1P SOMATOSTATIN SST_2 RECEPTOR KNOCK OUT MICE: PATTERNS OF SST_{1-5} RECEPTOR mRNA AND BINDING IN BRAIN BY *IN SITU* HYBRIDISATION HISTOCHEMISTRY AND RECEPTOR AUTORADIOGRAPHY

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Somatostatin (somatotropin release inhibiting factor; SRIF) via its receptors (sst_1-sst_5) regulates many physiological functions in the brain. Semi-quantitative analysis of sst_{1-5} receptor mRNA expression and SRIF receptor binding was established in sst_2 receptor knock-out (KO) mice using the reverse transcription-polymerase chain reaction (RT-PCR), *in situ* hybridisation and receptor autoradiography. Three to six month old C57B16 mice (10 wild-type (WT) controls (5M/5F) and 10 sst_2 KO (5M/5F)) were used. Experiments were performed as described (Fehlmann et al., 2000). Patterns of sst_{1-5} receptor mRNA expression in KO mice, for both RT-PCR and *in situ* hybridisation, were largely conserved for $sst_{1,3,4}$ and sst_5 selective oligonucleotide probes; whereas signals for the sst_2 oligoprobe were completely absent in KO mouse brain.

Autoradiographic analysis showed [^{125}I]-LTT $SRIF_{28}$ ([Leu^8,D-Trp^{22},^{125}I-Tyr^{25}] $SRIF_{28}$), [^{125}I]-CGP 23996 (c[Asu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser]) and [^{125}I]-Tyr^3-octreotide binding in wild type (WT) mouse brain sections. In KO mice, [^{125}I]-Tyr^3-octreotide binding (sst_2 and sst_5 selective ligand) was absent throughout the brain. In contrast, [^{125}I]-LTT $SRIF_{28}$ and [^{125}I]-CGP 23996 binding was present in KO mice, although to a lesser degree (especially in areas where [^{125}I]-Tyr^3-octreotide binding was found in WT animals).

The present data suggest that: 1) both sst_2 receptor protein and mRNA were completely absent in sst_2 KO animals brains; 2) there was no evidence of compensatory regulation, at the mRNA level of the other SRIF receptors resulting from the sst_2 receptor KO; 3) although [^{125}I]-Tyr^3-octreotide labels recombinant human and mouse sst_5 receptors, (Siehler *et al.*, 1998b; 1999a; Feuerbach *et al.*, 2000), the absence of any [^{125}I]-Tyr^3-octreotide binding in sst_2 KO mice brains, indicates that sst_5 receptors are not present, or that the low expression of sst_5 *vis-à-vis* sst_2 receptors, does not allow detection of specific sst_5 receptor binding in the brain; 4) [^{125}I]-LTT $SRIF_{28}$ and [^{125}I]-CGP 23996 label all five human and mouse SRIF receptors; evidence of their non-selective nature was apparent from their ability to label markedly more binding sites than those observed with [^{125}I]-Tyr^3-octreotide; 5) there were areas where the binding of [^{125}I]-LTT $SRIF_{28}$ and [^{125}I]-CGP 23996 was little affected by the sst_2 KO, suggesting that additional SRIF receptor subtypes may well contribute to the binding of the two radioligands. Finally, the relative distributions of these two ligands were not entirely superimposable, suggesting that their respective selectivity profiles towards the different SRIF receptor subtypes *in situ* are not identical.

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2P ELECTROPHYSIOLOGICAL ACTIONS OF GABA AND CLOMETHIAZOLE ON HUMAN RECOMBINANT $GABA_A$ RECEPTORS

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Clomethiazole (CMZ) a sedative-hypnotic and neuroprotective agent has a number of GABAmimetic actions but its molecular mechanism of action is incompletely defined (Green 1998). In this study, the actions of both GABA and CMZ were investigated quantitatively in patch clamp recordings, using recombinant $GABA_A$ receptors.

Standard whole-cell recordings were made at room temperature from mouse L(tk-) cells stably transfected with human $\alpha1\beta1\gamma2L$ or $\alpha1\beta2\gamma2S$ $GABA_A$ receptor subunits (Hadingham *et al* 1992) with a 140 mM CsCl-based intracellular solution. The extracellular solution containing (mM): NaCl (135), KCl (2.5), $MgSO_4$ (0.5), $CaCl_2$ (2.0), Na_2HPO_4 (1.0), HEPES acid (10) and glucose (10) was applied via a fast-perfusion system, with known concentrations of GABA and CMZ applied for 500 ms. Statistical analysis was performed using 2-way ANOVA.

Application of GABA or CMZ evoked large whole-cell currents in a concentration-dependent manner with reversal potentials indicative of chloride selectivity for both receptor subtypes. For $\alpha1\beta1\gamma2L$ $GABA_A$ receptors, the fitted EC_{50} was 2.3 μM for GABA (-30 mV, 95% C.I = 0.6–9 μM, n=8) and 300 μM for CMZ (-30 mV, C.I =150–600 μM, n=4). The response to GABA (10 μM) was abolished by bicuculline (100 μM, n=4) as was the response to CMZ (1 mM, n=3).

Application of GABA or CMZ to $\alpha1\beta2\gamma2S$ receptors gave significantly higher EC_{50} values compared to $\alpha1\beta1\gamma2L$ receptors (GABA: 10 μM C.I.=2.5-50 μM, F=[1,63] 13.57, $p<0.001$, -30 mV, n=3; CMZ: 1.5 mM C.I.=0.8-2.7 mM, F=[1,20] 30.18, $p<0.001$, -30 mV, n=3). Again, responses to GABA (10 μM, n=3) or CMZ (1 mM, n=3) were abolished by bicuculline (100 μM).

Electrophysiological studies using bovine chromaffin cells have previously shown CMZ to activate $GABA_A$ receptors directly only at millimolar concentrations (Hales & Lambert, 1992). Here we show that much lower concentrations of CMZ (<1 mM) can directly activate this receptor in the absence of GABA. We conclude that $\alpha1\beta1\gamma2L$ $GABA_A$ receptors have a greater sensitivity to CMZ than $\alpha1\beta2\gamma2S$ receptors. This is in line with previous findings that GABAmimetic action is dependent on receptor subunit composition (Slany *et al.*, 1995; Thompson *et al.*, 1996).

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3P EFFECTS OF SODIUM VALPROATE ON GABA AND GLUTAMATE RELEASE IN THE RAT ENTORHINAL CORTEX

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We have shown that the anticonvulsants, phenytoin and lamotrigine, modulate the release of glutamate and GABA in the entorhinal cortex (EC), decreasing the former and increasing the latter. Both effects were independent of any action at voltage-gated Na^+-channels (Cunningham *et al.*, 2000; Cunningham & Jones, 2000). Sodium valproate (NaVP) is a broad-spectrum anticonvulsant which is suggested to act primarily by enhancing GABAergic transmission, increasing synthesis and release and/or potentiating postsynaptic actions of the inhibitory transmitter (Löscher, 1999). In the present study we have determined the effect of NaVP on synaptic responses mediated by spontaneous release of glutamate and GABA in the rat EC *in vitro*.

EC slices were prepared from male Wistar rats (55-75g) Whole-cell patch clamp recordings were made from layer V neurones. Spontaneous excitatory postsynaptic currents (sEPSCs) mediated by glutamate acting at AMPA receptors were recorded using pipettes filled with a Cs-methanosulphonate based solution. To record spontaneous inhibitory postsynaptic currents (sIPSCs) mediated by GABA acting at $GABA_A$-receptors, pipettes were filled with a CsCl based solution, and AMPA and NMDA receptors were blocked with appropriate antagonists. All drugs were bath applied.

In 7 layer V neurones the mean (±SEM) inter-event interval (IEI) and amplitude of sEPSCs were 248.2 ± 7.3 ms and 11.4 ± 0.2 pA, respectively. During the bath application of NaVP (500μM) the IEI was increased to 390 ± 16.4 ms, reflecting a substantial reduction in frequency. There was no concurrent change in amplitude (11.9 ± 0.4 pA). Comparison of cumulative probability distributions of IEI using the Kolmogorov-Smirnov (KS) test showed that the change was highly significant ($P<0.0001$). In the presence of tetrodotoxin (TTX; 1 μM) the effect of NaVP on EPSC IEI was abolished (324 ± 18.4 ms *v* 336 ± 19.9 ms; n=4). Like-wise, the Ca^{++}-channel blocker, Cd^{++} (50μM) also abolished the effect of NaVP (398.5 ± 17.9 *v* 357.8 ±18.6; n=3). There were no changes in the rise or decay times of EPSCs in any condition.

In 8 layer V neurones the mean IEI and amplitude of sIPSCs were 157.3 ± 4.0 ms and 35.5 ± 0.7 pA. In the presence NaVP mean values were 195.6 ± 5.4 ms and 33.7 ± 0.7 pA. K-S analysis of cumulative probabilities showed that the increase in IEI was significant ($P<0.0001$). Again, TTX abolished this effect (173.5 ± 8.8 ms *v* 182.8 ± 9.0 ms). Finally, NaVP prolonged the decay time of the IPSCs both in the absence (7.4 ± 0.5 ms *v* 8.9 ± 0.5 ms), and presence of TTX (8.0 ± 0.2 ms *v* 9.7 ± 0.2 ms). Rise times were unaltered.

Thus, NaVP reduces the release of both glutamate and GABA, probably via blockade of presynaptic Na^+ or Ca^{++}-channels. In addition, it potentiated the postsynaptic effects of the inhibitory transmitter.

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4P DOES PHENYTOIN MODIFY GLUTAMATE AND GABA RELEASE VIA AN INTERACTION WITH PRESYNAPTIC $GABA_B$-RECEPTORS?

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Patch clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) have shown that the release of GABA can be enhanced by the anticonvulsant drug phenytoin (PHT) in the rat entorhinal cortex (EC) *in vitro* (Cunningham *et al.*, 2000). We have demonstrated a similar effect of the $GABA_B$-receptor antagonist, CGP55845, suggesting that GABA release is tonically inhibited via a presynaptic $GABA_B$ autoreceptor (Wood and Jones, 1999). We postulated that PHT might exert its effect at GABA synapses by an interaction with the autoreceptor. Thus, in the present study we sought to occlude the effect of PHT by prior treatment with CGP55845.

Whole-cell patch clamp recordings were made from layer V neurones in EC slices prepared from male Wistar rats (55-75g). sIPSCs mediated by GABA acting at $GABA_A$-receptors were recorded with pipettes were filled with a CsCl based solution containing QX-314 to ensure blockade of postsynaptic $GABA_B$-receptors. In other experiments, miniature excitatory postsynaptic currents (mEPSCs) mediated by glutamate release were recorded using pipettes filled with a Cs-methanosulphonate based solution in the presence of tetrodotoxin (0.5 μM). All drugs were bath applied.

In 5 neurones, CGP55845 (10 μM) increased the frequency of sIPSCs, reflected by a decrease in the mean (±s.e.m.) inter-event interval (IEI) from 179.2 ± 7.4 ms to 124.8 ± 4.2 ms. Comparison of cumulative probability distributions of pooled data (at least 150 sIPSCs per neurone in control and drug situations) using the Kolmogorov-Smirnov test showed the change to be significant ($P<0.00001$). The mean amplitude of sIPSCs was increased slightly from 46.1 ± 1.3 pA to 49.6 ± 1.3 pA, but this was not significant. With the addition of PHT (50 μM), the IEI was non-significantly increased (i.e. decreased frequency) to 141.4 ± 5.2 ms whereas the amplitude was slightly decreased to 43.6 ± 1.0 pA. These effects are opposite to the increased frequency and amplitude seen with phenytoin alone (Cunningham *et al.*, 2000).

The release of glutamate from excitatory synapses in the EC is decreased by PHT, reflected by a decrease in frequency of mEPSCs (Cunningham *et al.*, 2000). Surprisingly, we found that this effect was mimicked by CGP55845, the control IEI of 214.8 ± 6.2 ms increasing to 302.9 ± 9.5 ms in the presence of the antagonist (n=9). In 6 of these neurones CGP55845 prevented the decrease in frequency produced by PHT (CGP55845 291.6 ± 11.6 ms *v* 285.3 ± 12.6 ms with PHT).

The results suggest that PHT may increase GABA release by a direct interaction with the $GABA_B$ autoreceptor. The effect of CGP55845 on mEPSCs could result from increased spillover of GABA from adjacent synapses. In this scenario, the occlusion of the effect of PHT on glutamate release by CGP55845 could be secondary to an interaction at the autoreceptor rather than an interaction at the $GABA_B$ heteroreceptor.

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5P ACTIVATION OF THE NEUROKININ-3 (NK3) RECEPTOR ENHANCES RELEASE OF GLUTAMATE BUT NOT GABA IN THE RAT ENTORHINAL CORTEX

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Previous studies (Maubach *et al.*, 1998) have indicated that NK3 receptor activation may have a facilitatory effect on epileptiform activity in the rat entorhinal cortex (EC). It is possible that this could result from an alteration in the balance between excitatory and inhibitory transmission in cortical networks, since a metabotropic glutamate receptor antagonist reduced the effect. In the present study we have examined the effect of NK3 receptor activation on spontaneous glutamate and GABA release in rat EC *in vitro*.

EC slices were prepared from male Wistar rats (55-75g) Whole-cell patch clamp recordings were made from layer V neurones visualised using IR-DIC microscopy. Spontaneous excitatory postsynaptic currents (sEPSCs) mediated by glutamate acting at AMPA receptors were recorded using pipettes filled with a Cs-methanesulphonate based solution. To record spontaneous inhibitory postsynaptic currents (sIPSCs) mediated by GABA acting at $GABA_A$-receptors, pipettes were filled with a CsCl based solution, and AMPA and NMDA receptors were blocked with appropriate antagonists.

Bath application of the NK3 receptor agonist, senktide (200 nM) caused a robust increase in the frequency of sEPSCs (from a mean of 1.99±0.08 Hz to 11.37±0.49 Hz). The mean amplitude of sEPSCs was also increased from 13.83±0.29 pA to 22.82±0.56 pA. Cumulative probability distributions of pooled data (10 neurones, at least 200 events per neurone) were compared using the Kolgomorov-Smirnov test, and showed that the change in both parameters was highly significant ($P<0.005$). In contrast, application of senktide (up to 500 nM) had no significant effect on the frequency (3.37±0.13 Hz *v.* 3.09±0.14 Hz) or amplitude (28.42±1.09 pA *v* 32.33±1.22 pA) of $GABA_A$ sIPSCs (n=7). To determine the location of the NK3 receptors on glutamatergic neurones, tetrodotoxin (1μM) was used to suppress action potential dependent release and effectively isolate the presynaptic terminals. Miniature EPSCs (mEPSCs) recorded under these conditions result from random, monoquantal release. Senktide (200 nM) had no effect on mEPSC frequency (0.95±0.05 *v* 0.88±0.05 Hz) or amplitude 10.12±0.35 *v* 9.89±0.27 pA, n=5).

These data indicate that NK3 receptors are not present on GABAergic inhibitory neurones. The increase sEPSC frequency suggests that the NK3 receptor is present on glutamatergic neurones and acts to increase glutamate release. The experiments with mEPSCs suggest that the NK3 receptors are not located on glutamatergic terminals, rather they may be located on the soma, acting to enhance release through an action-potential dependent mechanism. The facilitation of glutamate release by the NK3 receptor may contribute to the "pro-convulsant" action previously reported (Maubach *et al.*, 1998). Thus, NK3 receptor *antagonists* may present a novel therapeutic target for the treatment of temporal lobe epilepsies.

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6P GROUP III METABOTROPIC GLUTAMATE RECEPTORS MODULATE EPILEPTIFORM ACTIVITY IN THE RAT ENTORHINAL CORTEX

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We have previously demonstrated that group III metabotropic glutamate receptors (mGluRs) enhance spontaneous glutamate release at terminals onto layer V neurones in the rat entorhinal cortex (Evans *et al.*, 2000). Enhancement of spontaneous release at excitatory terminals depends upon activation of protein kinase A and appears to be gated by the level of protein kinase C activity (Evans et al. 2001). In addition, we have shown that action-potential dependent, evoked glutamate release is suppressed by agonists at group III mGluRs, and that these same mGluRs inhibit spontaneous GABA release in layer V (Woodhall et al. 2001). We have hypothesised that these effects would lead to an anticonvulsant role for group III mGluR agonists in acute models of epileptiform activity, with a convulsant action of group III antagonists.

Experiments were conducted in brain slices containing EC from adult male Wistar rats (55-75g) prepared and maintained *in vitro* by conventional means. Extracellular field recordings were made from layers II and V in the EC and in area CA3 of hippocampus, using tungsten microelectrodes. Epileptiform activity was induced by bath application of the $GABA_A$ receptor antagonist, bicuculline (40μM), such that spontaneous discharges were elicited for 20-40 minutes prior to further pharmacological manipulations. In separate experiments, the group III agonists L-SOP and ACPT-1, and the antagonist CPPG were applied and ongoing spontaneous discharges were monitored. All drugs were bath applied.

During application of L-SOP (100μM), duration of epileptiform burst discharges was greatly reduced (from mean control 727 ± 73 ms to 250 ± 18 ms), as was the number of spikes within each discharge (from 4.97 ± 0.52 to 1.13 ± 0.14). These data proved to be significant when analysed with Student's t-test ($P<0.05$, n=5). Values returned to levels indistinguishable from control data upon washing (771 ± 106 ms burst duration, 5.88 ± 0.82 spikes per burst, $P>0.1$ compared to control data). Similar results were obtained with another group III mGluR agonist, ACPT-1 (n=5). In contrast, when we applied the group III mGluR antagonist CPPG, under similar conditions, an exacerbation of bursting activity was induced. Mean burst duration was increased to 1028 ± 141ms, and the number of spikes increased to 8.51 ± 0.94 ($P<0.05$, n=5). During simultaneous recordings in layers II and V we found that layer II burst activity and responses to drug application always followed that in layer V. However, when we recorded activity in layer V and CA3, changes in burst activity induced in layer V by CPPG were not mirrored in CA3. This suggests that group III mGluRs may have actions specific to the EC.

Overall, these data suggest that group III mGluRs play a role in epileptiform activity in the entorhinal cortex, and may represent potentially useful therapeutic targets.

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7P AN EVALUATION OF THE EFFECTS OF TNF-α ON LONG TERM POTENTIATION IN THE RAT DENTATE GYRUS *IN VITRO*

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The transcription factor NF-κB has been shown to be present in the neuronal cells of the hippocampus. The inactive form of NF-κB is located in the cytoplasm, bound to the inhibitory protein, IκB. Activation of NF-κB is due to phosphorylation and subsequent degradation of IκB that then enables the active form of NF-κB to translocate to the nucleus where it activates its downstream target genes. One such activator of NF-κB is the inflammatory cytokine TNF-α, whose levels are elevated in neurological conditions such as Alzheimer's disease, stroke, ischemia. These conditions are known to be associated with deficits in learning and memory.

