758 (EC_{50} 0.0027 ± 0.0005 µM) to the right of the controls. In contrast, L-NIO (100 µM) failed to modify the CRC to pinacidil (EC_{50} 0.73 ± 0.23 µM), KB-R6844 (EC_{50} 1.35 ± 0.28 µM), KB-R6907 (EC_{50} 4.82 ± 1.46 µM) and KB-R10757 (EC_{50} 2.08 ± 0.35 µM). Methylene blue (10 µM) also failed to modify the CRC to KB-R6844 (EC_{50} 1.24 ± 0.46 µM) and KB-R6907 (EC_{50} 5.76 ± 1.78 µM).

In conclusion, L-MMA can selectively modify the vaso-relaxant responses to pinacidil, KB-R6844, KB-R6907, KB-R10757. These effects appear to be independent of nitric oxide synthase and guanylate cyclase inhibitory properties. The re-placement of a Br for a CN group on the thiophene ring of this series of K_{ATP}COs may account for the sensitivity of the vaso-relaxant responses to L-MMA. The study suggests that the K_{ATP}COs do not possess a common pharmacophore and may interact with the 'K_{ATP}CO receptor' on the K_{ATP} channel in a different manner.

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29P IMPAIRED VASODILATION TO PEROXYNITRITE, ACETYLCHOLINE AND ISOPRENALINE IN ANAESTHETISED STREPTOZOTOCIN-INDUCED DIABETIC RATS.

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In both patients and animal models diabetes mellitus is associated with increased oxidative stress and impaired endo-thelium-dependent relaxation (Honing *et al*, 1998). Inactiva-tion of nitric oxide by superoxide anion may play a role in the attenuation of endothelium-dependent relaxation and the pro-duct of this reaction is the oxidant and vasoactive substance peroxynitrite. Peroxynitrite is a vasodilator which is subject tachyphylaxis *in vivo* and responses to the endothelium-dependent vasodilator acetylcholine are decreased after the in-duction of tachyphylaxis to peroxynitrite (Benkusky *et al*, 1998).

Peroxynitrite is a candidate mediator of diabetes-induced im-pairment of vasodilator function. We investigated the hypothe-sis that diabetes-induced endogenous production of peroxy-nitrite may lead to a reduced response to exogenous peroxynitrite in a model of experimental diabetes.

Rats were anaesthetised with ketamine and the jugular vein exposed. Diabetes was induced by injecting 55mg/kg of streptozotocin (STZ) into the jugular vein. After 4-6 weeks of diabetes rats were anaesthetised with pentobarbitone and cannulated in the femoral artery and vein for the measurement of blood pressure (MAP) and for i.v. administration of drugs. Regional blood flows (BF) were measured with pulsed doppler flow probes on the lower abdominal aorta, superior mesenteric artery and renal artery. Vascular resistance (VR) were cal-culated by the formula VR = MAP/BF. After 30 minute stabi-lisation period dose responses to peroxynitrite (2.5 μmol/kg-20 μmol/kg), acetylcholine (0.1 μmol/kg-5.0 μmol/kg) and isoprenaline (0.1 μmol/kg - 5.0 μmol/kg) were carried out in STZ and controls.

Peroxynitrite caused a dose-dependent fall in blood pressure and vascular resistances in control rats (n=6). The maximum fall in blood pressure was -52±2 mmHg and the maximum fall in hindquarter vascular resistance was -60±4%. In STZ-rats (n=6) the maximum blood pressure fall was reduced to -28±2 mmHG ($P<0.05$) and the maximum decrease in mesenteric resistance was significantly reduced (-39±4% $P<0.05$). Acetylcholine caused a dose-dependent hypotension (maximum response -48±1 mmHg) and vasodilation. For example, in the hindquarter bed the maximum fall in VR was 57±3%.

The dose-dependent responses to acetylcholine were significantly impaired in diabetic rats (max MAP -28±1 $P<0.05$; max hindquarter VR -39%±3 $P<0.05$). Isoprenaline caused dose-dependent falls in MAP (44±3 mmHg) and decrease in hindquarter VR (64%±4%) in controls. Theses responses were significantly impaired in diabetic rats (MAP 30±3 mmHg $P<0.05$; hindquarter VR -42%±4%).

STZ-diabetes reduces responses to peroxynitrite *in vivo*. This is consistent with the hypothesis that tachyphylaxis to endogenous peroxynitrite may occur in diabetes. In addition, responses to the vasodilators acetylcholine and isoprenaline are impaired in STZ diabetes and this may be due to the actions of endogenously produced peroxynitrite on these signal transaction pathways.

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30P DOSES OF BACTERIAL WALL FRAGMENT OF *S. AUREUS* THAT INDUCE DELAYED PRECONDITIONING DO NOT INDUCE HEME OXYGENASE-1 AND INDUCIBLE NITRIC OXIDE SYNTHASE.

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Brief periods of myocardial ischaemia and reperfusion are associated with enhanced myocardial protection. This phenomenon has been called "classic" ischaemic preconditioning (IPC) and lasts approximately 2 h (Murry *et al.*, 1991). It has been reported that a delayed phase of protection appears 24 h after IPC, referred to as delayed preconditioning (DP, Baxter *et al.*, 1996). Bacterial wall fragments of Gram-positive bacteria such as lipoteichoic acid (LTA) induce DP (Zacharowski *et al.*, 2000a). The protection afforded by LTA was only associated with a small increase in the mRNA levels of the cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The same study also demonstrated that the induction of TNF-α or IL-1β mRNA by LTA was only 50% or 20% of the induction afforded by lipopolysaccharide (LPS), respectively (Zacharowski *et al.*, 2000a).

The aim of this study was to investigate the relationship between two other markers of stress or inflammation and LTA-induced DP, namely inducible nitric oxide synthase (iNOS) and its product (NO), and the stress protein heme oygenase (HO) 1.

Male Wistar rats (220-260 g; n=3-4 each group) were pretreated with saline (1 ml kg^{-1} i.p.) or LTA (*S. aureus*, 1 mg kg^{-1} i.p.). Rats were anaesthetised (thiopentone sodium, 120 mg kg^{-1} i.p.) and hearts or plasma samples were taken after 2, 4, 8, 16 or 24 h, frozen and subjected to Northern blotting (HO-1 or iNOS, Frank *et al.*, 1999) or nitrate/nitrite (indicator of NO production; Zacharowski *et al.*, 2000b) analysis, respectively.

Data are expressed as mean±s.e.mean of *n* observations. ANOVA followed by Dunnett's test. * $P<0.05$ vs. saline control.

LTA did not cause an increase in any of the markers studied at any time-point (Table 1).

Table 1: Data of mRNA (HO-1 or iNOS, expressed as fold induction over saline control) and nitrate/nitrite (μmol l^{-1}).

	iNOS	nitrate/nitrite	HO-1
Control	1	40±2	1.0
LTA 2 h	<1	38±2	1.0±0.2
LTA 4 h	<1	40±3	0.6±0.1
LTA 8 h	<1	39±2	0.8±0.2
LTA 16 h	<1	39±2	0.7±0.2
LTA 24 h	<1	40±3	0.6±0.1

Surprisingly, although LTA is a bacterial wall fragment, it did not, unlike LPS (previous studies), induce the expression of either iNOS or HO-1. The mechanism(s) underlying the DP induced by LTA are therefore still unclear.

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31P *IN VIVO* IMAGING OF ET-1 BINDING TO ENDOTHELIN RECEPTORS USING [^{18}F]-ET-1 AND POSITRON EMISSION TOMOGRAPHY

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Molecular imaging with positron emission tomography (PET) is a sensitive and informative means to identify and study biological processes in normal and diseased tissue *in vivo*. The very potent vasoconstrictor endothelin (ET) plays an important role in maintaining vascular tone by action on its two receptors ET_A and ET_B. Alteration in ET function has been suggested to play a role in a number of human vascular diseases (Miyauchi & Masaki, 1999). The aim of this work has been to evaluate the potential of the ^{18}F-labelled endogenous peptide ET-1 (K_D=0.43 nM, Johnström *et al.*, 2000) to image ET receptors *in vivo*.

[^{18}F]-ET-1 was injected intravenously into isoflurane anaesthetised adult male Sprague Dawley rats (280-320 g, n=4). Blood samples were collected and at 60 min post injection the animals were killed and organs were removed for analysis. Samples of tissue were counted in a γ-counter and weighed. Storage phosphor imaging was used to analyse blood samples and to visualise distribution of radioligand in 30μm sections of tissue.

[^{18}F]-ET-1 kinetics and distribution in rat was also studied *in vivo* using PET (microPET, Concord Microsystems, USA). Images displaying the distribution of [^{18}F]-ET-1 in various organs were reconstructed from the acquired PET data. Regions-of-interest were drawn in the PET images to create curves for the accumulation of radioactivity in tissue as a function of time.

A biphasic blood curve was obtained, with the α-phase ($t_{½}$=2.9±1.0 min) likely to reflect loss of radioligand from the circulation due to binding to the ET receptor and the β-phase ($t_{½}$=50.4±13.5 min) reflecting elimination through excretion or metabolism. The apparent volume of distribution at injection was 14.9±2.7 ml (49.1±7.5 ml/kg) consistent with distribution of the label into the vascular compartment.

High uptake of [^{18}F]-ET-1 was found in lung as well as in liver and kidney (Table 1). As expected, very low levels of radioactivity were found in brain confirming that the radiolabelled peptide did not cross the blood-brain-barrier.

Table 1. In vivo biodistribution of [^{18}F]-ET-1 60 min post injection in various tissues expressed as % ID/g tissue (mean±s.e.mean)

Heart	Lung	Brain	Liver	Kidney	Muscle
1.4±0.3	19.8±1.2	0.05±0.01	10.8±2.5	12.4±2.2	0.21±0.07

Phosphor imaging of tissue sections showed the expected distribution of radioligand in the tissue, e.g. in kidney tissue high levels of radioactivity were found in the medulla and low levels of radioactivity were found in the cortex in agreement with previous *in vitro* results (Johnström *et al.*, 2000).