Long term changes in synaptic strength, both strengthening and weakening as seen in long term potentiation (LTP) and long term depression (LTD), respectively, are thought to be a cellular basis for learning and memory (Bliss & Collingridge, 1993). Evidence is emerging to suggest a role for NF-κB in these processes (Albensi & Mattson, 2000) and we address these possibilities in the present study. Recordings of field excitatory post-synaptic potentials (EPSPs) were made from the medial perforant path using standard methods. LTP was induced by high frequency tetanic stimulation as previously described (Coogan et al., 1999).

TNF-α has previously been shown to inhibit LTP (Cunningham *et al*, 1996) although the mechanisms underlying the process had not been explored. A similar result was reported for interleukin-1β, which shares the same pathway as TNF- α in NF-κB activation. The use of the p38 MAP kinase inhibitor was shown to abolish the inhibitory action of IL-1β on LTP (Coogan *et al.*, 1997). We report the same inhibitory effect as previously found with TNF- α on LTP (103±8%, n=7, $p<0.05$, 60 min post-tetanus compared to controls of 156±20%, baseline). We have extended this study by monitoring the electrophysiological responses of the slice to prolonged TNF-α exposure and observed a depressive effect (EPSPs 75±8%, 2 hrs post-tetanus; 65±6%, $p<0.05$, n=7, at 3 hrs). In an attempt to explore the possible signalling mechanisms underlying the inhibitory effects of TNF-α we have characterised the ability of this pro-inflammatory cytokine to activate NFκB in the dentate gyrus. Bath application of TNF-α resulted in pronounced immunostaining of activated NF-kB in the dentate granule cells of the hippocampus.

This study demonstrates an important correlation between the induction of a pro-inflammatory phenotype and reduced synaptic efficacy.

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8P THE EFFECTS OF THE L-TYPE CA^{2+} CHANNEL AGONIST BAY K8644 AND APV ON LTP IN THE HIPPOCAMPAL CA1 REGION *IN VIVO.*

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Long-term potentiation (LTP) is a form of use dependent synaptic plasticity that is often used as a cellular correlate of learning and memory. It has been known for some time that activation of N-methyl-D-aspartate receptor channel complex (NMDA) is required for LTP in the CA1 region. This form of synaptic plasticity relies on an increase in the concentration of calcium post-synaptically.

It has been shown using hippocampal slices that a second form of LTP can be induced in the presence of the NMDA receptor antagonist AP5 (Grover & Teyler, 1990). This form of plasticity is blocked by L-type voltage dependent Ca^{2+} channel blockers. We have demonstrated recently that LTP *in vivo* can also be reduced by i.p. injection of the L-type Ca^{2+} channel blockers, verapamil and diltiazem (Freir & Herron, 2000). Here we have investigated the effects of the L-type Ca^{2+} channel agonist BAY K8644 and also the effect of combined application of the NMDA receptor antagonist AP5 and the L-type blocker verapamil on LTP.

Male Wistar rats (150-200g) were anaesthetised with urethane (ethyl carbamate, 1.5 g / kg, i.p.) and injected icv with AP5 (5μg in 5μl), BAY K8644 (10 nmol in 5μl) or vehicle (67mM sodium phosphate buffer). Verapamil was administered i.p. at 10 mg/kg. Stimulating and recording electrodes were placed in the hippocampal CA1 region using standard procedures and excitatory post-synaptic potentials (EPSPs) were evoked. Changes in synaptic efficacy were determined from the EPSP slope.

The agents tested did not alter base-line synaptic responses (0.033Hz) when recorded for at least 30 minutes post injection. High-frequency stimulation (10 trains of 10 stimuli at 200 Hz, applied 3 times with a 30s interval) induced stable LTP (60 minutes post HFS, expressed as mean % baseline ± S.E.M.) in the control (vehicle, distilled water) group (178 ± 12; n=8). LTP was however, markedly reduced following injection with AP5 (139 ± 18; n=6) or verapamil (126 ± 33; n=6,$p<0.01$). The amplitude of LTP following co-injection of AP5 and verapamil was similar to that recorded in the presence of either agent on its own (130 ± 10 n= 5). The level of LTP recorded following icv injection of BAY K8644 in sodium phosphate vehicle (157 ± 17; n=6), was higher than that observed in vehicle alone (137 ± 20; n=6); however, the increase was not significant.

These results indicate that activation of L-type Ca^{2+} and NMDA receptor/channels are required for LTP in the CA1 region *in vivo*. This suggests that facilitation of post-synaptic calcium influx may aid the molecular processes involved in LTP.

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9P ACUTE STRESS BLOCKS BOTH NMDA RECEPTOR-DEPENDENT AND INDEPENDENT LTP IN RAT HIPPOCAMPUS AREA CA1 *IN VIVO*

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Acute inescapable stress dramatically alters synaptic plasticity in the hippocampus. Stress can completely block the induction of long-term potentiation (LTP) in the CA1 area. To date NMDA receptor-dependent LTP has been investigated. However, it is not known if stress also affects the induction of voltage-sensitive Ca^{2+} channel-dependent LTP. In the present study, we investigated the effect of stress on LTP induced by different high-frequency stimulation (HFS) protocols in area CA1 *in vivo*.

Experiments were carried out on urethane (1.5 g/kg i.p.) anaesthetized male Wistar rats (250-300 g). The animal care and experimental protocol were licensed by the Department of Health. Field EPSPs were recorded from the CA1 stratum radiatum in response to stimulation of the Schaffer collateral/commissural pathway. Test EPSPs were evoked at a frequency of 0.033 Hz and intensity to give an amplitude of 50% of maximum. The intensity was increased to give an EPSP of 75% of maximum amplitude during the HFS which consisted of 10 trains of 20 stimuli, inter-stimulus interval 5 ms (200 Hz) or 2.5 ms (400 Hz), inter-train interval 2 s. Drugs were injected via a cannula in a 5 µl volume into the lateral cerebral ventricle. Inescapable stress consisted of placing rats on an elevated platform for 30 min immediately prior to anaesthesia. After the stress some animals were allowed to escape to their home cage for 30 min before anaesthesia.

The NMDA receptor antagonist AP-5 (20 mM) completely blocked LTP induced by the standard 200 Hz HFS protocol. However a combination of AP-5 and the voltage-sensitive Ca^{2+} channel blocker mibefradil (10 mM) was required to prevent the induction of LTP with the strong HFS protocol (3 sets of 400 Hz HFS given 5 min apart). In control non-stressed rats stimulation with 200 Hz induced an LTP to 148±14% (mean±SEM% baseline measured 60 min post-HFS, n=4). Subsequent 3x400 Hz HFS further increased the EPSP amplitude (184±16, n=4 at 60 min after second HFS). Exposure of rats to inescapable stress completely blocked the induction of LTP in response to both 200 Hz HFS (97±4%, n=6, $p<0.001$, Student's t test) and subsequent 3x400 Hz HFS (101±5%, n=6, $p<0.001$). We also investigated the time window for LTP recovery after exposure to stress. When rats were allowed to escape to their home cage for 30 min after the platform, LTP induced by 200 Hz HFS was partially blocked (127±7%, n=4, $p<0.05$, compared to non-stressed controls). However, additional stimulation with 3x400 Hz HFS did not increase the amplitude of EPSPs any further (122±6%, n=4).

These results provide evidence that non-NMDA receptor-dependent LTP is very sensitive to the blocking effect of stress in the hippocampus.

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10P AN INHIBITORY ROLE FOR CYCLOOXYGENSE-2 IN LONG TERM DEPRSSION IN THE RAT DENTATE GYRUS *IN VITRO*

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Long term potentiation (LTP) and depression (LTD) are two forms of activity-dependent synaptic plasticity that are thought to be involved in learning and memory. The pro-inflammatory cytokine, IL-1beta, known to have a role in apoptosis and many neurological diseases, has previously been shown to inhibit LTP both *in vivo* and *in vitro*. We have previously reported that the p38 mitogen activated protein kinase (MAPK) inhibitor, SB203580, reverses an IL-1beta-induced inhibition of LTP (Coogan et al., 1999). There are many possible substrates for the p38 MAPK cascade including phospholipase A2 and arachidonic acid, which in turn may serve as a retrograde messenger during LTP expression. In this study we have used the specific p38 MAPK inhibitor, SB220025 and the COX-2 inhibitor, NS-398 on LTP and LTD in the rat dentate gyrus.

Experiments were performed on hippocampal slices containing the dentate gyrus (350µm) of young adult rats (40-90g). Recordings of field excitatory post-synaptic potentials (fEPSPs) were made from the medial perforant pathway using standard techniques. fEPSPs were evoked at a frequency of 0.05Hz, 25-35% maximum amplitude. LTP was induced by high frequency stimulation (8 trains of 8 Pulses at 200Hz at 2s intervals at test voltage). LTD was induced using a low stimulation protocol of 1Hz for 15mins. All drugs were added to the perfusion medium. FEPSP slope changes were analysed using winwcp software (J. Demster, Strathclyde) and presented as the mean±sem. Experiments were statistically analysed at 60 min. post LTP or LTD induction, using the Student's t-test.

IL-1beta (4ng/ml) significantly inhibited LTP as has been previously reported (112 ± 5% baseline compared to controls of 200 ± 5% at 60min, $P<0.01$, n=5). The same concentration of IL-1beta had no significant effect on LTD measured over a 2 hour period (57±2% compared to 65±8% in controls, n=6). We have previously reported that the COX-2 inhibitor NS-398 (1µM) had no significant effect on control LTP (Murray & O'Connor, 2001) but did reverse the inhibitory effect of IL-1beta on LTP. However NS-398 applied alone significantly attenuated control LTD (87±5% compared to controls at 60 min, n=6, $P<0.01$). Interestingly the specific p38 MAPK inhibitor, SB220025, applied alone, also significantly reversed LTD (88±4% compared to controls at 60min, n=6, $P<0.05$). These results demonstrate a major role for the p38 MAPK and cyclooxygenase in LTD in the hippocampus.

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11P LIPOXINS, LIPOXIN STABLE ANALOGUES AND ASPIRIN-TRIGGERED LIPOXINS STIMULATE PHAGOCYTOSIS OF APOPTOTIC PMN *IN VITRO* AND *IN VIVO*

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Lipoxins (LX), an acronym for lipoxygenase interaction products, are endogenously produced eicosanoids with anti-inflammatory actions. Through their inhibition of polymorphonuclear neutrophil (PMN) chemotaxis, adhesion and transmigration LX are braking signals in PMN-mediated inflammation. Recently we reported that LX modulate a second key phase in leukocyte trafficking namely, macrophage (Mϕ) phagocytosis of apoptotic PMN *in vitro* (Godson *et al.*, 2000). Efficient removal of activated leukocytes from an inflammatory focus is essential in the resolution of inflammation. LXA_4 ($1x10^{-9}$ M) and the LX stable synthetic analogues 15(*R/S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 ($1x10^{-11}$ M; 15 mins, 37°C) stimulated greater than a 2.5 fold increase in phagocytosis versus control (0.01% ethanol). LX-triggered phagocytosis was non-phlogistic; it did not provoke release of the proinflammatory mediators IL-8 and MCP-1 and was associated with the release of the prototypic anti-inflammatory cytokine TGF-β_1.

These data suggested that LX-stimulated phagocytosis of apoptotic PMN might promote the resolution of inflammation *in vivo*. Using an *in vivo* model of thioglycollate-induced peritonitis, we investigated the ability of LXA_4, 15(*R/S*)-methyl-LXA_4 and the aspirin triggered LX epimer 15-epi-LXB_4 to stimulate phagocytosis of apoptotic PMN *in vivo*. Ten week old male BALB/C mice (25-28g) were treated i.p. with 1ml of 3% (w/v) thioglycollate for 5 days prior to assay to induce an enriched population of inflammatory Mϕ. PMN were isolated and labelled with fluorescent CMO and aged in culture for 24 hrs to render a portion apoptotic. LX were administered in a 250 µl bolus i.p. for 15 mins prior to the instillation of labelled human apoptotic PMN (approx. 3:1 PMN to Mϕ). After 30 mins mice were sacrificed and peritoneal cells harvested by lavage. Cytospin preparations of the lavages were fixed and the number of Mϕ containing ingested CMO labelled PMN were counted.

Our results demonstrate that LXA_4 (1µg) stimulated phagocytosis *in vivo* (control 27.11 +/- 0.76, LXA_4 49.74 +/- 4.06* data are mean % phagocytosis +/-SEM *$p<0.005$, n=3). Interestingly, this effect was mimicked by the stable LX analogue 15(*R/S*)-methyl-LXA_4, (0.5µg) and by the aspirin triggered epimer 15-epi-LXB_4 (0.5µg) both of which stimulated phagocytosis *in vivo* (control 26.53 +/- 1.21, 15(*R/S*)-methyl-LXA_4 53.12 +/- 3.63**, 15-epi-LXB_4 47.48 +/- 3.27**, **$p<0.0005$ n=5).

In summary, our data demonstrate that LX stimulate Mϕ phagocytosis of apoptotic PMN *in vivo*. Complemented by our *in vitro* studies these results indicate that LX may act as novel anti-inflammatory mediators stimulating the removal of redundant cells from inflammatory sites thereby promoting the resolution of inflammation.

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12P SEX HORMONES AND NSAID ANALGESIC RESPONSE IN RATS

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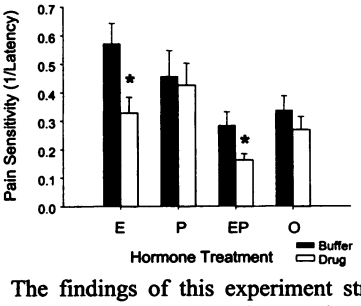
There are now several reports in the literature of gender differences in the way both humans and animals sense pain and respond to analgesic drugs (for review see Giles & Walker, 1999). Many studies suggest that sex hormones alter pain perception and symptom presentation. Others have shown that pain perception is altered with the phases of the menstrual cycle in humans or oestrus cycle in rodents (for review see Riley *et al.*, 1999). Surprisingly little has been done to examine the interaction between sex steroids and commonly employed analgesic drugs. The aim of this study was to examine the role of two female sex hormones, oestrogen and progesterone, in pain and analgesic response to ibuprofen in an animal model. Drawing on results from our human studies, we hypothesised that progesterone would antagonise the analgesic effects of ibuprofen.

Experiments were conducted with ethical approval from UNSW (ACEC99/38). The study was done in eighty ovariectomized female Sprague-Dawley rats (150-200g; ketamine-xylazine anaesthesia). The animals were allocated to one of four regimes: oestradiol (E, 5mg/kg s.c. daily); progesterone (P, 50 µg/kg s.c. daily); oestradiol+progesterone (EP, 5mg/kg and 50 µg/kg respectively s.c. daily) or oil vehicle (O). After four days' treatment, rats received either ibuprofen solution (70 mg/kg i.p.) or phosphate vehicle and pain threshold was determined at 0, 15, 30, 60 and 120 minutes post-treatment using a modification of the Tail Pinch method (Takagi et al., 1966). This involved application of an alligator clip to the base of the animal's tail and measuring the latency for reflex biting of the clip to occur. If the animal did not r damage to the tail (score then taken as 20s). Nociception was expressed as the reciprocal of response latency and results presented as mean ± SEM; values of $P< 0.05$ were considered statistically significant (ANOVA with post-hoc Bonferroni Correction)



The only significant analgesic response to ibuprofen was observed in rats treated with oestrogen (*ie* E and EP animals). Ibuprofen reduced pain sensitivity in these groups by 42% and 45% respectively (Figure 1).

The findings of this experiment strongly suggest that circulating sex hormones play an important role in exogenous analgesic response. Further research is required to determine whether this phenomenon is common to all NSAIDs. If this were the case, clinicians should take into account hormonal status when prescribing pain-relieving medication to female patients.

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13P DIFFERENTIAL UP-REGULATION OF PROSTAGLANDIN E RECEPTOR SUBTYPE mRNA IN SPINAL CORD IN ACUTE AND CHRONIC MODELS OF INFLAMMATION

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Prostaglandins (PGs), released by the action of cyclo-oxygenases on arachidonic acid, are implicated in spinal nociception and hyperalgesia (Malmberg and Yaksh, 1992), while elevated levels of spinal PGs have been described following peripheral inflammation (Hay *et al.*, 1997). Four PGE receptor subtypes have been cloned, which couple to different signal transduction pathways (Coleman *et al.*, 1994), and mRNA for each has been identified in spinal cord (Tai *et al.*, 1998). However, the distribution and function of EP receptor subtypes under inflammatory conditions is not well understood. This study set out to characterise the differential expression of EP receptor subtype mRNAs in spinal cord following acute (abdominal surgery) and chronic inflammation.

Lumbar spinal cord tissue was collected from two groups of animals. Group 1 comprised adult female sheep undergoing a midline laparotomy (for embryo retrieval) euthanased at 5 h (n = 6), 1 (n = 6), 2 (n = 6), 3 (n = 5) and 6 days (n = 6) after surgery, and age-matched healthy sheep (n = 6). Group 2 comprised sheep with chronic (> 3 months duration) unilateral inflammation of the mammary gland, mastitis (n = 5), and age-matched healthy animals (n = 5). Spinal cord tissues were processed for EP_1, EP_2, EP_3 and EP_4 mRNA using semi-quantitative real-time PCR (Heid *et al.*, 1996). Spinal cord tissue from mastitic animals was sectioned mid-line to compare ipsi- and contra-lateral sides. Data were analysed using analysis of variance with post-hoc Tukey's test.

No alterations in EP_1 mRNA occurred at any time point, and no changes in EP_1, EP_2, EP_3 or EP_4 mRNAs were detected at 5h or 1 day following surgery. EP_2 mRNA was significantly up-regulated at 2 days (over 50 fold; $p < 0.05$) and 3 days (over 100 fold; $p < 0.001$), while increased EP_3 mRNA was present at 3 days (over 200 fold; $p < 0.01$). By 6 days post-surgery, levels of EP_2 and EP_3 mRNA had returned to control levels. EP_4 mRNA was up regulated at 6 days (over 20 fold; $p < 0.01$). No change in spinal EP_1, EP_2 or EP_4 mRNA expression was evident in animals with chronic inflammation. However, there was a small but significant ipsilateral increase in EP_3 mRNA (over 3-fold; $p < 0.05$) in spinal cord relative to the contralateral side and compared to control animals.

These data indicate differential expression of EP receptor mRNAs in acute and chronic inflammation. The delayed onset of up-regulated $EP_{1,2}$ and $_3$ receptor expression following acute inflammation suggests that prostaglandin-mediated activity through these receptors may be involved in resolution of the inflammatory response. Up-regulation of EP_3 receptor mRNA following chronic inflammation confirms previous work (Dolan *et al.*, 2000), and it is now clear that other EP receptor subtypes are not involved in altered nociception associated with chronic inflammation.

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14P ROLE OF THE INDUCIBLE ISOFORM OF NITRIC OXIDE SYNTHASE (iNOS) IN A MODEL OF ESTABLISHED INFLAMMATORY PAIN IN THE RAT

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Nitric oxide (NO) has been linked with the development and maintenance of nociception (reviewed in Millan, 1999; Furst, 1999). Inhibition of NO production by N-nitro-L-arginine methyl ester prevents or reduces the hyperalgesia in chronic inflammatory pain models (Aley et al, 1998). A specific iNOS role has been shown in carrageenan induced hyperalgesia (Osborne & Coderre, 1999). Our aim was to study iNOS expression in the model of chronic inflammatory pain in rats by intraplantar injection of Freunds Complete Adjuvant (FCA) and to investigate whether (S)-2-amino-7-acetamidino-5-thioheptanoic acid (GW274150), a potent and selective iNOS inhibitor, is suitable as a treatment for this condition.

Male Random Hooded rats (200-250g) received 0.1ml FCA injection into the left hind paw. Hyperalgesia and oedema were assessed at different time points (10min-3weeks after injection) using respectively, a dual channel weight averager (Clayton et al, 1997) and a plethysmometer. Animals were perfused with Parafix 4% under pentobarbitone anaesthesia and the spinal cord, dorsal root ganglia, brain and left paw removed for iNOS immunostaining (standard avidin-biotin technique). The staining was quantified using image analysis. A second group received the FCA injection and 24h later (established inflammation) an oral dose of GW274150 (1-30mg/kg). Hyperalgesia and oedema were tested 10min-72h after dosing. Both paws and spinal cord were taken to measure nitrite levels by chemiluminescence.

iNOS immunostaining was detected locally in the paw 6h after FCA injection, with a plateau at 24-72h and falling slowly in the following weeks. This correlated with the late phase of hypersensitivity observed in the behavioural test. No central expression (brain or spinal cord) was detected. FCA injection caused an accumulation of nitrite in the left hind paw (FCA 23±3 vs. control 6.4±0.5pmol NO_2^-/mg dry weight, n=6, $p<0.01$). GW274150 (1-30mg/kg) significantly suppressed this accumulation at all the time points (e.g. FCA+inhibitor 24h post-dosing 8.4±3 vs. FCA+vehicle 23±3pmol NO_2^-/mg dry weight, n=6, $p<0.01$) indicating substantial iNOS inhibition. At the same time FCA-induced hypersensitivity and oedema were partially reversed in a dose-dependent manner. The effect (30mg/kg) on hypersensitivity was greater (e.g. 55±3% reduction 24h after dosing), with a faster onset (23±4% at 10min) and longer (54±6% at 72h) than the effect on oedema (26±4% reduction at 24h, beginning 1-3h after dosing and disappearing after 24h).

This study shows a role for iNOS in chronic inflammatory pain and the potential value of selective iNOS inhibitors in the treatment of these conditions.

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15P EFFECT OF A SELECTIVE iNOS INHIBITOR ON PROTEIN NITRATION, NEUTROPHIL ACCUMULATION AND PLASMA EXTRAVASATION IN A RAT MODEL OF THERMAL INJURY

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Reactive nitrogen species (RNS) cause protein nitration and have been implicated in the progressive tissue damage that follows thermal injury (Rawlingson *et al.*, 2000). Activation of inducible nitric oxide synthase (iNOS) is necessary for protein nitration under some circumstances (Setoguchi *et al.*, 1996). However, the importance of this enzyme in mediating protein nitration and plasma extravasation in the burn wound is not known. The aim of this study was to investigate the effect of the selective iNOS inhibitor, 1400W (Garvey *et al.* 1997), on protein nitration and vascular permeability in thermally injured rat skin. In addition, the effect of 1400W on neutrophil accumulation was examined.

Male Wistar rats (230-280 g) were anaesthetised with thiopentone sodium (Thiovet; 100 mg kg^{-1}, i.p.) in a non-recovery procedure. The abdominal skin was shaven and depilated. A cutaneous thermal injury (50°C, 10 min) was induced using a 1 cm diameter tem-perature-controlled skin heater placed onto the abdominal skin. 1400W was dissolved in saline and administered s.c. (10 mg kg^{-1}) in test animals 5 min prior to the induction of thermal injury. The animals were killed by cervical dislocation at 3 hr following burn. The skin was removed and control/burn sites were excised with a 16 mm diameter punch; snap frozen in liquid nitrogen and stored at -80°C prior to homogenisation. Protein nitration and neutrophil accumulation in homogenates were measured by 3-nitrotyrosine (3NT) ELISA (Khan *et al.*,1998) and myeloperoxidase assay (Pinter *et al.*, 1999) respectively. Plasma extravasation in skin sites was measured via the accumulation of [^{125}I]-albumin (Rawlingson *et al.*, 2000). All results are expressed as mean ±s.e.m. and were analysed by ANOVA followed by the Bonferroni's Test.

Thermal injury increased 3NT formation (1.6 ± 0.1 vs. 3.1 ± 0.2 nmol g $tissue^{-1}$; n=8; $p<0.001$), neutrophil accumulation (9.2 ± 1.2 vs. 40.4 ± 3.1 cells g $tissue^{-1}$ (10^6); n=8; $p<0.001$) and plasma extravasation (251.5 ± 72.3 vs. 1531.8 ± 292.1 µl g $tissue^{-1}$; n=4; $p<0.001$). 1400W attenuated protein nitration (3.1 ± 0.2 vs. 1.7 ± 0.1; $p<0.001$) and neutrophil accumulation (40.4 ± 3.1 vs. 22.8 ±1.7; $p<0.001$) compared to vehicle controls, but not plasma extravasation when examined at 3 hr post-burn (1531.8 ± 292.1 vs. 1633.9 ± 312.2).

We conclude that protein nitration and neutrophil accumulation in thermally injured rat skin occur in an iNOS dependent manner, and that microvascular dysfunction, as assessed by the measurement of plasma extravasation occurs independently of RNS activity in this model.

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16P ENDOTHELIAL NITRIC OXIDE MODULATES CAPSAICIN-SENSITIVE SENSORY NEUROTRANSMISSION IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

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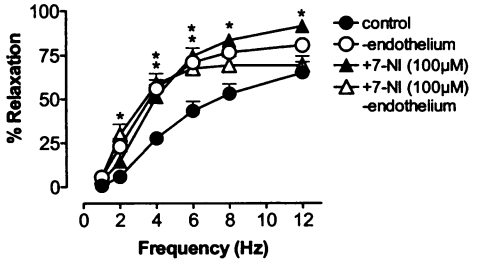
Nitric oxide (NO) can act as a neuromodulator in a variety of biological tissues, but it is unclear whether it can modulate sensory neurotransmission in the rat mesenteric arterial bed (Amerini *et al.*, 1993; Li *et al.*, 1993). Hence, the present study used two inhibitors of NO synthase (NOS), N^G-nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI), in order to investigate the possible involvement of NO as a modulator of capsaicin-sensitive sensory neurotransmission in this vascular preparation.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused via the superior mesenteric artery with oxygenated Krebs' solution at 5 ml min^{-1}, containing indomethacin (10µM), and guanethidine (5µM) to inhibit sympathetic neurotransmission (Ralevic *et al.*, 1995). After 30min equilibration, preparations were preconstricted with methoxamine (10-50µM) and electrical field stimulation (EFS; 1-12Hz, 60V, 0.1ms, 30s) was used to generate frequency-response curves. Calcitonin gene-related peptide (CGRP; 0.05-50pmol) was applied as 50µl bolus injections. Endothelium was removed with distilled water (7 min perfusion). Data reported are mean ± s.e.m. of % relaxations and were compared using ANOVA, with Tukey's *post-hoc* test. $P<0.05$ was considered significant.

EFS elicited frequency-dependent relaxations (n=8) that were augmented in the presence of L-NAME (300µM; n=11) and 7-NI (100µM; n=6) ($P<0.001$) (Figure 1). Relaxations at 4Hz were greater in the presence of L-NAME, at 51±7%, and 7-NI, at 51±7%, than in controls, at 28±3% ($P<0.01$). Dose-dependent relaxations to CGRP were not significantly different between control preparations (n=6), and in the presence of 7-NI (n=5) or L-NAME (n=10). pD_2 values were 11.6±0.1, 11.8±0.2 and 11.5±0.1, respectively. Maximal relaxations were 95±2%, 93±4% and 93±3%, respectively. After removal of the endothelium, relaxation to EFS was augmented, to a similar extent as in the presence of NOS inhibitors (n=4) (Figure 1). After endothelium removal there was no further augmentation by 7-NI of vasorelaxation to EFS (n=4) (Figure 1).

Figure 1 Effect of 7-NI and endothelium removal on EFS-mediated relaxation in rat mesenteric arterial bed



In conclusion, these data show that inhibition of NOS augments electrically-evoked sensory neurogenic relaxation of the rat isolated mesenteric arterial bed. The inhibitory effect of NO appears to be prejunctional as relaxation to exogenous CGRP was not affected. As endothelium removal mimicked the effect of NOS inhibition, and NOS inhibitors did not cause further augmentation of vasorelaxation, the likely source of the NO is the vascular endothelium.

Supported by the Royal Society.

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17P EFFECT OF NEUROKININ B ON PLASMA EXTRAVASATION AFTER INTRADERMAL AND INTRAVENOUS ADMINISTRATION IN TACHYKININ NK_1 WILDTYPE AND KNOCKOUT MICE

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It has been suggested that pre-eclampsia is associated with increased plasma levels of the tachykinin neurokinin B (NKB) (Page *et al.*, 2000). Major pathologies associated with pre-eclampsia include hypertension and systemic oedema. The aim of this study was to learn more about the receptor-dependent mechanisms via which NKB can mediate the plasma extravasation that leads to oedema formation, and to examine the role of the NK_1 receptor in these responses.

Sv129+C57BL/6 mice (both sexes, 25-35g), wild-type and NK_1 receptor knockout, were used. Anaesthesia was induced by urethane (100µl/10g; 25% w/v). Oedema formation was measured by the accumulation of ^{125}I-albumin, injected i.v (as Cao *et al.*, 1999). NKB (10-300 pmol, i.d; 30 nmol, i.v) or vehicle (Tyrode, i.d; saline, i.v) were injected and responses measured after 30 min. Results are expressed as plasma extravasation, mean ± s.e.mean and statistical analysis was by unpaired t tests.

The local cutaneous administration of NKB (10-300 pmol i.d.) produced dose-dependent oedema formation (e.g. 300 pmol 29.2±5.3µl/g vs. control, 1.8±4.2µl/g; $p<0.01$, n=8) at doses of 30 pmol or greater in wild-type mice. Over this dose range, it produced no oedema in NK_1 receptor knockout mice (300pmol, 6.7±3.9µl/g vs.control, 2.7±2.3µl/g; n.s., n=5). Knockout mice show pronounced oedema to other mediators (e.g. histamine, compound 48/80, Cao et al., 1999).

The systemic administration of NKB (30nmol i.v.) produced significant oedema in the ear, skin, lung and liver of wild-type mice. Oedema was also observed in the skin lung, kidney and liver in the NK_1 receptor knockout mice (see Table 1).

Table 1. Plasma extravasation induced by NKB in wild-type and NK_1 receptor knockout mice; *=$p<0.05$, **=$p<0.01$, compared to respective control values, n=6

Organ	Wild-type		NK1 receptor knockout	
	Control	NKB (30nmol)	Control	NKB (30nmol)
Ear	27+/-1	110+/-26**	34+/-5	40+/-1
Skin	23+/-1	97+/-24**	26+/-3	43+/-3**
Lung	309+/-48	5021+/-2059*	285+/-41	4090+/-943**
Kidney	288+/-27	509+/-146	296+/-29	411+/-16**
Uterus	126+/-23	277+/-100	139+/-25	144+/-18
Liver	176+/-22	715+/-233*	197+/-22	492+/-54**

These results demonstrate that NKB can induce NK_1-receptor-mediated oedema formation, after i.d. injection in the cutaneous microcirculation. However, significant tissue oedema is observed in the lung, kidney and liver of NK_1 knockout mice after systemic administration, suggesting the involvement of other receptor types (e.g. NK_2 or NK_3 receptors).

A.D. Grant is supported by a BHF studentship

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18P CB1 AND CB2 CANNABINOID RECEPTORS ARE IMPLICATED IN INFLAMMATORY HYPERSENSITIVITY TO PAIN

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It has been reported that cannabinoid receptor agonists (CB1 and CB2) have anti-nociceptive and anti-hypersensitivity effects in models of viseral and somatic pain (Jagger et al., 1998) as well as anti-inflammatory activity (Calignano et al 1998; Richardson et al., 1998). To determine the relative roles of CB1 and CB2 receptors in pain we have investigated the effect of the stable but non selective CB agonist HU210 and the selective CB2 agonist 1-2,3-Dichlorobenzoyl)-5-methoxyl-2methyl-(2-morpholin-4yl)ethyl)-1H-indole (GW405833) in models of nociceptive and inflammatory hypersensitivity (to mechanical stimuli; carrageenen) in the presence and absence of the selective CB1 (AM281) and CB2 (SR144528) antagonists. Male Random Hooded Rats (180g-220g) were fasted overnight. HU210 and GW405833 were administered 30mins before 100µl of 2% carrageenan into the left hind paw.

The effect of HU210 and GW405833 on carrageenan induced decrease in weight bearing on the inflamed left hind paw (dual channel weight averager, Clayton et al 1997) was determined 3 hours after the inflammatory insult. For antagonist studies AM281 (0.5mgkg^{-1}i.p.) and SR144528 (1.0-3.0mgkg^{-1} i.p.) were dosed 30mins before HU210 (10-30µgkg^{-1} i.p.) or GW405833 (3mgkg^{-1} i.p.). The effects of the compounds on carrageenan induced paw oedema were determined using a plethysmometer. Anti-nociceptive activity was determined by looking at the effect of HU210 on normal mechanical paw withdrawal thresholds using the algesymeter (Randall et al., 1957). Statistical analysis was carried out to determine if there was a significant difference between the vehicle treated group and the drug treated group using unpaired Student t test. ($p>0.05$).

HU210 produced a dose related increase in the normal mechanical paw withdrawal threshold (ED_{50}=100µgkg^{-1} i.p.), inhibited carrageenan induced decrease in weight bearing (ED_{50} =5.2µgkg^{-1} i.p.) and had significant anti-inflammatory activity. The CB1 antagonist AM281 (0.5mgkg^{-1}i.p.) inhibited the anti-hypersensitive effects and reduced the anti-inflammatory effects of HU210 (10-30µgkg^{-1}i.p.). In contrast the CB2 antagonist, SR144528 (1mgkg^{-1} i.p.) did not inhibit the anti-hypersensitive effects of HU210 (30µgkg^{-1} i.p.) but did inhibit the anti-inflammatory effects. The CB2 agonist GW405833 (0.3-3mgkg^{-1} i.p.) produced a dose related inhibition of the carrageenan induced decrease in weight bearing and reduced the paw oedema. These effects were blocked by the CB2 antagonist SR144528 (3mgkg^{-1} i.p.). SR144528 (3mgkg^{-1} i.p.) alone exacerbated the hypersensitivity and paw oedema induced by carrageenan. suggesting an endogenous cannabinoid tone.

In conclusion, these studies demonstrate that CB1 receptors are important in mediating nociceptive pain and that CB1 and CB2 have a significant role in mediating inflammatory hypersensitivity to pain. Activation of CB1 receptors is likely to produce unwanted CNS side effects as they have a wide distribution in the brain e.g. psychoactivity, sedation and dependence. CB2 receptors are expressed exclusively in peripheral tissues and are not associated with any such side effects. CB2 agonists therefore have the potential to provide safe and effective treatment of chronic inflammatory pain conditions.

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19P AN ELECTROPHYSIOLOGICAL INVESTIGATION INTO MECHANISMS VIA WHICH THE VENOM FROM THE *Phoneutria nigriventer* (PNV) SPIDER ACTIVATES THE RAT VAGUS NERVE

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In Brazil, attacks by spiders *Phoneutria* are frequent and associated with fatal consequences (Bucaretchi et al., 2000). Local pain is the main local clinical manifestation after this spider sting with local oedema and hyperemia. In experimental models, the PNV mediates neurogenic oedema formation (Costa et al., 2000). To test whether PNV contains a chemical activator of sensory C-fibres we used the grease-gap technique to compare the actions of PNV with those of capsaicin on the rat vagus nerve.

Male Wistar rats were killed humanely by exposition to $C0_2$ and the excised nerves were placed in buffer (mM): 130 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 3 KCl, 11 glucose, 5 HEPES; pH 7.4. The nerves were desheathed and transferred to grease-gap recording chambers. The DC potential between the ends of the nerve was measured using extracellular Ag/AgCl electrodes. Results are mean values ± S.E.M. for *n* animals. Statistical analysis was carried out using (ANOVA) followed by Student's unpaired t-test where appropriate. *P<0.05 was taken as significant. PNV depolarised the nerve in a dose-dependent manner (0.18 ± 0.3, 0.27 ± 0.07 and 0.62 ± 0.13 mV at 0.3, 3 and 10 μg ml^{-1} respectively, n=11). Homologous desensitisation was not observed when PNV (10 μg ml^{-1}) was re-applied 30 min after the first application. At 0.3 μM capsaicin depolarized the nerve (0.63 ± 0.10 mV, n=5) but had no effect on subsequent PNV responses.

At 5 μM, capsaicin desensitised the nerve to subsequent application of PNV. Ruthenium red (n=5) inhibited capsaicin (0.3 μM) responses (from 0.55 ± 0.16 mV to 0.49 ± 0.13, 0.26 ± 0.13* and 0.17 ± 0.05* mV at 1, 3 and 10 μM, respectively) but did not reduce PNV responses. The Na^+ channel blocker tetrodotoxin (TTX, n=4) inhibited the PNV-induced depolarisation (from 0.30 ± 0.05 mV to 0.18 ± 0.02, 0.15 ± 0.05* and 0.05 ± 0.03* mV at 1, 3 and 10 μM respectively) but had no effect on capsaicin responses. The 5-HT_4 receptor antagonist RS39604 (n=7) abolished the PNV depolarisation (from 0.61 ± 0.11 to 0.23 ± 0.09* and 0.07 ± 0.02* mV, at 1 and 10 μM respectively) and significantly inhibited 5-HT (3 μM, n=5) response (from 0.71 ± 0.06 mV to 0.46 ± 0.04* and 0.39 ± 0.07* mV, at 1 and 10 μM respectively). Both Na^+ channel agonist veratridine (30 μM) and KCl (10 mM)-induced depolarisation were unaffected by RS39604.

In the vagus nerve the PNV-induced depolarisation involves activation of Na^+ channels and 5-HT_4 receptors. The lack of effect of PNV after a high capsaicin concentration suggests that the venom acts on capsaicin-sensitive C-fibres. The evidence indicates that PNV contains a previously unidentified 5-HT_4 agonist that may have functional relevance in mediating the pain observed after spider bites.