In the PET images uptake of [^{18}F]-ET-1 in various organs could be visualised. For example the expected difference of ET receptor density in kidney with low levels of [^{18}F]-ET-1 uptake in cortex and high levels of uptake in medulla/papilla could be delineated. A fast accumulation of radioactivity in tissue was observed which rapidly reached a steady state with no decrease in the level of radioactivity over time (up to 1 hr) indicating that the uptake reflected binding of [^{18}F]-ET-1 to ET receptors.

In conclusion, our results indicate that [^{18}F]-ET-1 is a potential radioligand for *in vivo* imaging of ET receptors in normal and diseased tissue in animals and humans using PET.

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33P A SIMPLE COMPUTATIONAL METHOD TO QUANTIFY ARRHYTHMIAS THAT INVOLVE CONTRACTILE VARIABILITY

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The relationship between force-interval and arrhythmogenesis has been well documented for the isolated heart. However, there are still difficulties in the classification, quantification and analysis of arrhythmias (Walker et al., 1988). We propose a quick and simple method (Peakfinder1) for non-clinical evaluation of anti-arrhythmic (or pro-arrhythmic) effects of drugs, which can quantify all arrhythmias that involve contractile variability in the Langendorff (retrograde) perfused isolated heart.

Male Wistar rat (250-300g) hearts were retrogradely perfused with a modified Krebs-Henseleit solution. Arrhythmias were induced by the addition of 10 μM tefluthrin (from a 10 mM stock solution in dimethylsulfoxide (DMSO)) to the perfusate (known to induce arrhythmias in this preparation (Spencer et al., 2001)). Cardiac contractile force was measured with a force transducer attached to the ventricular apex by silk thread. The electrocardiogram (ECG) and force of contraction (FOC) were continuously displayed and recorded using Chart v3.4.8.1 ® software (PowerLab ®, ADInstruments, USA) at a sampling rate of 400Hz.

The maximum peaks of the FOC during control and arrhythmic periods were calculated off-line using Peakfinder1 (from an ASCII file of the FOC trace) and the coefficient of variation (CV) was calculated. The CV can be regarded as a measure of the degree of arrhythmicity: the greater the value for the CV the more arrhythmic the heart. A paired Student's t-test was used to calculate the statistical significance between

Peakfinder1's accuracy in determining the maximum peak FOC during control periods was found to be 99.00% +/- 0.02% (n=40). Analysis of 22 hearts revealed that for a 10 minute control period, the mean % CV of the peak FOC was 0.72 +/- 0.07. To demonstrate how Peakfinder1 analyses different types of arrhythmic episodes, 1 minute periods of Ventricular Tachycardia (VT), Ventricular Fibrillation (VF), and Salvos (identified according to the Lambeth Conventions; following ECG analysis) were subjected to Peakfinder1 analysis. Peakfinder1 accurately calculated the peak FOC of the most common arrhythmic episodes, except for VF where the FOC decreased to a minimum (also a feature heart block). This however is not a limitation of Peakfinder1 since in the presence of VF or heart block FOC is zero. In 5 hearts, the % CV of the mean peak FOC of the control period (0.75 ± 0.18 %; n=5) markedly increased (1.57 ± 0.1 %; n=5) in the presence of tefluthrin (10 μM). The results show that arrhythmic periods increase the % CV of the mean peak FOC significantly ($P<0.05$).

Our method is an ideal approach for quick quantification identification of pro- or anti-antiarrhythmic effects of drugs where a "yes" or "no" answer is required, for example in secondary evaluation of pro- or anti-arrhythmic effects of compounds in a drug discovery programme.

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34P PPADS, RESPIRATORY CHEMOREFLEXES AND CAROTID SINUS NERVE DISCHARGE IN ANAESTHETISED RATS

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We have previously shown that the P2 purinoceptor (nucleotide receptor) antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) blocks some of the respiratory reflexes evoked by the P2X agonist α,β-methylene-ATP (αβMeATP), but not the increase in carotid sinus nerve discharge (McQueen et al, 1998). Zhang et al (2000) however showed that suramin, a relatively less selective P2 receptor antagonist partially blocks the neural response to hypoxia in cultured rat chemoreceptor cells. Furthermore, during recent studies, we noticed that PPADS appeared to block the ventilatory response to hypoxia and sodium cyanide in rats and therefore investigated it further.

Adult male wistar rats were anaesthetised with pentobarbital; the trachea and femoral vessels were cannulated and blood pressure, tidal volume and minute volume were recorded. Drugs were dissolved in normal saline. In 10 rats we studied the effect on minute ventilation of αβMeATP 2.0×10^{-9} – 5.9×10^{-8} mol, and in 9 rats the effect of 10% O_2. We made multiunit (2-5 fibres) neural recordings from the carotid sinus nerves in 6 rats paralysed with gallamine (8mg iv): following αβMeATP 2.0×10^{-9} – 5.9×10^{-8} mol; during 3 minutes of hypoxia (10% O_2); during 30s of asphyxia (respiratory pump stopped) and following sodium cyanide (100mg iv). All these studies were repeated following PPADS 10mg.kg^{-1} iv. Data were analysed using Microsoft Excel v2000 and GraphPad Prism v2.01. Values are shown as mean±sem, we rejected the null hypothesis at p=0.05.

PPADS significantly reduced the increase in minute ventilation evoked by hypoxia (from 188±22 to 70±11ml.min^{-1}) and significantly reduced the hyperventilatory response to αβMeATP (apparent ED_{50} increased from 3.28×10^{-8}mol to 1.15×10^{-7}mol). In neural recordings, PPADS significantly reduced basal neural discharge (from 7.2±1.8 to 1.0±0.3 impulses per second (ips)), significantly reduced the neural response to sodium cyanide (from 637±127 to 140±90 impulses) and significantly reduced the increase in basal discharge evoked by hypoxia and asphyxia (from 31.1±1.7 to 7.1±8.8 and from 46.7±11.4 to 16.8±8.6 ips respectively). However its action was variable; in 2 rats PPADS almost completely abolished the response to hypoxia, asphyxia and cyanide whereas in the other rats its effects were more modest.

We have shown that in anaesthetised rats PPADS is able to antagonise the hyperventilation evoked by hypoxia, asphyxia and P2X antagonists. That this effect is mediated, at least in part, at the level of the carotid body. There is however considerable variability between animals and in previous studies we did not detect this effect, which may be partly related to the short duration of action of PPADS and its relative lack of selectivity. Our results support evidence of an important role for ATP in chemoreception in the rat.

McQueen DS et al 1998 J. Physiol; 507:843-55
Zhang M et al 2000 J. Physiol; 535:143-58

35P ABNORMAL VASOMOTOR RESPONSES IN PATIENTS WITH THE VASODEPRESSOR FORM OF CAROTID SINUS SYNDROME

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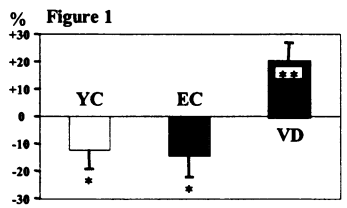
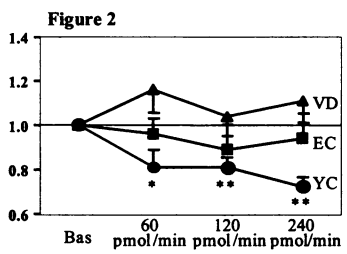
Despite the clinical relevance of the vasodepressor form of carotid sinus syndrome (VD), it is unknown whether the primary abnormality involves the baroreflex pathways in the brainstem and/or peripheral adrenoreceptors. We addressed this issue by measuring forearm arterial blood flow (FABF) during manoeuvres designed to induce either generalised sympathetic activation (lower body negative pressure, LBNP) or direct adrenoreceptor stimulation via local administration of noradrenaline (NA).

FABF was measured in both left (L) and right (R) upper limbs by venous occlusion plethysmography in 11 young controls (YC, age 24.2±0.7 years, mean±SEM), 8 elderly controls (EC, 74.1±0.6 years), and 8 patients with VD (79.0±1.7 years). Measurements were performed during baseline conditions, LBNP (-15 mmHg), and intra-arterial NA infusion in the L brachial artery at 3 progressively increasing rates (60, 120, and 240 pmol/min).

Data are expressed as percent changes in combined L and R FABF vs baseline (LBNP) and L:R FABF ratios (NA infusion). During LBNP, FABF significantly decreased in YC (-12±6%) and EC (-14±7%) but increased in VD (+20±6%) (Figure 1, * p<0.05, ** p<0.01 vs baseline).



During NA infusion, a significant forearm vasoconstriction occurred in YC whereas no significant FABF changes were observed in EC and VD (Figure 2, * p<0.05, ** p<0.01 vs baseline).



VD is associated with: i) a paradoxical vasodilatation during LBNP; ii) a marked impairment of NA-mediated vaso-constriction, which is partly explained by ageing. These vasomotor responses might be secondary to an abnormal central signal processing following baroreceptor deactivation, a reduced α_1-adrenergic activity, and/or an imbalance between α_1- and β_2-adrenergic response in the peripheral vasculature.

36P THE CYCLOPENTENONE PROSTAGLANDIN 15d-PGJ$_2$ REDUCES THE EXPRESSION OF iNOS AND OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 CAUSED BY ISCHAEMIA-REPERFUSION IN THE HEART.

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The endogenous prostaglandin D_2 metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$) is a ligand for the peroxisome-proliferator activator receptor-γ (PPAR-γ) (Ricote *et al.*, 1998) and reduces myocardial infarct size (as determined by macrochemical staining) in the anaesthetised rat (Wayman & Thiemermann, 2001). This study investigates the effects of 15d-PGJ$_2$ on (i) the increase in the plasma levels of troponin T (TnT) caused by myocardial ischaemia and reperfusion (MI/R). In addition, we have investigated whether 15d-PGJ$_2$ attenuates the expression (in the heart) of inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein-1 (MCP-1) caused by MI/R.