This study was supported by FAPESP

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20P BETA ADRENOCEPTOR FUNCTION IN TRANSGENIC MICE WITH CARDIAC-SPECIFIC OVEREXPRESSION OF THE BETA 2 ADRENOCEPTOR

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In left atrial strips from transgenic (TG4) mice with cardiac-directed overexpression (~200 fold) of the β_2 adrenoceptor (β_2AR), the βAR agonist isoprenaline (ISO) elicits both positive and negative inotropy (Prendergast *et al.*, 2000). Here, the nature of these responses has been further characterized.

Isolated, mouse (16 male and 13 female TG4, weight 28±0.9g, killed by cervical dislocation) left atria were bisected, and each strip suspended in 32°C Krebs solution (applied force 0.8g), bubbled with 5% CO_2 in O_2 for isometric force of contraction (Fc) recording (as Prendergast *et al.*, 2000). Strips were electrically stimulated at 1Hz, 1ms, 130% threshold V. Preparations were exposed to antagonists (≥90min) before cumulative addition of ISO concentrations in half log molar increments. Non-linear regression was used to fit $p[A]_{50}$ and range parameters. Comparisons were by unpaired t-test.

In 6/11 TG4 controls (no antagonist), ISO elicited an increase then a decrease in Fc, whilst 5/11 showed only a decrease. These were classified "biphasic" and "monophasic" subgroups respectively. There were no differences between them in basal Fc (182±41mg, 144±40mg), or in the range (70±12mg, 46±10mg) and $p[A]_{50}$ (6.61±0.16, 6.54±0.14) of the negative inotropic effect. The mean increase in Fc was 17±7mg (n=11 including zero values), with a mean $p[A]_{50}$ of 9.52±0.08 (n=6).

In the presence of ICI-118,551 (ICI; 100nM), basal Fc was significantly reduced (14±5%; P<0.01). This could be due to a reversal of β_2AR constitutive activity by the inverse agonist properties of ICI (Bond *et al.*, 1995). In addition, every ISO curve was biphasic, and both phases were shifted equivalently: "up" phase $p[A]_{50}$ 7.91±0.05, range 61±22mg, pA_2 8.60±0.07; "down" phase $p[A]_{50}$ 5.14±0.22, range 67±23mg, pA_2 8.45±0.19. The range of the "up" phase, but not the "down", was significantly greater (P<0.05) than control.

Unlike ICI, the β_1AR antagonist CGP 20712A (100nM) had no significant effect on any parameter. It was thus reasoned that the effects of ISO were β_2AR-mediated.

β_2AR signalling has been reported to switch from Gs to Gi coupling upon phosphorylation by protein kinase A (PKA; Daaka *et al.*, 1997). As phosphorylation is also implicated in desensitisation, it was investigated whether agonist pre-treatment could modulate the change in coupling.

Acutely, 10nM ISO (~10min) elicited an increase in Fc (16±5mg, n=7), that was not different (P>0.05) from the effect of 10nM ISO as part of a cumulative curve (30±7mg, n=10). The "up" phase of the ISO curve had a $p[A]_{50}$ of 9.49±0.07, and a range of 31±7mg; the "down" phase had a $p[A]_{50}$ of 6.77±0.05, range 63±7mg. However, after acute exposure to 10nM ISO, and extensive wash out, cumulative ISO did not elicit any quantifiable concentration-dependent increase in Fc, although the $p[A]_{50}$ and range of the "down" phase (6.69±0.04 and 59±8mg, respectively; P>0.05) were not affected.

It is suggested that the change in cumulative ISO curve shape, caused by acute pre-exposure to agonist, may be due to an increase in the proportion of PKA-phosphorylated β_2AR.

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21P EFFECTS OF ADENOSINE POLYPHOSPHO GUANOSINES (Ap_nGs) AND GUANOSINE POLYPHOSPHO GUANOSINES (Gp_nGs) IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

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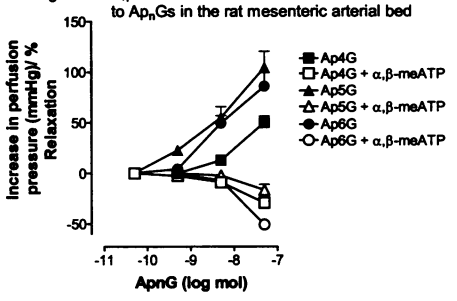
Diadenosine polyphosphates (Ap_nAs), adenosine polyphospho guanosines (Ap_nGs) and guanosine polyphospho guanosines (Gp_nGs) are naturally occurring signalling molecules released from platelets and other cells (Schlüter *et al.*, 1994). In blood vessels, the potency and type of response of Ap_nAs is determined by the number of phosphates (n) in the polyphosphate chain (Ralevic *et al.*, 1995). In general, Ap_nAs with a phosphate chain length of n=2-3 are vasodilators, acting via P2Y and P1 receptors, whilst Ap_nAs with n=4-6 are constrictors, acting via P2X receptors. Ap_nGs (n=4-6) constrict, via $P2X_1$ receptors, rat mesenteric artery segments (Lewis *et al.*, 2000), but whether they and/or Ap_nGs (n=2-3) can mediate vasorelaxation is unclear. The present study investigated the effects of Ap_nGs and Gp_nGs in the rat isolated mesenteric arterial bed.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused via the superior mesenteric artery with oxygenated Krebs' solution (37°C) at 5ml min^{-1} (Ralevic *et al.*, 1995). After 30min equilibration, preparations were preconstricted with methoxamine (10-50μM) and responses to bolus injections (50μl) of Ap_nAs/Ap_nGs/Gp_nGs were investigated in the absence and presence of antagonists (incubated for >20 min). Data are means±s.e.m. of 4-6 experiments and were compared by ANOVA, with Tukey's *post hoc* test.

Ap_nGs and Ap_nAs with a phosphate chain length of n=2-3 elicited dose-dependent vasorelaxation, and those with n=4-6 evoked dose-dependent vasoconstriction. The constrictor potency order of Ap_nGs was $Ap_5G \geq Ap_6G > Ap_4G$ (pD_{30} values 8.9±0.2, 8.7±0.2 and 7.8±0.1, respectively). A similar contractile potency order was observed for Ap_nAs, where $Ap_5A \geq Ap_6A > Ap_4A$ (pD_{30} values 8.5±0.2, 8.2±0.1 and 7.9±0.1, respectively). Pyridoxalphosphate-6-azophenyl-2'-4'-disulphonic acid (PPADS; 10μM), a P2 receptor antagonist, inhibited contractions to Ap_nGs and Ap_nAs (n=4-6) and relaxations to Ap_3G and Ap_3A (P<0.05). In the presence of α,β-methylene ATP (α,β-meATP; 10μM), to desensitize P2X receptors, contractions to Ap_nGs and Ap_nAs (n=4-6) were blocked and rapid relaxations revealed (Figure 1). Except for Gp_2G, which elicited α,β-meATP-sensitive contraction (29±5mmHg at 50nmol), Gp_nGs were inactive.

Figure 1 α,β-meATP blocks contraction and reveals relaxation to Ap_nGs in the rat mesenteric arterial bed



These data show that phosphate chain length influences the activity of Ap_nGs; those with n=4-6 are vasoconstrictors, via $P2X_1$-like receptors, and those with n=2-3 are vasodilators in the rat mesenteric arterial bed. However, Ap_nGs and Ap_nAs (n=4-6) can also mediate relaxation when contraction is blocked by desensitization of P2X receptors. At least one adenosine is crucial for vasomotor activity, as Gp_nGs generally had little or no effect on vascular tone.

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22P EVIDENCE FOR THE SUSTAINED PHASE OF ATP-INDUCED VASODILATION BEING MEDIATED BY ENDOTHELIAL DERIVED HYPERPOLARISING FACTOR

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Endothelium-derived hyperpolarising factor (EDHF) is a nitric oxide (NO) and prostacyclin independent vasodilator pathway believed to be important in the maintenance of small vessel tone. Previously, we have described a biphasic relaxant response induced by ATP in the rat isolated mesenteric artery bed, consisting of a transient first phase, mediated by NO followed, by a previously unidentified, sustained phase mediated by an unknown factor (Stanford and Mitchell 1998, Ralevic, 2001). We have investigated the possibility that this second sustained phase is mediated by EDHF.

Male Wistar rats (250-300g) were anaesthetised with sodium pentobarbitone (100 $mgkg^{-1}$; *i.p.*) and sacrificed by cervical dislocation. The mesenteric artery was cannulated and the mesentery excised. The bed was perfused at a constant rate with Kreb's buffer heated to 37°C and gassed with 95% O_2, 5% CO_2. Test drugs were added to the Krebs buffer. Perfusion pressure, measured by an arterial cannula, was raised to approx. 120 mmHg by methoxamine ($1x10^{-6}$M – $1.2\ x10^{-5}$). The effects of 1-3μl volume injections of ATP ($1x10^{-7}$ and $3x10^{-7}$ M) were recorded. In some experiments the endothelium was removed by perfusing the bed with deoxycholate detergent (5mM, 45 sec). Effective removal was determined by the absence of a dilator response to acetylcholine ($1x10^{-8}$M). All data is shown as mean±S.E.Mean.

Endothelial removal abolished the second sustained phase of ATP-induced dilation (10^{-7} and $3x10^{-7}$ M; n=5; Figure 1). Similarly, in the presence of 'high' potassium chloride (60-120 mM) the sustained phase of ATP-induced dilation was also abolished (n=4; Figure 1). By contrast, when NO and prostacyclin were both inhibited by co-administration of indomethacin (10μM) and L-NAME (1mM), the sustained phase of ATP-induced dilation was not reduced (n=4; Figure 1).

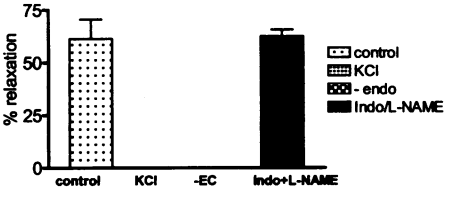

Figure 1. Effect of high KCl (60-120mM), endothelial removal (-EC) or combined NOS and COX inhibition (Indo+L-NAME) on the sustained phase of ATP (10^{-7}mol) induced relaxation (n=4-5).

These data show that the sustained phase of ATP-induced dilation is endothelium dependent, NO/prostacyclin independent and abolished with high potassium. These observations suggest that ATP activates a distinct pathway, linked to the exclusive generation of EDHF.

Stanford S.J and Mitchell J.A (1998) *Br J. Pharmacol.* **125,** 94P

Ralevic V (2001) *Br J Pharmacol.***132,** 685-92

23P THE SECOND PHASE OF ATP-EVOKED RELAXATION IN THE ISOLATED RAT MESENTERIC ARTERY BED MAY BE INDEPENDENT OF P2Y RECEPTORS

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Previously we have identified the ability of ATP to elicit both a rapid and a sustained phase of relaxation in the isolated rat mesenteric artery bed (Stanford & Mitchell, 1998). The rapid phase of relaxation induced by ATP is well studied and believed to be mediated through the activation of the P2Y receptor family. However, the potential involvement of P2Y receptors in mediating the sustained phase of ATP-induced dilation has not been addressed. In addition to ATP, other phosphate nucleotides activate P2Y receptors to cause vasodilatation. Therefore, in this study we have used ADP and UTP in comparison with ATP to characterise the receptor through which the sustained phase is mediated.

Male Wistar rats (250-300g) were anaesthetised with sodium pentobarbitone (100 $mgkg^{-1}$; *i.p.*) and sacrificed by cervical dislocation. The mesenteric artery was cannulated and the mesentery excised. The bed was perfused at a constant rate with Kreb's buffer heated to 37°C and gassed with 95% O_2, 5% CO_2. Perfusion pressure, measured by an arterial cannula, was raised to approx. 120 mmHg by methoxamine ($1x10^{-6}$M – $1.2\ x10^{-5}$) added to the perfusate. The effects of 1-3µl volume injections of ATP, ADP and UTP ($1x10^{-11}$ - $3x10^{-7}$ M) were recorded.

ATP, ADP and UTP all caused a rapid and transient relaxation in the pre-constricted rat mesenteric bed. ADP and UTP were equipotent and both more potent that ATP (Fig 1A).

By contrast, only ATP induced a subsequent prolong phase of dilation (Fig. 1B).

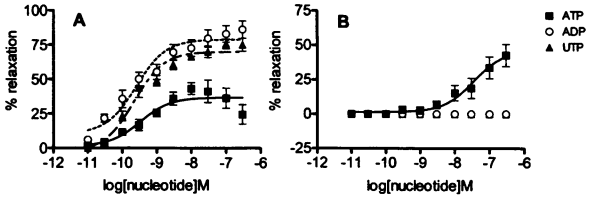

Figure 1. Effect of ATP (filled squares), ADP (open circles) and UTP(filled triangles) on the first phase (A) and second phase(B) relaxation of the isolated rat mesenteric artery bed. n=5-7.

ATP activates all P2Y receptors. At $P2Y_4$ and $_6$ UTP>ATP and at $P2Y_2$ ATP=UTP. At $P2Y_1$ receptors UTP is inactive but ADP=ATP (Virgilio *et al.*, 2001). Thus, it is likely that the transient phase of ATP induced dilation is mediated by one of the classical P2Y receptors, without the availability of ant-agonists it is not possible to suggest a subtype. However, because only ATP induced a sustained phase of relaxation, it is likely that this part of the response is not mediated by $P2Y_{1,2,4}$ or $_6$ receptors. At $P2Y_{11}$ ATP is more potent than ADP and UTP is inactive. However, since ADP was totally inactive as an inducer of a sustained phase of dilation, even $P2Y_{11}$ receptors can be ruled out. Thus, the sustained phase of ATP-induced dilation appears to be independent of classical P2Y receptor activation.

Stanford S.J and Mitchell J.A, (1998) *Br J. Pharmacol.* **125**, 94P

Di Virgilio F. *et al* (2001) *Blood* 97, 587-600

24P NUCLEOTIDE REGULATION OF PROLIFERATION IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

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Intimal proliferation of vascular smooth muscle (VSM) plays a central role in the pathology of cardiovascular diseases and restenosis following angioplasty. We have previously shown (White *et al.*, 2000) that extracellular nucleotides acting through their native P2Y receptors modulate the proliferative response to PDGF in primary cultures of human VSMC. This study has further characterised the proproliferative action of ATP and the antiproliferative effect of UTP and asked whether these nucleotides exert their primary control at the level of the cell cycle.

Studies were performed on quiescent near confluent primary cultures (p3-p7) of human saphenous vein (SV) VSM cells in 6 or 96 well plates. Incorporation of [^{3}H]-thymidine into DNA following a 1 hour pulse stimulation with PDGF-BB, nucleotides or both was used as an index of proliferation along with a colourimetric cell proliferation assay. Concentration response curves were constructed in the presence of an appropriate regeneration system to minimise interconversion of nucleotides. Following a 1 hour exposure to agonists, samples for western blot were prepared 8-24 hours later in ice-cold lysis buffer, separated by electrophoresis and the blots probed with antibodies specific for cyclins D1 and E and the cell cycle inhibitor $p27^{kip1}$.

PDGF-BB (100pM-30nM) stimulated a concentration dependent increase in [^{3}H]- thymidine incorporation ($logEC_{50}$ -8.43±0.12) which was further enhanced by 300µM ATP at every concentration, (30nM PDGF 7993±538; 30nM PDGF+300µM ATP 15880±1099 dpm/µg protein $p<0.001$). Additionally, increasing concentrations of ATP (100nM-300µM) significantly increased PDGF mediated (1nM) incorporation of [^{3}H]- thymidine in a concentration dependant manner ($logEC_{50}$,-6.35±0.27) with a maximal increase of 73.61 ± 10.8% at maximal concentrations of ATP (300µM). In contrast, UTP and UDP (100nM-300µM) significantly inhibited the PDGF response (log IC_{50} UTP -4.20±0.36; UDP -3.84±0.40) by 46.20± 2.95% and 46.32 ±3.04% respectively at maximal concentrations. Interestingly, 300µM UTP and UDP significantly decreased PDGF mediated [^{3}H]- thymidine incorporation near the $logEC_{50}$ of PDGF (3nM PDGF 14835±708; +300µM UTP, 10286±816; +300µM UDP, 6209±822; dpm/µg protein $p<0.001$). At maximal concentrations of PDGF there was no significant difference in response when UTP or UDP were present.

In protein equalised whole cell extracts, $p27^{kip1}$ was present in the unstimulated controls and downregulated by PDGF (1nM) between 18-24 hours. Levels of $p27^{kip1}$were further decreased by the addition of ATP (300µM) with an earlier onset than PDGF alone. UTP had no effect on $p27^{kip1}$ levels either alone or in the presence of PDGF. Cyclin D1 was almost undetectable in unstimulated controls with a small increase following PDGF application between 8-24 hours. Simultaneous application of PDGF with ATP or UTP did not alter levels of cyclin D1. Cyclin E levels were easily detectable and remained unchanged following stimulation with PDGF alone or with nucleotides.

ATP acts as a pro-proliferative regulator of human VSM cells derived from saphenous vein, which is coupled to reduction of $p27^{kip1}$ and increase of cyclin D1 leading to progression through the cell cycle. UTP and UDP exert strong antiproliferative effects, which appear to be independent of these regulators of the cell cycle.

White PJ, Kumari R, Porter KE, London NJM, Ng LL, Boarder MR. Antiproliferative effect of UTP on human arterial and venous smooth muscle cells. *Am J Physiol.* 2000; 276: H2735-H2742.

25P ANANDAMIDE-INDUCED RELAXATION IN RAT CORONARY ARTERY IS NOT MEDIATED BY THE VANILLOID RECEPTOR VR1

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The activation of vanilloid receptors (VR1) and the subsequent release of calcitonin gene-related peptide from sensory nerve terminals has been shown to mediate relaxation to the putative endocannabinoid anandamide in some vessels including the rat mesenteric artery (Zygmunt *et al.*, 1999). In this study, we examined the involvement of VR1 receptor in anandamide-induced relaxation in isolated rat coronary artery.

Male Wistar rats (300-400 g) were killed with an overdose of sodium pentobarbitone (60 mg kg^{-1}, i.p.). Segments (2 mm) of the coronary artery (Otley *et al.*, 1996) or third generation mesenteric artery (White & Hiley, 1997) were mounted in a wire myograph under normalised tension in oxygenated Krebs-Henseleit solution containing indomethacin (10 μM). Mesenteric arteries were precontracted with methoxamine (10 μM), and >90% and <10% relaxation to 10 μM carbachol were designated as endothelium-intact and –denuded respectively, while coronary arteries were precontracted with 5-hydroxytryptamine (3 μM), and >60% and <20% relaxation to 10 μM carbachol were designated as endothelium-intact and –denuded respectively. Responses to cumulative addition of anandamide and capsaicin to precontracted vessels are given as mean ± s.e.m. Statistical significance was determined by two-way analysis of variance.