Twenty-seven male Wistar rats (230-290 g) were anaesthetised with thiopentone sodium (120 mg kg^{-1} i.p.). All animals were tracheotomised and ventilated (inspiratory oxygen concentra-tion: 30%; tidal volume: 8-10 ml kg^{-1}; respiration rate: 70 strokes min^{-1}). The carotid artery was cannulated to measure mean arterial blood pressure (MAP), the jugular vein for the administration of drugs. The chest was opened (left sided thoracotomy) and a 6-0 silk thread placed around the left anterior descending coronary artery (LAD). The animals were allowed to recover for 15 min. 15d-PGJ$_2$ (300 μg kg^{-1}, n=10) or its vehicle (10% v v^{-1} dimethyl sulphoxide, DMSO, 1 ml kg^{-1} n=17) was then administered as an i.v. bolus. Thirty minutes later, the LAD was occluded for 25 min and then reperfused for 2 h. In one series of experiments (n=21) the LAD was re-occluded, and 1 ml of Evans Blue dye (2% w v^{-1}) was injected into the jugular vein to determine the non-perfused (area at risk, AR). Infarct size (IS) was determined by incubation of small pieces of the AR with nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1} at 37°C for 40 min). In separate experiments, (n=6) the heart was excised and the AR and a non-ischaemic section of the heart were frozen in liquid nitrogen for the determination of iNOS and MCP-1 mRNA expression by RT-PCR analysis. All data are expressed as mean±s.e.mean. Statistical differences between groups were analysed by ANOVA followed by a Bonferroni's test. A probability of <0.05) was considered to be statistically significant.

When compared to rats, which had been subjected to thoracotomy, but not LAD-occlusion (sham-operation, n=7, IS: 1±1%,), occlusion and reperfusion of the LAD of vehicle-treated rats resulted in an infarct size of 47±4% (n=7) of the AR. When compared to sham-operated rats (TnT; 1 ng ml^{-1}), occlusion and reperfusion of the LAD of vehicle-treated rats resulted in an increase in the plasma levels of TnT to 7 ng ml^{-1} (n=7). Pre-treatment of rats with the PPAR-γ ligand 15d-PGJ2 caused a reduction in infarct size from 47±4% (vehicle-control) to 25±3% ($p<0.05$) and abolished the rise in the plasma levels of TnT (2 ng ml^{-1}, n=7). In addition, MI/R resulted in a significant increase in the expression of iNOS and MCP-1 mRNA in the AR, while no such increase was seen in the non-ischaemic myocardium. There were no significant differences for MAP or heart rate in any of the experimental groups studied.

Thus, the PPAR-γ ligand 15d-PGJ$_2$ causes a significant reduction in myocardial tissue injury (macrochemical staining with NBT and determination of plasma levels of TnT) caused by regional MI/R in the anaesthetised rat. In addtion, 15d-PGJ$_2$ reduced the expression of the pro-inflammatory proteins iNOS and MCP-1 caused by MI/R.

Ricote, M. Li, AC. Willson, TM. *et al.*, (1998). *Nature,* **391,** 79-82.

Wayman, N. & Thiemermann, C. (2001). *Br. J. Pharmacol.*, **133,** 34P.

37P EFFECTS OF THE CANNABINOID RECEPTOR ANTAGONIST, AM251, ON THE CARDIOVASCULAR RESPONSES TO THE CANNABINOID RECEPTOR AGONIST, WIN 55212-2, AND TO ANANDAMIDE, IN CONSCIOUS RATS

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In conscious rats, WIN 55212-2 (WIN) i.v. causes a rise in mean arterial blood pressure (BP), falls in renal (R) and mesenteric (M) vascular conductances (VC), and a rise in hindquarters (H)VC. Ganglion blockade abolishes the pressor and vasoconstrictor effects of WIN, but not the vasodilatation (Gardiner *et al.*, 2001). In anaesthetised rats, WIN causes hypotension which is sensitive to cannabinoid receptor antagonism (Lake *et al.*, 1997), but the possible involvement of cannabinoid receptors in the regional cardiovascular effects of WIN in conscious rats has not been explored. We have now examined the effects AM 251 (N-(piperidin-1-yl)-5-(4-iodo phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (Gatley *et al.*, 1997) on the cardiovascular effects of WIN in conscious rats. For comparison, the effects of AM 251 on responses to the endocannabinoid, anandamide, were also assessed. Under anaesthesia (fentanyl and meditomidine, 300 μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg kg^{-1} of each s.c.), male, Sprague Dawley rats (380-450g) had pulsed Doppler flow probes and, at least 14 days later, intravascular catheters, implanted. On the day following catheterisation, measurements of heart rate (HR), BP and RVC, MVC and HVC were made in conscious, freely-moving animals. WIN (50 and 150 μg kg^{-1}, in saline containing 5% propylene glycol and 2% Tween 80) and anandamide (1 and 3 mg kg^{-1}) were given i.v., starting 30 min after administration of AM 251 (3 mg kg^{-1}) or its vehicle (as for WIN).

Table 1. Effects of WIN 55212-2 (150 μg kg^{-1} at 60 and 180s) or anandamide (3 mg kg^{-1} at 10 and 30s) after administration of vehicle (VEH) or AM 251 (3 mg kg^{-1} (AM). Values for HR (beats min^{-1}), BP (mmHg) and VC (%) are mean ± s.e.mean, n=7. * $P < 0.05$ *vs* baseline (Friedman's test)

WIN 55212-2

VEH	**HR**	**BP**	**RVC**	**MVC**	**HVC**
60s	-17±13	+24±2*	-33±4*	-48±3*	+16±10
180s	+3±13	+20±3*	-27±5*	-42±4*	+55±8*
AM	**HR**	**BP**	**RVC**	**MVC**	**HVC**
60s	-4±2	+4±2	-4±3	-7±4	+3±3
180s	+5±8	+3±2	-6±4	-13±2*	+7±4

Anandamide

VEH	**HR**	**BP**	**RVC**	**MVC**	**HVC**
10s	-166±25*	-6±11	-11±5*	-38±6*	-47±6*
30s	-36±12*	+19±2*	-27±3*	-44±3*	+38±13*
AM	**HR**	**BP**	**RVC**	**MVC**	**HVC**
10s	-190±26*	-16±8	-10±3*	-51±7*	-58±8*
30s	-25±24	+22±4*	-41±7*	-57±5*	+27±9*

AM 251 markedly inhibited the cardiovascular effects of WIN, but not anandamide (Table 1), suggesting that the former, but not the latter, involve cannabinoid receptors.

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Gatley, S.J. *et al.* (1997) Life Sci., **61,** PL191-197
Lake, K.D. *et al.* (1997) J. Pharmacol. Exp. Ther., **281,** 1030-1037
Supported by the British Heart Foundation

38P CARDIOVASCULAR EFFECTS OF CORTICOTROPIN RELEASING FACTOR (CRF) COMPARED WITH HUMAN UROTENSIN II (h-UII) IN CONSCIOUS RATS

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In a recent description of the regional haemodynamic effects of h-UII (Gardiner *et al.*, 2001), we alluded to some similarity between the haemodynamic profile of h-UII and that of CRF. To quantify this observation, we have now assessed the cardiovascular effects of CRF given at the same doses, and under the same conditions, as in our experiments with h-UII. Under anaesthesia (fentanyl and meditomidine, 300 μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg kg^{-1} of each s.c.), male Sprague Dawley rats (380-450g) had pulsed Doppler flow probes and, subsequently, intravascular catheters implanted in a two-stage procedure, separated by at least 14 days. On the day following catheterisation, measurements of heart rate (HR), mean arterial blood pressure (BP) and renal (R), mesenteric (M), and hindquarters (H) vascular conductances (VC) were made in conscious, freely-moving animals, before and after i.v. administration of CRF (3, 30, 300 and 3000 pmol kg^{-1} given in ascending order).

At the two lower doses, CRF had no significant cardiovascular effects. At doses of 300 and 3000 pmol kg^{-1}, CRF, like h-UII (Gardiner *et al.*, 2001), caused dose-dependent tachycardia, hypotension and increases in MVC and HVC (Table 1). There was a fall in RVC following administration of CRF, which was not seen following h-UII (Gardiner *et al.*, 2001). Whilst the effects of CRF and h-UII on HR and BP were similar, there were differences in the time-course and magnitude of the changes in MVC and HVC (Table 2).

Table 1. Cardiovascular effects of CRF (pmol kg^{-1}) in conscious rats. Values (mean ± s.e.mean; n=8) represent the areas under or over the curves (AUC, AOC) between 0 and 30 min for HR (beats), BP (mmHg min), and VC (% min).

CRF	HR	BP	RVC	MVC	HVC
300	+455±142	-50±19	-107±38	+203±44	+69±22
3000	+3126±473	-276±76	-426±104	+648±154	+310±54

Table 2. Cardiovascular effects of CRF and h-UII (3000 pmol kg^{-1}) in conscious rats. Values (mean ± s.e.mean) are peak changes (Δ) in HR (beats min^{-1}) BP (mmHg) and VC (%) and time to peak (t min). * significantly different from CRF (Mann-Whitney U test)

	CRF (n=8)		h-UII (n=8)	
	Δ	t	Δ	t
HR	134±14	6±1	113±12	3±4
BP	-15±2	5±1	-20±3	6±1
MVC	90±8	1±0.3	63±8*	4±0.4*
HVC	50±6	49±11	83±7*	8±1*

The haemodynamic profile of CRF described here, extends previous findings in other strains of rat (Gardiner *et al.*, 1988). Although the haemodynamic effects of CRF are qualitatively similar to those of h-UII, the much earlier time to peak of HVC following h-UII makes it unlikely that this effect is due to h-UII releasing endogenous CRF (Gardiner *et al.*, 2001).

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Gardiner, S.M. *et al.* (2001) Br. J. Pharmacol., **132**, 1625-1629.

39P SENSORY NERVES INNERVATING BLOOD VESSELS INDUCE CARDIOVASCULAR AND RESPIRATORY REFLEXES IN RESPONSE TO ALGOGENS IN ANAESTHETISED RATS

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Arteries and veins receive a rich sensory innervation (Millen, 1948), mainly comprised of fine peptidergic nerves, but their precise function has yet to be identified. In addition to nociception, we hypothesise that they also play a role in the local and reflex regulation of the cardiovascular and respiratory (CVR) systems. We measured CVR reflexes evoked by administration of various pharmacological stimuli into the hindlimb vasculature of anaesthetised rats. We simultaneously recorded activity of sensory afferents on the surface of blood vessels within the hindlimb.