Anandamide produced endothelium-independent relaxation in both mesenteric and coronary arteries, but was about 5-7 fold less potent and produced a lower maximum percentage relaxation in coronary arteries (Table 1). Capsaicin pre-treatment (10 μM for 30 min; $n = 3$; $P < 0.01$) or capsazepine (competitive antagonist of VR1 receptor, 3 μM; n = 4; $P < 0.01$) inhibited the relaxation to anandamide in the mesenteric artery, consistent with previous findings (Zygmunt *et al.*, 1999).

Table 1 Anandamide-induced relaxation in mesenteric and coronary artery.

		EC_{50} (μM)	R_{max} (%)	*n*
Mesenteric artery	+ EC	0.16 ± 0.01	94.3 ± 2.5	6
	- EC	0.21 ± 0.01	84.3 ± 2.6	5
Coronary artery	+ EC	0.80 ± 0.30	53.6 ± 11.5	6
	- EC	1.43 ± 0.28	54.5 ± 9.1	6

In coronary artery, capsaicin produced endothelium-independent relaxation (endothelium-intact vessels: EC_{50} = 13.7 ± 1.3 μM, R_{max} = 88.7 ± 5.9%; n = 6 vs endothelium-denuded vessels: EC_{50} = 10.5 ± 0.9 μM, R_{max} = 95.4 ± 7.8%; n = 4) which was sensitive to 5 μM capsazepine (endothelium-denuded vessels: EC_{50} = 23.9 ± 3.7 μM, R_{max} = 64.1 ± 8.3%; n = 4 in the presence of capsazepine; $P<0.01$), consistent with the presence of a functional VR1 system. However, capsaicin pre-treatment (10 μM for 30 min; $n = 4$) or capsazepine (5 μM; $n = 3$) had no significant effect on relaxation to anandamide in the coronary artery.

This study has demonstrated that anandamide-induced relaxation in rat coronary artery, unlike that in the rat mesenteric artery, is not mediated by the vanilloid receptor VR1.

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26P EFFECTS OF ANOXIA ON MECHANISMS OF RELAXATION TO ISOPRENALINE IN RAT SMALL MESENTERIC ARTERY

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Isoprenaline relaxes precontracted, small mesenteric arteries from the rat by activating β_1-adrenoceptors (Graves & Poston, 1994; White et al., 2000). Previously, we found relaxations to isoprenaline in oxygenated conditions were insensitive to the K_{ATP} inhibitor, glibenclamide, and the protein kinase A inhibitor, Rp-cAMPS (White et al., 2000). Since reducing oxygenation changes the metabolic balance of smooth muscle cells, we have re-investigated the effects of these inhibitors on the responses to isoprenaline in anoxic vessels.

Segments (2 mm) of third generation mesenteric arteries from male Wistar rats (250-300g) were mounted in a wire myograph under a normalised tension in oxygenated Krebs-Henseleit buffer at 37 C as described by Omar *et al.* (2000). The endothelium was removed by rubbing the intimal surface with a human hair and the bathing solution made anoxic by bubbling with 5% CO_2/95% N_2. After 1h, vessels were precontracted with methoxamine (10 μM), and a concentration/relaxation curve to isoprenaline determined in the presence or absence of glibenclamide (10 μM) or Rp-cAMPS (50 μM) added 30 min before determination of the responses to isoprenaline. Data were fitted to a logistic equation to determine EC_{50} and the maximal response (R_{max}) and comparison between values was by Student's t-test (Omar *et al.*, 2000).

No significant change in the potency of isoprenaline was seen between oxygenated (EC_{50} = 39±10 nM, E_{max} =82±5%, n = 10) and anoxic conditions (EC_{50} = 48±14 nM, E_{max} = 89±7%, n = 4). Unlike in oxygenated conditions however (White et al., 2000), Rp-cAMPS significantly inhibited isoprenaline relaxation in anoxia (E_{max} = 35±3%, n = 8). Glibenclamide also inhibited the effects of isoprenaline (E_{max} = 46±6%, n = 8), and the effects of Rp-cAMPS and glibenclamide were not additive (isoprenaline relaxation in the presence of both inhibitors, E_{max} = 47±3%, n = 6). Another protein kinase A inhibitor, H-89 (5 μM) also inhibited isoprenaline relaxation in anoxic vessels (E_{max} = 51±2%, n = 5), again contrasting with its lack of effect in oxygenated tissues (White *et al.*, 2000).

Anoxia did not significantly alter the relaxant effects of levcromakalim (K_{ATP} activator), NS1619 (BK_{Ca} activator) or the cyclic AMP analogue, dibutyryl cyclic AMP.

In conclusion, anoxia 'unmasks' the ability of inhibitors of K_{ATP} and protein kinase A to attenuate isoprenaline relaxation, which is not seen under oxygenated conditions (Omar et al., 2000). Since the potencies of levcromakalim, NS1619 and dibutyryl cyclic AMP were unchanged, it is possible that the effect of anoxia involves inhibition of an additional protein kinase A- and K_{ATP}-independent vasorelaxant mechanism of isoprenaline which predominates in oxygenated conditions.

RW is a Junior Research Fellow of Sidney Sussex College, Cambridge.

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27P VASCULAR ENDOTHELIAL GROWTH FACTOR-B DEFICIENT MICE EXHIBIT NORMAL ENDOTHELIUM-DEPENDENT VASORELAXATON BUT SUBNORMAL RESPONSE TO CHRONIC HYPOXIA

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Vascular endothelial growth factor-A (Vegf-A) is important for growth and differentiation of vascular endothelial cells and angiogenesis; it has been postulated to have a role during the development of hypoxic pulmonary hypertension (Christou *et al.*, 1998). The physiological/pathological functions of the related peptide, Vegf-B, are largely unknown, with the only role thus far identified being an influence on coronary vascular development (Bellomo *et al.*, 2000). In this study, mice (strain,129T2/SvEms; male and female; 15-28 g) genetically deficient in Vegf-B (*Vegfb*$^{-/-}$) have been compared with wild-type mice (*Vegfb*$^{+/+}$) with respect to (i) endothelium-dependent vasorelaxation to acetylcholine (ACh; cumulative addition) on isolated artery rings (modified Krebs solution; 37°C; 95%O_2/5%CO_2) submaximally contracted with phenylephrine (0.1 or 0.3 μM), and (ii) the development of chronic hypoxic pulmonary hypertension (hypoxic mice housed in 10% O_2 for 4 weeks; normoxic mice housed in room air, 21% O_2). Neg log EC50 values for ACh on aorta and main and intralobar pulmonary arteries (n=4-6) from *Vegfb*$^{-/-}$ mice (6.82±0.15, 6.61±0.10 and 6.74±0.20, respectively) were not different (P>0.05) from corresponding data in *Vegfb*$^{+/+}$ mice (6.87±0.13, 6.65±0.19 and 6.61±0.10); maximal relaxations (% reversal of pre-contraction) were also not different in *Vegfb*$^{-/-}$ mice (57±15, 52±6 and 41±9) and *Vegfb*$^{+/+}$ mice (55±7, 44±11 and 26±6). Chronically hypoxic *Vegfb*$^{+/+}$ mice (n=8-9) had elevated right ventricular systolic pressure (RVSP, mmHg; measured under pentobarbitone anaesthesia, 60 mg kg^{-1} i.p.: normoxic 12±1.2; hypoxic 20±1.1; P<0.001) and right ventricular hypertrophy, i.e. an increase in the ratio of the weights of right ventricle/left ventricle + septum (RV/[LV+S]: normoxic 0.27±0.02; hypoxic 0.45±0.02: P<0.001). Also, medial thickness of intralobar pulmonary arteries (measured in histological sections of left lung lobe and expressed as a % of vessel diameter; n=19=28 vessels, 150-400 μm o.d.) was significantly increased (normoxic 3.1±0.2; hypoxic 4.2±0.2; P<0.01). These features are characteristic of pulmonary hypertension. Hypoxic *Vegfb*$^{-/-}$ mice (n=8-9) did not have elevated RVSP (mmHg: normoxic 15±1.7; hypoxic 16±1.5; P>0.05) nor any increase in medial thickness (normoxic 3.1±0.2; hypoxic 3.4±0.1; P>0.05; n=15-35 vessels); furthermore, right ventricular hypertrophy (RV/[LV+S]: normoxic 0.28±0.01; hypoxic 0.39±0.01; P<0.001) was significantly (P<0.05) less pronounced than in *Vegfb*$^{+/+}$ mice.

We conclude that (i) Vegf-B deficient mice have normal endothelial vasorelaxant function and (ii) Vegf-B may have a role in the development of hypoxic pulmonary hypertension in mice. The mechanism of this role remains to be identified.

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28P EXPRESSION OF HEME OXYGENASE-1 IN AN EXPERIMENTAL MODEL OF ANGIOPLASTY AND VEIN GRAFTING: MODULATION BY CHOLESTEROL AND VITAMIN E

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Heme oxygenase-1 (HO-1) is a stress inducible enzyme whose primary function is the metabolism of heme. Furthermore exogenous induction of HO-1 has recently been shown to protect against intimal hyperplasia in an experimental model of angioplasty (Tulis *et al.*, 2001). The aim of this study was to determine the influence of dietary vitamin E and cholesterol on the expression of HO-1 post vein graft surgery and angioplasty in a rabbit model.

Four groups of male New Zealand white rabbits (2-2.5 kg) were examined. Group A was fed a standard diet without supplements. Group B was supplemented with 200 mg/kg vitamin E. Group C was fed a diet containing 0.5 % cholesterol, supplemented with 200 mg/kg vitamin E. Group D was fed a diet containing 0.5 % cholesterol. Cholesterol- and vitamin E-supplemented groups were housed for 28 days and the standard diet group for 7 days, prior to surgery. During this period pre-operative supplements of cholesterol and vitamin E were administered to the appropriate groups. During surgery, the right jugular vein was harvested, reversed and anastomosed, end on side, to the right common carotid artery. The left common carotid was balloon injured using a 2F Fogarty balloon. Cholesterol and vitamin E were administered daily during the 28 day follow up period. Serum was taken at appropriate time points, frozen and later analysed for thiobarbituric acid-reactive substances (TBARS). Tissue was harvested 28 days post surgery, immediately frozen in liquid nitrogen and analysed for HO-1 expression by Western blotting. Data from Western blotting and TBARS assays are presented as arbitrary densitometric units and μM malondialdehyde (MDA) respectively (mean ± s.e.m. of 3-5 (TBARS) or 3 (Western blotting) observations. The significance of differences between treatments was assessed by ANOVA.

Expression of HO-1 was increased in excised vein grafts from standard diet animals when compared to the corresponding untreated jugular vein (1984 ± 367 vs. 630 ± 131, p<0.05). The increase in HO-1 expression observed in vein grafts from standard diet animals was further increased by hypercholesterolaemia (4056 ± 838, p<0.01), with vitamin E attenuating this rise (866 ± 181, p<0.0001). Arterial angioplasty increased HO-1 expression when compared with an untreated carotid artery (4098 ± 588 vs. 1247 ± 583, p<0.001). A further increase was observed in balloon-injured vessels from the hypercholesterolaemic group (5834 ± 588, p<0.05), which was attenuated by vitamin E supplementation (4067 ± 756, p<0.05). Hypercholesterolaemic animals had significantly higher levels of serum MDA than animals from the standard diet group (27.65 ± 2.0 vs. 7.85 ± 0.69 μM, p<0.0001).

To conclude, HO-1 is induced in experimental models of both angioplasty and vein grafting and this induction can be modified by dietary factors. The induction of HO-1 by vascular surgery may be a physiological mechanism for the prevention of intimal hyperplasia and restenosis.

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29P FROM GENE TO FUNCTION: RECENTLY ADOPTED ORPHAN RECEPTOR FOR THE NOVEL VASODILATOR PEPTIDE GHRELIN IS UPREGULATED IN HUMAN ATHEROSCLEROTIC CORONARY ARTERY.

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The 28 amino acid peptide ghrelin (Kojima *et al.*, 1999) was recently identified as the endogenous ligand for the orphan G protein-coupled, growth hormone secretagogue receptor. The Ser^3 residue is *n*-octanoylated, a unique post-translational modification amongst naturally occurring peptides which is essential for binding. The pharmacology of this receptor system is not known. We have discovered for the first time that ghrelin is a potent vasodilator of human arteries (Wiley & Davenport, this meeting), but it is not yet determined if this receptor system is altered with vascular disease. We hypothesised that receptor density in the vasculature for this novel vasodilator may be altered in conditions associated with enhanced vasoconstriction; atherosclerosis in coronary artery (CA) and accelerated atherosclerosis in saphenous vein (SV) graft obtained at the time of heart transplantation. Receptors for vasodilator peptides are known to be present in cardiac muscle and mediate inotropic actions. We therefore determined whether ghrelin receptors are altered in cardiac muscle of the left ventricle (LV) from patients transplanted for dilated cardiomyopathy (DCM) and ischaemic heart disease (IHD), two major causes of heart failure.

Human tissues were obtained with approval from the local ethics committee. Following optimisation of binding conditions (50mM Tris, 10mM EDTA, 10mM EGTA, 1mM 4-(2-aminoethyl) benzenesulfonyl fluoride, pH 7.2, 25 min incubation at 23°C) saturation binding experiments were carried out using 0.01-1.5.nM [^{125}I-*His*^{9}]ghrelin. Non-specific binding was determined using 1μM ghrelin. For *in-vitro* receptor autoradiography, 0.15nM [^{125}I-*His*^{9}]ghrelin was incubated in the absence or presence of unlabelled ghrelin (1μM). Values of affinity (K_D) and receptor density (B_{max}) were determined using the non-iterative curve fitting KELL suite of programmes (Biosoft, Cambridge, UK).

Table 1. Ligand affinity, receptor density and Hill coefficient (n_H) for [^{125}I-*His*^{9}]ghrelin binding to human cardiovascular tissue. Values are mean ± s.e.mean, (n=4-6 individuals).

Tissue	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n_H
Normal CA	0.22 ± 0.08	6.2 ± 2.1	0.96 ± 0.03
Diseased CA	0.44 ± 0.07	28.8 ± 6.9 *	0.99 ± 0.07
Normal SV	0.29 ± 0.10	7.2 ± 2.7	1.04 ± 0.05
Diseased SV	0.25 ± 0.03	23.7 ± 1.7 *	0.89 ± 0.04
Normal LV	0.55 ± 0.14	8.1 ± 1.3	0.98 ± 0.08
DCM LV	0.44 ± 0.03	7.2 ± 1.4	1.16 ± 0.07
IHD LV	0.55 ± 0.10	5.5 ± 0.9	1.08 ± 0.05

*P<0.05 Student's unpaired *t*-test, compared to normal vessel

Ghrelin receptor density was significantly upregulated (P<0.05, approximately 4-fold) in atherosclerotic coronary artery compared with normal vessels (Table 1). Arterialisation following saphenous vein grafting into the coronary circulation also resulted in a significant increase (P<0.05, approximately 3-fold) in ghrelin receptor density compared with non-diseased saphenous veins. However, we saw no significant alteration of receptor density in diseased compared to normal left ventricle (Table 1).

We have discovered that receptors for this novel vasodilator peptide are upregulated in human atherosclerosis of coronary artery and accelerated atherosclerosis in saphenous vein grafts, suggesting a role, potentially beneficial, in human vascular pathology. Ghrelin receptors may therefore represent a novel, as yet unexploited therapeutic target in the treatment of cardiovascular disease.

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30P INCREASED POTENCY OF NEUROPEPTIDE Y TO ATTENUATE CONTRACTION VIA Y_2 RECEPTORS DURING THE DEVELOPMENT OF CARDIOMYOCYTE HYPERTROPHY

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Although a causal role for circulating neuropeptide Y (NPY) in hypertension and heart failure has not been established, a consistent finding is a correlation between the plasma concentration of the peptide and the severity of left ventricular hypertrophy (LVH) (Hulting *et al.*, 1990). In the spontaneously hypertensive rat (SHR), a model displaying many similarities with human essential hypertension, elevated systolic blood pressure is evident by 12 weeks of age and cardiac hypertrophy develops subsequently as a result of pressure overload (Doggrell & Brown, 1998.). We have shown previously in normal myocytes that NPY, acting at Y_2 receptors, attenuates the positive contractile effect of isoprenaline (McDermott *et al.*, 1997). The aim of this study was to investigate whether the response mediated by the cardiac Y_2 receptor is altered with the progression of ventricular cell hypertrophy, using the Y_2 receptor selective agonist, PYY_{3-36} and Y_2 receptor selective antagonist BIIE246 (Doods et al., 1999).

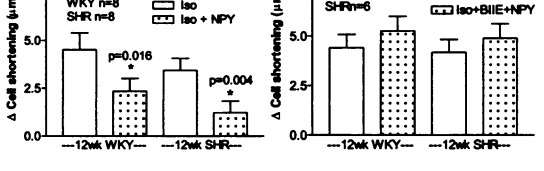
Ventricular myocytes were isolated by enzymatic digestion from excised hearts of male SHRs and control Wistar Kyoto (WKY) rats (200-400g) of 12, 16 and 20 weeks of age. Cells were stimulated at 0.5Hz with biphasic pulses of 0.5ms duration at 60V in Tyrode's buffer, and visualised at x1280 magnification. Contractile amplitude was assessed by means of video edge detection and analysed using WCP software (University of Strathclyde). Peptide concentration response relationships (10^{-12} to $3x10^{-9}$M) were obtained in a non-cumulative manner using a protocol where cells were initially challenged with isoprenaline (10^{-8}M, 4min), returned to the basal amplitude under control conditions, and then treated with either (i) peptide (15min) followed by isoprenaline (10^{-8}M) + peptide or (ii) peptide + antagonist (10^{-7}M) (15min) followed by isoprenaline (10^{-8}M) + peptide + antagonist (10^{-7}M). Data were expressed as mean±s.e.m. and analysed by two-way ANOVA with *post hoc* analysis (Bonferroni).

Cell width was greater (p<0.0001) in SHRs at 16 and 20weeks (31.2μm± 0.6 and 37.9μm±0.7, respectively) compared to WKY rats (23.6μm±0.4 and 26.4μm±0.5, respectively). The potency of NPY and PYY_{3-36} to depress amplitude in WKY myocytes was unaltered with age. There was no difference in responses to either peptide between SHR and WKY myocytes at 12weeks. However, in SHR myocytes there was a decrease in EC_{50} values at 16 and 20wks compared to age matched WKY myocytes for both NPY and PYY_{3-36} responses. Both peptides exhibited enhanced potency in 20wk SHR myocytes compared to 12wk SHR myocytes (Table).

	EC_{50} (NPY)		EC_{50} (PYY_{3-36})	
Strain	WKY n=8	SHR n=8	WKY n=8	SHR n=8
12wk	$6.24x10^{-10}$M	$2.66x10^{-10}$M	$9.78x10^{-10}$M	$1.61x10^{-10}$M
16wk	$2.46x10^{-10}$M	$6.75x10^{-11}$M ♦	$1.34x10^{-9}$M	$2.80x10^{-11}$M
20wk	$7.48x10^{-10}$M	$4.31x10^{-12}$M *♦	$6.07x10^{-10}$M	$4.32x10^{-12}$M

*=p<0.05 vs. 12wk SHR ♦=p<0.05 vs. age matched control

BIIE246 abolished the negative contractile effects of both NPY and PYY_{3-36} in myocytes from 12week (graph) and also in 16 and 20week WKY and SHR rats.



These findings demonstrate (i) evidence for the development of concentric hypertrophy in the ageing SHR, (ii) that both NPY and PYY_{3-36} exhibit increased potency to attenuate isoprenaline-stimulated contraction in ageing SHR during the active phase of ventricular cell growth, and (iii) that the negative contractile effect of NPY is mediated solely via the Y_2 receptor. In conclusion, this enhanced Y_2 receptor-mediated effect may be a compensatory mechanism to maintain normal cardiac output in the presence of increased sympathetic neuronal activity.

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31P EVIDENCE FOR ENHANCED ENDOTHELIN (ET_B) RECEPTOR AFFINITY DURING THE PROGRESSION OF HYPERTENSION- INDUCED VENTRICULAR CARDIOMYOCYTE HYPERTROPHY.

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Endothelin-1 and its receptors are up-regulated in pressure overload–induced hypertrophy *in vivo* (Arai et al., 1995). Although mixed ET_A/ET_B receptor antagonists have been shown to prevent hypertrophy *in vivo* (Karam et al., 1996), the relative contribution of the ET_B receptor subtype to the hypertrophic process remains unclear. The hypothesis that alterations occur in ETreceptors during the progression of cardiomyocyte hypertrophy *in vivo* in response to pressure overload was tested using the ageing Spontaneously Hypertensive rat (SHR) along with a normotensive control strain, the Wistar Kyoto rat (WKY). Cardiomyocyte hypertrophy was characterised by a significant increase in cellular protein in SHRs compared to age matched WKY rats (table 1). ETreceptors were characterised by radioligand binding and functional response to ETreceptor stimulation was defined in terms of effects on *de novo* protein synthesis.

Adult male SHRs or WKY rats aged 12, 16 or 20 weeks and weighing 200-400g were subjected to deep anaesthesia using isofluorane. Excised hearts were digested with collagenase (400$\mu g.ml^{-1}$) in a calcium-free Krebs Ringer buffer solution using a Langendorff perfusion apparatus. For radioligand binding, dissociated and purified ventricular cardiomyocytes were homogenised and centrifuged (20000rpm, 30min). Harvested sarcoplasmic membranes (20$\mu g.ml^{-1}$) were incubated with ^{125}ET-1 (20pM) and ET-3 (0.1-200pM) for 2hrs at 37°C in Tris/HCL 20mM, EDTA 5mM (pH 7.4). An excess of cold ET-1 (200pM) was used to measure non-specific binding (9.4 ± 0.9%, n=22). Binding was expressed as a percentage of specific binding and analysed by non-linear regression (GraphPad Prism). For functional studies, ventricular cardiomyocytes were incubated with l-U-[^{14}C]-phenylalanine (0.1$\mu Ci.ml^{-1}$ in Carnitine,Creatine,Taurine containing medium) and the appropriate concentrations of agonist for 24hrs at 37°C. The incorporation of l-U-[^{14}C]-phenylalanine into *de novo* cellular protein was quantified by counting the radioactivity of cellular protein and correcting for DNA content, measured spectrophotometrically by the incorporation of bisbenzamide dye into DNA. Statistical analysis of data was by Student's unpaired t-test and 1 and 2-factor ANOVA as required. All data are given as mean ± s.e.m. * Denotes $p<0.05$.

	12week SHR	16week SHR*	20week SHR*
protein:DNA	39.3±3.6 (n=4)	39.7±2.5 (n=6)	48.6±2.9 (n=4)

(table 1)

Displacement of ^{125}ET-1 binding by ET-3, which has a high affinity (pM) for the ET_B receptor and a low affinity for the ET_A receptor, indicated one site, of low affinity, in 12 and 16 week WKY rats (1.91±0.11nM, n=4 and 2.74± 0.06nM, n=4 respectively). In contrast, a high affinity (ET_B) and a low affinity binding site (ET_A) were obtained in 20 week WKY rats (123.7± 18.9pM and 5.3±0.5nM, n=3) respectively). Two sites were obtained in SHRs at all ages. The affinity of the ET_B receptor was increased at 16 and 20 weeks compared to 12 week SHRs (table 2)

AGE (wks)	STRAIN	n	IC_{50} (pM) ET_B	IC_{50} (nM) ET_A	$\%ET_B$
12	SHR	3	137.5 ± 12.4	3.8 ± 0.1	25 ± 4
16	SHR	4	12.9 ± 5*	2.5 ± 0.2	18 ± 3
20	SHR	4	8.2 ± 3*	3.9 ± 0.8	24 ± 3

(table 2)

Incorporation of l-U-[^{14}C]-phenylalanine into *de novo* protein in response to the ET_B receptor selective agonist sarafotoxin S6c (100pM-100nM) was enhanced in SHRs by comparison with age-matched WKY rats (table 3).

AGE			S6c response (% of basal value)			
(wks)	STRAIN	n	0.1nM	1nM	10nM	100nM
12	WKY	6	3.0 ± 3.0	7.5 ± 1.5	5.4 ± 1.7	8.7 ± 4.0
12	SHR*	12	14.8 ± 2.0	14.4 ± 2.6	17.4 ± 2.2	21.2 ± 3.6
16	WKY	11	3.1 ± 4.8	0.6 ± 3.6	2.3 ± 3.4	2.5 ± 2.6
16	SHR*	6	9.7 ± 3.4	13.0 ± 2.3	19.5 ± 2.4	15.3 ± 1.4
20	WKY	8	5.0 ± 3.5	8.1 ± 4.6	8.9 ± 2.8	7.6 ± 2.6
20	SHR*	8	7.0 ± 5.1	11.5 ± 4.5	20.9 ± 5.4	18.5 ± 4.9

(table 3)

In conclusion, ET_B receptors were detected in SHR but not WKY myocytes as early as 12 weeks. Furthermore, the enhanced affinity of ET-3 for ET_B receptors at 16 and 20 weeks compared to 12 week SHRs, coupled with increased responsiveness of SHR compared to WKY cardiomyocytes to sarafotoxin S6c at all ages, support the early and sustained involvement of ET_B receptors in the pathogenesis of hypertrophy in response to pressure overload.

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32P STRUCTURE ACTIVITY RELATIONSHIPS IN THE MODULATION OF GLUTAMATE TRANSPORT IN SYNAPTOSOMES AND HEK_{GLT1} CELLS BY FATTY ACIDS

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The polyunsaturated fatty acid, arachidonic acid has been shown to inhibit the transport of glutamate in synaptosomes (Lundy and McBean 1995), but to activate the GLT1 (EAAT2) subtype of transporter (Zerangue *et al.*, 1995). In this study, we have investigated the structural specificity of a range of polyunsaturated fatty acids for their ability to alter the activity of glutamate transporters in synaptosomes, and the rat GLT1 transporter expressed in HEK cells.

Synaptosomes from rat (male Wistar, 220 –240 g) brain were purified by Percoll density-gradient centrifugation. Sodium-dependent uptake of D-[^{3}H]aspartate was performed at 25°C for 4 min using 0-40 μM D-aspartate under conditions of initial velocity (K_m = 3.71 ± 0.67μM and V_{max} = 1.64 ± 0.09 nmol mg $protein^{-1}min^{-1}$). Stable cell lines (HEK_{GLT1}) were grown in 12-well plates, and when 80 % confluent, sodium-dependent transport of D-[^{3}H]aspartate (1-500 μM) was measured for 10 min at room temperature (K_m = 459.9 ± 83.9 μM, and V_{max} = 20.9 ± 2.2 nmol mg $protein^{-1}min^{-1}$). Fatty acids were added at either the same time as D-aspartate ('co-incubation') or at timed intervals prior to the commencement of the transport assay ('pre-incubation'). The effects of fatty acids on transport were assessed in the presence of 0.4 μM (synaptosomes) or 10 μM (HEK_{GLT1} cells) D-aspartate.

The following order of potency in inhibition of synaptosomal transport was observed: docosahexaenoic acid (DHA; 22:6) > arachidonic acid (20:4) = eicosapentaenoic acid (EPA; 20:5) > γ-linolenate (18:3 n-3) = linolenate (18:3 n-6) = linoleic (18:2) = oleic (18:1) > palmitoleic (16:1). Fatty acids possessing 3 or fewer double bonds did not significantly alter uptake of D-[^{3}H]aspartate at concentrations below 50 μM. Anandamide (arachidonylethanolamide) was inactive. After 10 min pre-incubation, 10 μM DHA reduced transport in synaptosomes to 56.8 ± 2.6 % of control, compared to 68.3 ± 3.0 % of control in co-incubation experiments ($P< 0.02$, n = 6).

In HEK_{GLT1} cells, DHA was again the most potent of the fatty acids in stimulating D-aspartate transport. Pre-incubation of the cells with 100 μM DHA increased transport to 146 ± 6 % of control, which was significantly greater than the increase obtained with co-incubation (118 ± 4 %; $P < 0.05$, n = 6).

These results show that in both synaptosomes and HEK_{GLT1} cells, DHA is more potent than arachidonic acid in its modulation of D-aspartate transport. As with arachidonic acid, an increased potency of DHA is observed when cells/ synaptosomes are pre-incubated with the fatty acid prior to measuring uptake.

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33P EFFECT OF ARCAINE AND RO 25-6981 ON LOCOMOTOR ACTIVITY IN NAÏVE AND RESERPINISED MICE

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There is great potential for the development of NMDA receptor subunit selective drugs for the treatment of Parkinson's Disease (Mutel *et al.*, 1998; Nash *et al.*, 1999). Ifenprodil and eliprodil are NR2B receptor antagonists that block polyamine binding in a non-competitive manner (Nash *et al.*, 1999). These drugs have shown some promise in animal models of Parkinson's Disease (Nash *et al.*, 1999).

In this study, it was of interest to investigate the anti-parkinsonian potential of the potent ifenprodil-based NR2B antagonist Ro 25-6981 (Mutel *et al.*, 1998) and the competitive polyamine antagonist/ partial agonist arcaine (Williams *et al.*, 1991). Locomotor count was recorded every 10 minutes over a period of 2 hours using a Panlab Actisystem, which detects horizontal movement. Significance of data was assessed using 2-way ANOVA and post-hoc analysis, and results were expressed as mean ± s.e.m. Reserpine (5 mg/kg, s.c.) was administered 24 hours prior to the study. Reserpinised mice displayed classical akinesia and tremors.

Arcaine (10, 25μg or 50μg, i.c.v.) was administered in a volume of 5μl to naïve (n=7/dose) or reserpinised (n=6/dose) male TO mice (20 – 25 g). The mice were briefly anaesthetised with isoflurane (5% gas mixture) for the injection. Ro 25-6981 (1, 5, 10, 20 or 40 mg/kg, s.c.; n=6/dose) was administered alone in naïve and reserpinised male TO mice (30 – 40 g).

Ro 25-6981 caused a significant dose-dependent stimulatory effect on locomotor activity in naïve mice, without any noticeable adverse effects. The mean locomotor count at 120 min for each of the above doses was 100 ± 3, 490 ± 21 ($P< 0.05$), 515 ± 26 ($P< 0.05$), 741 ± 68($P< 0.0001$) and 780 ± 61 ($P< 0.0001$) respectively, compared to the control locomotor count of 110 ± 9. However, in reserpinised mice, Ro 25-6981 failed to reverse akinesia or tremors (locomotor count of 2±0 with 40 mg/kg dose at t=120 min, compared to 0±0 in control animals).

The isoflurane anaesthetic had a very short-lived effect (<5 minutes) on control naïve mice. Normal levels of locomotor activity were observed throughout the 2 hour period of the study. Reserpinised control mice displayed akinesia and tremors that were indistinguishable from animals that had not received anaesthetic. Arcaine (10 or 25μg) in naïve mice failed to produce any significant effect on locomotor activity (locomotor count of 99±40 with 25μg dose at t=120 min, compared to 94±30 in control animals), but at 50μg, a small, but significant decline in locomotor count (62±20, $P< 0.05$) was observed. In reserpinised mice, arcaine (10, 25 or 50 μg) failed to reverse akinesia or tremors (locomotor count of 0±0 with 50 μg dose, compared to 40±2 in control animals at t=120 min). In summary, Ro 25-6981 stimulated locomotor count in naïve but not reserpinised mice. Arcaine had no stimulatory effect in either naïve or reserpinised animals and in fact, caused sedation at the highest dose tested in naïve animals.

These results suggest that non-competitive polyamine antagonists / NR2B antagonists may have better potential than arcaine as anti-parkinsonian drug treatments. However, the effect of Ro 25-6981 needs to be further explored.

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34P COMPARISON OF AMPHETAMINE DERIVATIVES IN THEIR ACTIONS AT ALPHA2-ADRENOCEPTORS

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We have recently shown that ecstasy (3,4-methylenedioxymethamphetamine; MDMA) has major agonist actions at prejunctional alpha2-adrenoceptors on noradrenergic nerves (Lavelle et al., 1999). We wished to examine whether this action is shared by a number of amphetamine-like agents: MDA, MDEA and the active constituent of Khat, cathinone.

We have studied prejunctional alpha2-adrenoceptors in Wistar rat and wild-type C57B mouse vas deferens (alpha2D adrenoceptors) and additionally in the alpha2A/D knockout mouse (Hein et al., 1999), in which a prejunctional alpha2C-adrenoceptor is expressed. Epididymal portions of rat (250-350g) vas deferens and whole mouse (18-28g) vas deferens were employed. Experiments were carried out in the presence of nifedipine (10μM) to reduce postjunctional actions of agonists. Tissues were electrically stimulated (supramaximal voltage, 0.5ms) with a single stimulus (rat) or 10 Hz 4 sec (mouse) to produce isometric contractions at intervals of 5 min. Drug concentrations were administered just after a stimulation and 5 min before the next.

In rat vas deferens, MDMA, cathinone, MDA and MDEA reduced stimulation-evoked contractions with pD2 values (± s.e.m., -logIC50) of 5.88±0.16, 5.80±0.31, 5.67±0.28 & 4.95±0.17 (-logM), respectively (n=4, each). In mouse vas deferens, responses to a single stimulus were small and inconsistent so that responses to 10Hz 4 sec were obtained. In the presence of nifedipine 10Hz stimulation produced isometric contractions of 0.12±0.03g (n=9) and 0.20±0.05g (n=8), in vas deferens from wild-type and knockout, respectively (response significantly greater in knockout, ANOVA, $P<0.01$). Agonists had similar potencies in vas deferens from wild-type (prejunctional α2A/D-adrenoceptor) and α2A/D-knockout mice (prejunctional α2C-adrenoceptor), except that MDEA was significantly less potent in knockout (<3.5) than wildtype (4.28±0.07).

It is concluded that MDMA, cathinone and MDA have similar potencies as agonists at alpha2D-adrenoceptors, with MDEA somewhat less potent. All of these agonists also have agonist actions at alpha2C-adrenoceptors, although MDEA was again less potent. It is likely that this action at alpha2-adrenoceptors contributes to the abuse potential and morbidity of these agents.

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35P A COMPARISON OF THE *IN VITRO* EFFECTS OF (±)-4-METHYLTHIOAMPHETAMINE AND (±)-MDMA ON 5-HT RE-UPTAKE IN THE BRAIN AND ON VASCULAR RESPONSES TO 5-HT

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4-Methylthioamphetamine (4-MTA) reportedly has psychoactive properties similar to those of 3,4-methylenedioxymethamphetamine (MDMA) (Huang *et al*, 1992). The aims of this study were to compare effects of 4-MTA and MDMA on *in vitro* vascular responses to 5-HT and on re-uptake of 5-HT into rat brain synaptosomes.

4-MTA and MDMA were synthesised according to published procedures (Butterick *et al.*, 1974; Braun *et al.*, 1980, respectively). Synaptosomes isolated from rat (male Wistar, 220-240 g) brain were purified by Percoll density-gradient centrifugation. Specific uptake of [^{3}H]5-HT was performed at 37°C for 4 min using 0-500 nM 5-HT under conditions of initial velocity. Aortic rings dissected from descending rat thoracic aorta were mounted in a tissue bath and maintained at 37°C in Krebs' bicarbonate medium continuously bubbled with 95% O_2/5% CO_2. Rings were pre-loaded to a basal tension of 1.3 g. 5-HT-mediated increases in isometric tension were measured after 15 min pre-incubation ± drug. Data were analysed using the Students' t test, $p<0.05$ was considered significant. All data are expressed as mean ± s.e.m (n=4-8).

At 100 μM, neither 4-MTA nor MDMA significantly altered the K_m for synaptosomal re-uptake of 5-HT, but reduced the V_{max} from 25.16 ± 2.46 to 6.90 ± 5.08 and 4.57 ± 0.48 pmol mg protein^{-1}min^{-1} respectively. The IC_{50} for 4-MTA and MDMA were determined in the presence of 50 nM 5-HT as 1.35 μM and 0.38 μM respectively, showing 4-MTA to be more potent. There was no significant variation of individual IC_{50} values with 5-HT concentration.

There was no significant difference in maximal contractions (E_{max}) to 5-HT in the absence and presence of 100 μM MDMA (0.93 ± 0.04 and 0.83 ± 0.06 g respectively). At 100 μM, 4-MTA was a potent inhibitor of 5-HT-mediated contraction, significantly reducing the E_{max} to 71% (0.84 ± 0.03 g) that of control rings (1.20 ± 0.05 g) The EC_{50} values for 5-HT in the presence of 4-MTA or MDMA were not significantly different from controls. At 100 μM, neither MDMA nor 4-MTA induced aortic contraction.

These results demonstrate that 4-MTA and MDMA inhibit 5-HT transport in rat brain. Furthermore, 4-MTA significantly attenuates constrictor responses to 5HT in the peripheral vasculature. Thus it appears that 4-MTA not only mimics MDMA effects in the brain, but may have added potential to compromise vascular reactivity.

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36P EFFECT OF A PRIOR NEUROTOXIC DOSE OF MDMA ON THE EXPRESSION OF ARC mRNA IN RESPONSE TO ACUTE MDMA ADMINISTRATION

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Arc (activity-regulated cytoskeleton associated gene) is an effector immediate early gene whose mRNA is selectively localised in neuronal dendrites. Its expression is induced by neuronal excitation and by stimulation of 5-HT_{2A} (Pei et al., 2000) and D_1 receptors (Fosnaugh et al., 1995). MDMA is an amphetamine analogue which induces the release of monoamines, principally 5-HT and dopamine (DA), in rat brain (Shulgin, 1986). At high doses it is also neurotoxic, causing a lasting decrease in cortical 5-HT content and selective degeneration of serotonergic nerve terminals (Colado et al., 1995). In this study we have investigated whether prior exposure to a neurotoxic dose of MDMA modifies the subsequent arc mRNA response to a second challenge dose of MDMA.