Experiments were licensed under U.K. Home Office regulations. Male Wistar rats (352 ± 13 g, n = 6) were anaesthetised with sodium pentobarbitone (60 mg kg^{-1} i.p., maintained with a 0.5 mg $kg^{-1}hr^{-1}$ i.v. infusion). A tracheal cannula was inserted and connected to a pneumotachograph head linked to an electrospirometer and a computerised recording system for measuring and recording tracheal airflow, and respiratory minute volume (RMV). Animals breathed room air spontaneously. The right carotid artery was cannulated for measurement of mean arterial pressure (MAP) via a blood pressure transducer. A left femoral artery catheter was advanced into the abdominal aorta to allow close i.a. administration of drugs into the right hindlimb. A suction electrode (tip I.D. ~200μm) was placed on the adventitial surface of the right femoral artery in order to record the neural activity of arterial sensory afferents. Signals were analysed off-line using a pulse-height voltage discriminator (Digitimer D130) linked to a personal computer operating Spike2 software (Cambridge Electronic Design). Neural activity and CVR reflexes evoked by bolus injections (0.3 ml i.a. over 2 sec) of capsaicin (0.3-10 nmoles), bradykinin (0.9-9 nmoles) and α,β–methylene ATP (20-60 nmoles) were investigated. Rats were killed by an overdose of anaesthetic.

Experimental data for single doses of agonists are presented in Table 1. Capsaicin and bradykinin caused a dose-dependent reflex fall in MAP, whilst α,β–methylene ATP caused a pressor response. All three agonists caused an increase in RMV. An increase in neural discharge was observed during administration of capsaicin and α,β–methylene ATP. Bradykinin evoked firing (18.4 impulses s^{-1}) in only 1/5 afferents tested, with a response latency of 5.4 s.

Table 1. Changes in blood pressure, ventilation, and sensory discharge evoked by intra-arterial injection of pharmacological stimuli.

Drug	delta MAP (mmHg)	delta RMV (ml min^{-1})	Afferent discharge (impulses s^{-1})	Afferent sensitivity (*n*/*n*)
Saline	0 ± 2	1.6 ± 2.4	0.4 ± 0.1	-
Capsaicin (0.3)	-22 ± 4 **	86.4 ± 24.3 **	12.5 ± 5.5 *	100% (6/6)
αβMe-ATP (60)	40 ± 2 **	88.1 ± 27.3 **	2.1 ± 1.1 *	50% (3/6)
Bradykinin (0.9)	-27 ± 9 **	61.4 ± 9.9 **	N/A	20% (1/5)

Values are shown as mean ± s.e.mean. Dose of drugs shown in (nmoles). MAP = mean arterial pressure, RMV = respiratory minute volume. Afferent discharge measured during 5 s immediately post-injection. * P 0.05; ** P 0.01; versus saline vehicle, unpaired Student's t test (n= 5-6 for CVR parameters, n= 3 or 6 for neural).

These data demonstrate that stimulation of sensory nerve endings within the hindlimb vasculature can induce cardiovascular and respiratory reflexes in the rat.

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40P PULMONARY VASOCONSTRICTION BY DEXFENFLURAMINE IS NOT MODIFIED BY α_1-ADRENOCEPTOR ANTAGONISM OR PRE-TREATMENT WITH AN SSRI OR SNRI IN THE WISTAR RAT LUNG

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Intake of the 5-hydroxytryptamine (serotonin; 5HT)-releasing agent dexfenfluramine (dFEN), is a risk factor for the development of pulmonary hypertension (PH; Abenhaim *et al.*, 1996). Whilst dFEN is a substrate for the 5HT transporter and facilitates the release of 5HT (Rothman *et al.*, 1999), the causative link between dFEN-induced PH and 5HT, or any other vasoconstrictor, has not been proven. We have shown that dFEN causes vasoconstriction in the isolated blood-perfused Wistar rat lung preparation (IPL), which is unaffected by $5HT_2$ receptor blockade (Sisodiya *et al.*, 2000). In the current study, we have questioned whether pre-treatment with citalopram (CIT), a selective serotonin reuptake inhibitor (SSRI) or venlafaxine (VEN), a mixed serotonin and noradrenaline reuptake inhibitor (SNRI), could influence the dFEN-induced increase in pulmonary arterial pressure (Ppa). In addition, the response has been examined in the presence of prazosin, an α_1-adrenoceptor antagonist.

Male Wistar rats (8-12 weeks) were anaesthetised with sodium pentobarbitone (Sagatal; 60mg.kg^{-1}, intraperitoneally). Their lungs were isolated *in situ*, ventilated (air + 5% CO_2) and perfused with autologous blood at a constant flow (20 mL.min^{-1}, 39°C). Changes in Ppa, which was measured continuously (Acqknowledge v 3.0.1), reflect changes in pulmonary vascular tone. A cumulative dose response curve to dFEN (0.4-144.4 μM, ≤ 0.1 mL) was constructed, either alone (n= 5) or after pre-treatment with CIT (0.25 μM, 0.3 mL; n=6), VEN (3 μM, ≤ 0.3 mL; n=7) or prazosin (0.3 μM, ≤ 0.3 mL; n=5). Saline was given as a volume control. Statistical analysis employed ANOVA with Tukey's post-hoc test.