Male Dark Agouti rats (190-210g) were administered (i.p.) either saline (1ml/kg) or MDMA (12.5 mg/kg). Rats were left for 3 weeks to allow serotonergic neurodegeneration to occur, and then given a second injection of either saline (1ml/kg) or MDMA (12.5 mg/kg) and killed 2 hours later. Brains were isolated and frozen in cooled isopentane and stored at -70°C prior to use. Arc mRNA expression was analysed by in situ hybridisation histochemistry using [^{35}S]-dATP labelled oligonucleotide probe as described previously (Pei et al., 2000). Relative abundance of arc mRNA in selected areas was determined by densitometric quantification of autoradiograms using the NIH-Image system. Statistical analysis of the results was made using ANOVA and the Bonferroni post-hoc test.

Acute administration of MDMA (SM) induced a significant increase in arc mRNA expression in cortex, striatum and CA1 region of hippocampus compared to control animals (SS). Administration of MDMA 3 weeks after the neurotoxic dose (MM) induced similar increases in arc mRNA expression which were not significantly different from acute administration (SM). Animals that received saline 3 weeks following the initial neurotoxic MDMA dose (MS) showed similar arc mRNA expression to control animals except in the CA1 region which was significantly increased compared to control animals (SS).

Table 1: Effect of MDMA on arc mRNA expression.

Treatment 1	Treatment 2	Cingulate Cortex	Orbital Cortex	Striatum	CA1
S	S	100 ± 26	100 ± 17	100 ± 30	100 ± 10
S	M	238 ± 29*	200 ± 25*	648 ± 65*	176 ± 11*
M	M	309 ± 27*	250 ± 25*	697 ± 73*	162 ± 9*
M	S	142 ± 9	133 ± 13	88 ± 26	154 ± 13*

Values are expressed in terms of % Control (saline/saline) response as mean ± SEM for n=6 per group. * $P<0.05$ v Control, Bonferoni's test. S = Saline ; M = MDMA

We conclude that the expression of arc mRNA in rat brain in response to acute MDMA administration is not affected by prior exposure to a neurotoxic dose of MDMA. However, 3 weeks after the intial dose of MDMA arc mRNA expression in the hippocampal CA1 region remained significantly increased. The molecular mechanisms underlying this sustained activation of arc mRNA expression are currently under investigation.

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37P EVIDENCE FOR A CRITICAL ROLE OF BODY TEMPERATURE IN THE MODULATION OF MDMA NEUROTOXICITY BY DRUGS OF ABUSE

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The ring-substituted amphetamine derivative 3,4-methylenedioxy-methamphetamine (MDMA, 'Ecstasy') is a popular drug of abuse, which produces acute hyperlocomotion and hyperthermia and long-term decreases in markers of forebrain 5-HT function in rodents (White et al., 1996). The current study aimed to investigate the effects of common drugs of abuse on the acute hyperthermic and long-term neurotoxic actions of MDMA (20 mg/kg) in rats.

Male Wistar rats (200 – 250g) received two i.p. injections: saline or DRUG (see Table 1), followed by saline or 20 mg/kg MDMA. The acute hyperthermic effects of drug treatments were measured using aural temperature recording and area under the curve (AUC) analysis calculated from time vs. temperature plots. The long-term neurotoxic effects of drug treatments were assessed at 14 days post-injection, using h.p.l.c. to determine 5-HT and 5-HIAA concentrations, and [^{3}H]paroxetine binding to measure 5-HT transporter density in the rat frontal cortex (O'Loinsigh et al., 2001). The average decrease in these three markers of 5-HT nerve terminal integrity was then calculated to give a single regional marker of 5-HT neurotoxicity.

Administration of 20 mg/kg MDMA produced an acute hyperthermic response ($p < 0.01$ vs. saline-treated animals, 2-way ANOVA followed by Newman-Keul's test) during the 5 h post-injection. MDMA also produced long-term reductions in frontal cortex 5-HT ($p < 0.01$) and 5-HIAA concentrations ($p < 0.05$) compared to saline-treated animals (2-way ANOVA, followed by Fisher's LSD test). The average % decrease in the three markers of 5-HT nerve terminal integrity was 15.23 ± 2.10 % (n = 10).

The effects of co-treatment with various drugs of abuse on MDMA-induced acute hyperthermia and long-term decreases in 5-HT markers are displayed in Table 1. Linear regression analysis revealed a direct correlation between the magnitude of acute hyperthermia and the extent of 5-HT neurotoxicity in the cortex ($r = 0.7547$, $p < 0.05$) after DRUG/MDMA co-treatments. These results extend previous findings that acute hyperthermia plays a critical role in the production of long-term serotonergic neurotoxicity by MDMA (Malberg and Seiden, 1998). They further indicate that the development of serotonergic neurotoxicity in human ecstasy users may be influenced by co-use of other psychoactive drugs of abuse.

Table 1: The effect of co-treatment with drugs of abuse on MDMA-induced hyperthermia and long-term serotonergic neurotoxicity.

Co-treatment	Hyperthermia (AUC)	% Decrease in frontal cortex 5-HT markers
MDMA alone (20 mg/kg)	368.4 ± 25.3	15.23 ± 2.10
+ Caffeine (5 mg/kg)	476.2 ± 40.3**	23.87 ± 3.33
+ Amphetamine (1 mg/kg)	445.2 ± 38.3	19.40 ± 3.93
+ Diazepam (5 mg/kg)	339.9 ± 61.2	12.47 ± 1.83
+ LSD (0.1 mg/kg)	323.6 ± 57.3	10.43 ± 3.10
+ Ethanol (1.5 g/kg)	266.9 ± 48.9*	8.97 ± 2.82
+ Ketamine (15 mg/kg)	265.0 ± 51.1*	7.07 ± 2.39
+ Δ^9-THC (5 mg/kg)	243.0 ± 42.4*	17.33 ± 1.00

Data are expressed as mean ± SEM (n = 6 - 10). * $p < 0.05$, ** $p < 0.01$ vs. MDMA alone group (2-way ANOVA followed by Newman-Keul's test).

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38P METHYLENEDIOXYMETHAMPHETAMINE (MDMA; "ECSTASY") SUPPRESSES THE PRIMARY IgG RESPONSE TO AN ANTIGENIC CHALLENGE IN RATS

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Methylenedioxymethamphetamine (MDMA; "Ecstasy") is a widely abused amphetamine derivative in humans. We previously demonstrated that MDMA suppresses T-lymphocyte and macrophage activity in rats (Connor et al., 2000a,b). In the present study we examined the effects of acute MDMA administration on responsiveness to an *in vivo* immune challenge with the soluble protein antigen keyhole limpet haemocyanin (KLH) in female Sprague-Dawley rats (220-250 g). MDMA (2.5, 5 or 10 mg/kg; i.p.) was co-administered with KLH (100 μg/kg; i.p.). Serum IgM was measured 7 days, and serum IgG was measured 14 days following the KLH challenge by ELISA. MDMA did not alter the KLH-specific IgM response. In contrast, MDMA (5 and 10 mg/kg) significantly reduced KLH-specific IgG production. Thus MDMA administration did not alter the initial generation of the antibody response but rather inhibited antibody class switching from IgM to IgG. Two pathways for the genetic switch from IgM to IgG production were investigated. One pathway requires the Th_1 type cytokine IFN-γ to stimulate IgM-secreting cells to switch to IgG_{2a}-secreting cells. Another pathway requires the Th_2 type cytokines IL-4 and IL-6 to stimulate IgM-secreting cells to switch to IgG_1-secreting cells. IgG_1 and IgG_{2a} levels were measured to determine if these two pathways were differentially affected. Only IgG_{2a} levels were decreased following MDMA administration (Table 1). The effect of MDMA on the secondary immune response to KLH was also examined.

MDMA (10 mg/kg; i.p.) was co-administered with KLH (100 μg/kg; i.p.) on day 1, and KLH was administered again on day 35. IgM and IgG were measured on day 42 in order to determine the effect of MDMA on the secondary antibody response to KLH. However the secondary antibody response was not effected by MDMA administration.

Table 1:
Effect of acute MDMA administration on KLH-specific antibody production

Treatment	IgM	IgG	IgG_1	IgG_{2a}
Vehicle	100 ± 20	100 ± 9	100 ± 12	100 ± 12
MDMA (2.5 mg/kg)	110 ± 23	86 ± 12	108 ± 20	80 ± 16
MDMA (5 mg/kg)	102 ± 20	73 ± 8 *	86 ± 16	60 ± 10 *
MDMA (10 mg/kg)	88 ± 20	65 ± 5 **	108 ± 17	48 ± 8 **

*Results expressed as % Control. Data expressed as group mean ± SEM (n=8-9). * P < 0.05, ** P< 0.01 vs. Vehicle (One-way ANOVA followed by Fisher's LSD).*

In conclusion, these data indicate that MDMA suppresses the primary IgG response to antigen. This results from an inability to switch from IgM to IgG_{2a} production following MDMA administration. However MDMA does not alter the secondary (recall) immune response to antigen.

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39P (±)-3,4 METHYLENEDIOXYMETHAMPHETAMINE (MDMA) STIMULATES ELECTROGENIC ION TRANSPORT IN RAT COLONIC MUCOSA *IN VITRO*.

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(±)-3,4,-Methylenedioxymethamphetamine (MDMA) is a widely abused drug which is structurally related to amphetamine. Many studies which have examined the pharmacology and toxicology of MDMA have focussed on the central nervous system and peripheral actions of MDMA remain relatively unexplored. As the gastrointestinal tract contains an extensive neuronal secretomotor network, we chose to examine the effects of MDMA on colonic epithelial ion transport.

Colonic samples were obtained from male Wistar rats (200-250g) after death by stunning and decapitation. Epithelial sheets were dissected free of underlying smooth muscle and mounted on Ussing chambers. Both the apical and basolateral domains were separately bathed with identical bicarbonate-buffered physiological solutions which were maintained at 37°C and circulated and oxygenated using a gas-lift ($95\%O_2/5\%CO_2$). Tissues were voltage clamped to zero transepithelial potential difference and the short circuit current (SCC; a measure of net electrogenic ion transport) was monitored continuously. Cumulative concentration response curves were generated and compared by one-way analysis of variance. Significant differences are identified where $P<0.05$.

Addition of MDMA to the basolateral bathing solution evoked a rapid-onset inward SCC which was concentration dependent (EC_{50}=7.8±2.0nM; maximal $\Delta SCC=53\pm5\mu A/cm^2$; n=30). Amphetamine evoked a qualitatively similar SCC response which was lower in magnitude than responses to MDMA (EC_{50}=15±2nM; maximal $\Delta SCC=16\pm3\mu A/cm^2$; n=14).

Apical application of MDMA or amphetamine at concentrations up to 10μM were without effect (n=6).SCC responses to MDMA and amphetamine were significantly attenuated by bumetanide (1μM; n=4; $P<0.05$) but were unaffected by the presence of amiloride (1μM; n=5).

Tetrodotoxin (TTX; 1μM) significantly attenuated SCC responses to MDMA (1nM-100μM; n=6; $P<0.05$) but not to amphetamine (1nM-100μM) or to the directly acting secretagogue forskolin (1μM; n=6). In separate experiments, SCC responses to MDMA were unaffected by atropine (10μM) which virtually abolished SCC responses to carbachol (1-10μM; n=6).

These data support the hypothesis that MDMA stimulates electrogenic chloride secretion in colonic epithelia via activation of lamina propria nerves. Such an ion transport response would, *in vivo*, generate electrical and osmotic gradients to favour cation and water secretion. Our findings may account for some of the electrolyte and fluid imbalances which accompany MDMA use in humans.

40P PAROXETINE DOWN-REGULATES 5-HT_7 RECEPTORS IN RAT HYPOTHALAMUS BUT NOT HIPPOCAMPUS FOLLOWING CHRONIC ADMINISTRATION

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The suprachiasmatic nucleus of the hypothalamus functions as the primary pacemaker for circadian rhythms. Receptor localisation and pharmacological studies suggest that 5-HT_7 receptors are involved in circadian rhythm control and represent a potential therapeutic target for the treatment of sleep disorders and depression (eg. Hagan *et al.*, 2000). In relation to this, a number of studies have reported that the density of 5-HT_7 receptors in rat hypothalamus is reduced following chronic administration of antidepressants including fluoxetine, imipramine and mianserin (Sleight *et al.*, 1995; Mullins *et al.*, 1999). These studies utilised the non-selective agonist radioligands [^{3}H]-5-HT or [^{3}H]-5-CT, necessitating inclusion of blocking agents to prevent labelling of non-5-HT_7 sites. In the present study we have used the 5-HT_7 receptor-selective antagonist radioligand, [^{3}H]-SB-269970 (Thomas *et al.*, 2000), to investigate the effect of chronic administration of the antidepressant paroxetine on the density of 5-HT_7 receptors in rat hypothalamus and for comparison, hippocampus.

Rats (male, Sprague Dawley; n = 6 – 8 per group) were dosed with vehicle or paroxetine (10 or 30 mg kg^{-1} p.o.) acutely, or chronically, once daily for 21 days. Tissues were removed 2 h following acute treatment, or 24 h after the final dose for chronic treatment. [^{3}H]-SB-269970 binding to washed membranes prepared from rat hypothalamus and hippocampus was carried out according to the method of Thomas *et al.* (2000). 10μM 5-HT was used to define non-specific binding. [^{3}H]-SB-269970 bound saturably and monophasically to rat hypothalamic and hippocampal membranes. Non-specific [^{3}H]-SB-269970 binding increased with radioligand concentration in both tissues. K_d values for [^{3}H]-SB-269970 binding to rat hypothalamus and hippocampus were comparable to that reported at the human cloned 5-$HT_{7(a)}$ receptor and were not significantly altered following chronic paroxetine in either tissue. In contrast, chronic paroxetine treatment produced a dose-related reduction in [^{3}H]-SB-269970 binding density (B_{max}) in rat hypothalamus compared to vehicle treatment (Table 1.), an effect that was not seen after acute treatment (data not shown). Furthermore, chronic treatment did not alter the B_{max} for [^{3}H]-SB-269970 binding measured in hippocampus.

Table 1. Effect of chronic paroxetine administration on [^{3}H]-SB-269970 binding to rat hypothalamic and hippocampal membranes. (Data are the mean ± s.e.mean; n ≥ 3 experiments).

Brain region	Dose (mg kg^{-1} p.o.)	K_d (nM)	B_{max} (fmoles mg $protein^{-1}$)
Hypothalamus	Vehicle	2.0 ± 0.2	85 ± 14
	10	2.2 ± 0.6	57 ± 3.8
	30	3.4 ± 0.8	*41 ± 7.1
Hippocampus	Vehicle	2.2 ± 0.6	24 ± 3.5
	10	3.0 ± 0.3	34 ± 6.4
	30	2.5 ± 0.4	24 ± 7.0

* $p<0.05$ vs vehicle treatment, one-way Anova

In summary, these data suggest that chronic paroxetine administration may produce a selective down-regulation of 5-HT_7 receptors in hypothalamus and therefore provide supportive evidence that hypothalamic 5-HT_7 receptors may play a role in the antidepressant activity of therapeutic agents.

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41P PHARMACOLOGICAL CHARACTERISATION OF OREXIN RECEPTOR SUBTYPE SELECTIVE LIGANDS

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The orexins are a recently discovered family of neuropeptides which have been linked to a range of physiological functions, most notably regulation of the sleep-wake cycle and feeding (Jerman & Smart, 2001). However, there are two orexin receptor subtypes, OX_1 and OX_2, and it has been difficult to elucidate which is involved in a given function, as orexin-A is equipotent at both, whilst orexin-B is only 10-fold selective for OX_2, (Smart *et al.*, 1999). To address this, the present study describes the pharmacological characterisation of four orexin receptor subtype-selective ligands, SB-284422 (1-(4-Methylsulfanyl-phenyl)-3-quinolin-4-yl-urea), SB-334867 (1-(2-Methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea), SB-408124 (1-(6,8-Difluoro-2-methylquinolin-4-yl)-3-(4-dimethylamin-phenyl)-urea) and SB-668875 (RSGPPGLQ GRAQRLLQASGNHAAGILTM-amide, Asahi *et al.*, 1999).

$CHO\text{-}OX_1$ and $CHO\text{-}OX_2$ cells were cultured as described previously (Smart *et al.*, 1999). Cells, seeded (20,000 cells per well) into black walled clear-base 96 well plates (Costar UK), were incubated with MEM-Alpha medium containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, Texas) and 2.5 mM probenecid at 37°C for 60 min. The cells were then washed three times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid and 0.1% gelatine, before being incubated for 30 min at 37°C with either buffer alone (control) or buffer containing compound (0.1nM - 10 μM). The plates were then placed into a FLIPR™ (Molecular Devices, UK) to monitor fluorescence (λ_{ex} = 488 nm, λ_{EM} = 540 nm) (Smart *et al.*, 1999) before and after the addition of orexin-A, orexin-B (10 pM - 1 μM), or compound (100 pM - 10 μM).

Orexin-A and -B and SB-668875 caused a concentration-dependent increase in $[Ca^{2+}]_i$ in $CHO\text{-}OX_1$ cells, with pEC_{50} values of 8.38 ± 0.04, mean ± s.e.m., 7.26 ± 0.05 and 5.62 ± 0.04 respectively (n=12). Similarly, Orexin-A and -B and SB-668875 increased $[Ca^{2+}]_i$ in $CHO\text{-}OX_2$ cells, with pEC_{50} values of 8.20 ± 0.03, 8.26 ± 0.04 and 7.64 ± 0.04 respectively (n=8). SB-284422, SB-334867-A and SB-408124 were devoid of agonist activity, but did inhibit orexin-A (10nM)-induced Ca^{2+} responses in $CHO\text{-}OX_1$ cells, with pK_B values of 7.86 ± 0.06 (n=15), 7.49 ± 0.04 (n=13) and 7.78 ± 0.04 (n=17) respectively. These compounds also acted as weak antagonists at OX_2, with pK_B values of <5.7, 5.73 ± 0.08 and 5.77 ± 0.04 (n=7) respectively.

In conclusion, we have characterised 3 non-peptide OX_1-selective antagonists and an OX_2-selective peptide agonist, which will aid in the elucidation of the receptor subtypes underlying the actions of the orexins *in vivo*.