The doses of CIT and VEN were based on their affinities for the transporters (Owens *et al.*, 1997). 0.3 μM prazosin was chosen because it selectively abolished the increase in Ppa to phenylephrine, an α_1 agonist. Results are presented as the mean change in Ppa (ΔPpa ± SEM) from the pre-dFEN baseline.

dFEN caused a dose-dependent sustained increase in Ppa in all test groups (Table 1; ANOVA $p < 0.05$). Both CIT and VEN caused a small rise in Ppa (ΔPpa ± SEM CIT 1.4 ± 0.4; VEN 1.0 ± 0.1), but did not modulate the pulmonary pressor effects of dFEN. The pressor responses were also not altered by α_1-adrenoceptor blockade.

Table 1: Cumulative (mean ΔPpa ± SEM mmHg) to dFEN is not affected by pre-treatment with CIT, VEN or prazosin

		Pretreatment		
Cumulative dFEN (μM)	dFEN alone	CIT	VEN	Prazosin
Saline	0.1 ±0.2	0.0 ± 0.1	-0.2 ± 0.0	-0.2 ± 0.1
0.4	1.8 ±0.5	1.0 ± 0.3	1.6 ± 0.5	1.7 ± 0.2
4.4	2.8 ±0.7	2.3 ± 0.5	2.6 ± 0.7	3.2 ± 0.4
44.4	6.3 ±0.9	5.0 ± 0.8	5.8 ± 1.0	5.4 ± 0.6
144.4	10.3 ± 1.1	8.2 ± 1.0	9.5 ± 1.3	7.5 ± 0.8

In summary, these data suggest that dFEN does not require the 5HT/noradrenaline transporters or α_1-adrenoceptor stimulation to mediate its pulmonary vasoconstrictor action.

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41P TRIFLUOROMETHYLPHENYLIMIDAZOLE (TRIM) PRODUCES SELECTIVE INHIBITION OF CAPACITATIVE CALCIUM ENTRY IN SMOOTH MUSCLE

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Capacitative (store-operated) calcium entry (CCE) may play an important role in excitation-contraction coupling in several smooth muscles (Gibson et al., 1998). However, research into CCE is hindered by a paucity of drugs that selectively inhibit store-operated channels (SOCs) over other calcium entry processes. In this study, we report that trifluoromethylphenylimidazole (TRIM) produces selective inhibition of thapsigargin (Tg)-induced CCE in mouse anococcygeus, and that this effect is independent of its previously reported ability to inhibit nitric oxide synthase (Moore & Handy, 1997).

Anococcygeus muscles were dissected from male mice (LACA; 25-35 g) and set up for the recording of isometric tension responses to drugs and field stimulation (Wallace *et al.*, 1999). The Krebs solution contained phentolamine (1 μM), L-N^G-nitroarginine (L-NOARG; 50 μM) and verapamil (10 μM); in addition, muscles were pre-incubated with guanethidine (30 μM; 10 min). Verapamil was omitted when responses to high K^+ Krebs (60 mM) were to be investigated; L-NOARG was absent in studies involving field stimulation. Single smooth muscle cells were freshly dispersed from the mouse anococcygeus, loaded with FURA-2, and fluorescence ratio ($R_{340/380}$) recorded by methods described fully elsewhere (Wallace *et al.*, 1999). Results are given as mean ± s.e.m with a minimum n value of 5; analysis was by Student's t test.

Tg (100 nM) produced sustained contractions (550 ± 55 mg), which we have shown to be dependent on CCE (Wallace et al., 1999). Field stimulation (10 Hz for 10 s every 100 s) of the contracted tissue caused nitrergic relaxations which were abolished by 50 μM L-NOARG, but were unaffected by TRIM (1-333 μM). Rather, TRIM produced clear concentration-related relaxation of Tg-induced tone (pD_2, 4.38 ± 0.07; 94 ± 3% relaxation at 333 μM). High K^+ caused a similar sustained contraction (440 ± 40 mg) but in this case TRIM had little effect on tone, producing only a small relaxation (12 ± 5%) at the highest concentration used. As a comparison, papaverine was equally effective as a relaxant of tone induced by either Tg (pD_2, 5.55 ± 0.07) or high K^+ (pD_2, 5.75 ± 0.09).

In FURA-2 loaded smooth muscle cells bathed in calcium-free medium, 100 nM Tg caused a small, transient increase in fluorescence ratio; subsequent re-admission of 2.5 mM calcium caused a large, sustained increase (0.23 ± 0.02) which was significantly reduced by 200 μM TRIM (by 35 ± 12%). High K^+ did not produce the transient effect seen with Tg, but calcium again caused a sustained increase in fluorescence ratio (0.27 ± 0.02) which was, however, unaffected by TRIM.

Thus, TRIM provides a novel, selective inhibitor of CCE and should be useful in improving our understanding of this ubiquitous calcium entry pathway. It may also act as a template for the production of more potent analogues, free from the complication of nitric oxide synthase inhibition.

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