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42P FUNCTIONAL EXPRESSION AND PHARMACOLOGICAL CHARACTERISATION OF THE GOLDFISH SOMATOSTATIN SST5 RECEPTOR

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Somatostatin (somatotropin release inhibiting factor = SRIF) exerts its effects via specific G-protein-coupled receptors of which 5 subtypes have been cloned in mammals ($sst_{1\text{-}5}$). Recently SRIF receptors have also been cloned from fish tissues. In this study, goldfish sst_5 receptors were stably expressed and characterised in the Chinese hamster lung fibroblast cell line, which harbours the luciferase reporter gene driven by the serum responsive element (CCL39-SRE-Luci). The agonist radioligands [^{125}I]-LTT-$SRIF_{28}$ and [^{125}I][Tyr^{10}]CST_{14} labelled similar receptor densities with high affinity and in a saturable manner (pK_d: 9.99±0.19, 9.71±0.08; B_{max}: 302.6±15.1, 347.9±22.8 fmol mg^{-1}, mean ± s.e.m., n=4 respectively). The GTP analogue 5'-Guanylyl-imidodiphosphate inhibited [^{125}I]LTT-$SRIF_{28}$ and [^{125}I][Tyr^{10}]CST_{14} binding to some extent (38.5±3%, 57.9±4.5%, n=3, respectively), suggesting binding to G protein–coupled and –uncoupled sites.

In competition radioligand binding studies the pharmacological profile of SRIF ligands defined with [^{125}I]LTT-$SRIF_{28}$ and [^{125}I][Tyr^{10}]CST_{14} correlated significantly (r^2=0.97, n=20) and displayed the following rank order: LTT-$SRIF_{28}$ > $SRIF_{28}$ = goldfish $SRIF_{28}$ $SRIF_{25}$> $SRIF_{14}$ > [Pro^2]$SRIF_{14}$ = CST_{17} = RC 160 = BIM 23052 = [Tyr^{10}]CST_{14} > CST_{14} > BIM 23014 [Tyr^3]Octreotide = CGP 23996 = Octreotide = Seglitide > BIM 23056 = L3622,855 > Cycloantagonist SA = BIM 23030. Pharmacological profiles of human and mouse sst_5 receptors expressed in CCL39 cells (Siehler *et al.*, 1999, Feuerbach *et al.*, 2000) correlated markedly less with that of the goldfish sst5 profile (r^2=0.52-0.78).

Functional expression of the receptor was examined by measurement of agonist-induced luciferase expression and stimulation of [^{35}S]GTPγS binding as previously described (Siehler *et al.*, 1999, Feuerbach *et al.*, 2000). Profiles were similar to those of radioligand binding (r^2=0.81-0.93), although potency (pEC_{50}) was reduced by a factor of 10-100. By contrast, relative efficacy profiles between luciferase expression and GTPγS binding, were rather divergent (r^2=0.48): thus natural SRIF ligands acted as full agonists in both, whereas synthetic analogues were more efficacious in stimulating GTPγS binding. RC 160, BIM 23052, BIM 23014, and L362,855 acted as full agonists in GTPγS binding, but only as partial agonists (E_{max} 15-70%) at luciferase expression. BIM 23056 acted as an inverse agonist on stimulation of GTPγS binding (E_{max}: -43.9±6.5). Pre-incubation of pertussis toxin (PTX) (100 ng ml^{-1}, 18 h) abolished the effect of $SRIF_{14}$ on luciferase expression suggesting the receptor to be coupled to G_i/G_o proteins.

In summary the present study shows that the goldfish sst_5 receptor has a pharmacological profile which is similar to mammalian sst_5 receptors. The receptor is able to stimulate luciferase expression via the SRE in a PTX sensitive manner. Dependent on the functional assay used the SRIF ligands demonstrated different relative efficacy profiles.

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43P PHARMACOLOGICAL CHARACTERIZATION OF THE TRUNCATED, SOLUBLE *N*-TERMINUS OF THE TYPE 1α METABOTROPIC GLUTAMATE RECEPTOR: COMPARISON TO THE NATIVE FULL-LENGTH RECEPTOR

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Metabotropic glutamate (mGlu) receptors are members of the family 3 sub-group of G protein-coupled receptors (GPCRs). All eight mammalian mGlu receptors possess a large extracellular N-terminus, which contains the ligand binding site (O'Hara *et al.*, 1993). In the present study we have investigated the pharmacological binding properties of a hexahistidine-tagged, truncated form of the human mGlu1 receptor (mGlu1-$^{1-592}$NT), whose amino acid sequence terminates at residue 592, just prior to the first transmembrane domain.

Expression of mGlu1-$^{1-592}$NT by Chinese hamster ovary cells was enhanced with 2 mM sodium butyrate for 5 days before harvesting the mGlu1-$^{1-592}$NT protein from the culture medium. mGlu1-$^{1-592}$NT was precipitated from the crude cell culture medium with Ni-NTA superflow beads (Qiagen, Crawley, UK) (1:15, bead:medium ratio) for 2 h at 4°C and then washed with 3 x 30 ml ice-cold assay buffer (20 mM HEPES, 2 mM $MgCl_2$, pH 7.4). The beads were incubated with 1-50 nM, or 5 nM [^{3}H]-quisqualate for 45 min at room temperature for saturation and competition binding assays respectively. Assays were terminated by filtration through GF/B filter papers and pulse-washed with ice-cold assay buffer (5 x 2 ml). Non-specific binding was defined in the presence of 10μM quisqualate. Experiments using BHK-mGlu1α membranes (50 μg assay^{-1}, prepared as described in Akam *et al.*, 1997) were performed under identical conditions, except that 10 nM [^{3}H]-quisqualate was used in competition binding studies.

A range of B_{max} values (0.2-2.4 pmol ml^{-1} of crude medium) for the truncated receptor were obtained from saturation binding experiments. Such variation was probably due to differences in seeded cell density. Despite such variation in B_{max} values, the K_d estimates for [^{3}H]-quisqualate binding to mGlu1α-$^{1-592}$NT remained remarkably constant, producing a value of 5.6 ± 1.8 nM (n=4). This is somewhat lower than the K_d obtained for BHK-mGlu1α receptors of 10.0 ± 1.0 nM (B_{max}, 3.8 ± 0.3 pmol mg^{-1} protein; n=3).

The pharmacological profile of the mGlu1α receptor was generally maintained by mGlu1-$^{1-592}$NT, although the agonist quisqualate, and the antagonist LY367385 (*S*-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid), displayed greater affinity for the truncated compared to the intact receptor (1.9 and 7.6 fold, respectively; see Table 1). [^{3}H]-quisqualate binding was only displaced at very high concentrations by group II- and III-selective mGlu receptor agonists (DCG-IV (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine) (10 μM), 19 ± 5%; L-AP4 (L(+)-2-amino-4-phosphonobutyric acid) (300 μM), 19 ± 5% inhibition of specific binding, respectively). Finally, the binding affinities of neither L-glutamate nor quisqualate for mGlu1-$^{1-592}$NT were influenced by Ca^{2+} (-Ca^{2+}, K_i, 250 ± 28 and 1.9 ± 0.5 nM; +Ca^{2+} (1.3 mM), K_i, 180 ± 48 and 2.6 ± 0.9 nM for L-glutamate and quisqualate, respectively) suggesting that, in contrast to GABA(B) receptors (Wise et al., 1999), mGlu receptors do not 'sense' extracellular Ca^{2+} through modulation of agonist binding.

Table 1 Competition binding affinities for quisqualate, L-glutamate and LY367385 at soluble mGlu1-$^{1-592}$NT and BHK-mGlu1α cell membranes

	K_i (nM) (means ± S.E.M. n = 4-6)	
	mGlu1-$^{1-592}$NT	BHK-mGlu1α
quisqualate	1.8 ± 0.4	3.3 ± 0.2
L-glutamate	323 ± 165	--
LY367385	60.3 ± 10.6	459 ± 49

In conclusion the soluble *N*-terminus of mGlu1α displays pharmacology, determined by agonist binding, characteristic of the mGlu1 receptor, and this is unaffected by the presence of physiological concentrations of Ca^{2+}.

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44P BINDING SITE SELECTIVITY OF EPIBATIDINE AT THE MUSCLE NICOTINIC ACETYLCHOLINE RECEPTOR

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The foetal muscle nicotinic acetylcholine receptor (AChR) possesses two binding sites for acetylcholine, formed at the interface of the two alpha subunits and their neighbouring gamma or delta subunits. These binding sites differ in their affinity for a number of ligands (Sine *et al.*, 1995). Since the alpha subunits are identical, such affinity differences must be due to the sequence differences of the gamma and delta subunits. The agonist (-)-epibatidine has been shown to exhibit >200-fold greater affinity for the alpha-gamma site compared to the alpha-delta site, when the receptor is desensitised (Prince *et al.*, 2000). Recent studies using gamma-delta subunit chimeras have shown that sequence differences in the amino acids 104-117 in gamma and 106-119 in delta are largely responsible for this difference in affinity (Prince *et al.*, 2000).

In this study, single or multiple residues in gamma were mutated to their equivalent in delta and *vice-versa*, to further delineate the contributions of individual amino acids in the region 104-117 to the site selectivity of epibatidine. Subunit omitted receptors of the form $\alpha_2\beta\chi_2$ (where χ is either wild type gamma or a point mutant of gamma or delta) were transiently expressed in HEK 293 cells (Sine, 1993). Receptors were desensitised with 100 μM proadifen, and the affinity of epibatidine determined by competition against the initial rate of [^{125}I] α-bungarotoxin binding. Analysis was performed on the log shift of the binding curves normalised to a gamma curve, using a Student paired t-test. Values were Considered statistically different from each other when P≤0.05. Data are expressed as mean ± SEM of at least 3 binding curves.

Ki values for wild type delta and gamma were 445.8 ± 68.3 nM and 1.910 ± 0.155 nM, respectively (P<0.001).

The mutation γL104Y produced a 3-fold decrease (6.18 ± 0.85 nM, P=0.05) in affinity, while γY117T produced a 3.5-fold decrease (6.68 ± 0.71 nM, P<0.05) in affinity for epibatidine compared to γ wild-type. γI116V had no significant effect. However, combining all three mutations produced a further reduction in affinity (11.6 ± 3.6 nM, P<0.01). Similarly, the equivalent δ point mutants at residues 106 and 119 both produced a 3-fold increase in affinity (P<0.005), compared to the δ wild type, having Ki values of 138 ± 22nM and 136 ± 21 nM, respectively. When all three were combined a 50-fold increase in affinity (8.30 ± 0.90, P<0.005) was observed. Residues γ116/δ118 and γ117/δ119 have previously been identified as important determinants of binding site selectivity in the foetal muscle AChR (Sine, 1993). It is probable that a number of residues participate in the site selectivity of epibatidine at the foetal muscle AChR. Further studies using point mutants should help pinpoint their location.

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45P THE ROLE AND PREDICTED PROPENSITY OF CONSERVED PROLINE RESIDUES IN THE MOUSE 5-HT_{3A} RECEPTOR

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The 5-HT_3 receptor is a member of the cys-loop family of ligand-gated ion channel, proteins which possess a number of highly conserved proline (P) residues. As these residues impose considerable restraint on protein folding, they are often critical for the structure and/or function of the molecule. To examine the role of conserved proline residues in this family of proteins, we changed them to alanine. To explore the conformational preference (propensity) of these residues we also examined the proportion and immediate neighbours of cis proline residues in the protein database (PDB).

Amino acid residues in the mouse 5-$HT_{3A(b)}$ receptor subunit subunit were mutagenised using the Kunkel method (Kunkel, 1985) in the expression vector pcDNA3 (Clontech), transiently transfected into HEK 293 cells using calcium phosphate precipitation (Chen & Okayama, 1988), and examined using radioligand binding assays, immunocytochemistry and electrophysiology (Spier & Lummis, 2000). Cis peptide bonds were identified in the PDB as those where the ω dihedral fell between -20 and 20°.

Mutation of P^{56}, P^{104}, P^{123} and P^{170} resulted in ablation of radioligand binding. Receptors containing P^{104}, P^{123} and P^{170} were expressed, but not at the plasma membrane. Mutation of P^{257} and P^{301} resulted in receptors that bound [^{3}H]granisetron with K_d values (0.27 ± 0.05nM and 0.20 ± 0.07nM respectively, mean ± s.e.m., n=3), not significantly different (Student's t test) to wild type receptors (0.19 ± 0.03nM, mean ± s.e.m., n=4). They were expressed at the plasma membrane but were non-functional.

550 cis peptide bonds were identified in the PBD and 4.7% of prolines were in the cis conformation. Comparison of the proportion of each amino acid located immediately prior to cis vs trans prolines revealed 5 residues (G,P,Y,W & F) found more frequently than expected (Chi squared, $p < 0.05$), with 5 residues being found less frequently (V,I,L,D & T). A similar plot for those residues located subsequent to a cis proline showed 3 residues whose occurrence was greater than expected (P,Y & F) and 2 residues (D & E) whose occurrence was less. Of the 20 prolines in the 5-HT_{3A} receptor subunit, only one, P^{170}, has favourable adjacent residues. Mutating these to non-favourable residues resulted in ablation of ligand binding whereas replacement with other favourable residues did not.

Thus the data suggest that conserved prolines in the N terminal domain may be involved in forming the correct receptor structure, whilst P^{257} and P^{301} are necessary for the function of the protein. We also suggest that P^{170} which is part of the characteristic cys-cys loop found in this family of proteins, may be in the cis conformation

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46P CO-EXPRESSION AND FUNCTIONAL CHARACTERISATION OF RAT $P2X_4$ AND $P2X_6$ SUBUNITS IN MAMMALIAN CELLS

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Heteromeric combinations of rat $P2X_4$ and $P2X_6$ receptors have been described both immunochemically (Torres *et al.*, 1999) and functionally (Lê *et al.*, 1998), but the latter only in non-mammalian systems. The aim of this study was to co-express and functionally characterise $P2X_{4+6}$ receptor subunits in mammalian cells and to compare their pharmacological properties with homomeric $rP2X_4$ expressed in the same system.

Whole cell recordings were made from rafts of HEK-293 cells stably expressing either rat recombinant $P2X_4$ or N-terminal-his tagged $P2X_6$ receptors, or both, using Cs-aspartate containing electrodes (3-8MΩ), in a HEPES-buffered extracellular medium, as previously described (Jones *et al.*, 2000). Data are mean ± SEM. In a total of 62 experiments, at a holding potential of -90mV, 10μM ATP induced inward currents in 100% of cell rafts expressing $rP2X_4$ or $rP2X_{4+6}$ receptors.

Agonist-induced currents recorded from single cells expressing $rP2X_{4+6}$ receptors undergo rundown. This rundown of response was not significantly different from that observed at homomeric $rP2X_4$ and was overcome by patching rafts of 4 or more electrically coupled cells. In keeping with previous reports, cells expressing homomeric $P2X_6$ receptors did not display significant responses to application of ATP (100μM). Electrophoretic and confocal analysis of these cells indicated the presence of $P2X_6$ receptors on the cell surface.

Cells co-expressing $rP2X_{4+6}$ receptors were found to have a significantly higher current density ($P<0.05$) than those expressing $rP2X_4$ alone (-46 ± 4pA/pF and −33 ± 7pA/pF respectively, in response to 100μM ATP, n=20). Responses to ATP at $rP2X_{4+6}$ were otherwise qualitatively similar to those at $rP2X_4$, being rapidly activating and relatively non-desensitising over the 2s agonist application period, with EC_{50} values of 3.0 ± 0.4μM (n=8) and 4.3 ± 0.5μM (n=7) for $rP2X_{4+6}$ and $rP2X_4$, respectively.

αβmeATP, a stable analogue of ATP, is commonly used to identify P2X mediated responses in native brain tissue. Heteromeric $rP2X_{4+6}$ receptors were no more sensitive to this agonist that $P2X_4$ alone, with 100μM α,β-meATP eliciting 11% of the maximum response to ATP (n=6), compared to 7% at cells expressing $rP2X_4$ (n=6). However, 2MeSATP (100μM) elicited a significantly higher maximum response ($P<0.05$) at $rP2X_{4+6}$ than at $rP2X_4$ (57% and 38% of the maximum response to ATP, EC_{50} = 11.2 ± 1.9μM and 7.5 ± 1.4μM, respectively, n=4).

Adenosine tetraphosphate acted as a partial agonist with respect to ATP at both $rP2X_4$ and $rP2X_{4+6}$ receptor expressing cells, with 100μM eliciting approximately 50% of the maximum response to ATP in both cell types. ATPγS (100μM) also acted as a partial agonist with respect to ATP and failed to distinguish between the homomeric and heteromeric receptors, eliciting 21% and 16% of the maximum response to ATP at cells expressing $rP2X_{4+6}$ and $rP2X_4$, respectively. Cells expressing homomeric and heteromeric receptors were also found to be insensitive to the P2 receptor agonists, αβmeADP, diadenosine tetraphosphate and AMP, as well as the antagonists PPADS and suramin (100 μM).

The functional characteristics of $P2X_6$ and heteromeric combinations thereof may be regulated by phosphorylation or glycosylation events. However, using heterologously expressed $P2X_4$ and $P2X_6$ subunits in this study, it is clear that, while small but significant differences can be observed between homomeric $P2X_4$ and heteromeric $P2X_{4+6}$, these differences cannot account for α,β-meATP-sensitive central P2X responses described to date.

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47P SYNAPTOTAGMIN I RESCUES MUSCARINIC ACETYLCHOLINE RECEPTOR INTERNALIZATION INHIBITED BY EITHER DOMINANT-NEGATIVE ARRESTIN OR DOMINANT-NEGATIVE DYNAMIN

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Synaptotagmin is believed to play a major role in the Ca^{2+}-dependent exocytosis of neurotransmitters (Fernández-Chacón *et al.*, 2001). Intriguingly, this protein also appears to function as a membrane receptor for the clathrin adaptor protein AP-2 during endocytosis (Hauke *et al.*, 2000). In this study, we investigated the role of synaptotagmin in the agonist-stimulated internalization of the M_4 muscarinic acetylcholine receptor by examining the effect on receptor internalization of wild type synaptotagmin I tagged at its C-terminus with green fluorescent protein (GFP) and of GFP-tagged synaptotagmin with a mutated C2B domain that cannot bind AP-2.

Cells (tsA-201) were transfected with the following constructs, by calcium phosphate precipitation: M_4 muscarinic receptor tagged at its N-terminus with a haemagglutinin (HA) epitope, wild type and dominant-negative (319-418) arrestin-2, HA-tagged wild type and dominant-negative (K44A) dynamin, and wild type and mutant (K326,327A) synaptotagmin I-GFP. Proteins were localized by laser scanning confocal immunofluorescence microscopy. Receptor internalization was triggered by the muscarinic agonist carbachol (1 mM), and quantified through the binding of the membrane-impermeant radioligand [^{3}H]N-methylscopolamine (Koenig & Edwardson, 1994). Data are means ± s.e.m. The statistical significance of differences between means was assessed using Student's *t*-test for unpaired data.

Over a 30-min incubation of control transfected cells with carbachol, 27±2% (n=18) of the receptors initially at the cell surface became internalized. In cells also expressing dominant-negative arrestin-2, internalization was reduced to 12±3% (n=6, $P<0.001$). Dominant-negative dynamin caused a greater reduction in internalization, to 3±1% (n=9, $P<0.001$, compared with control). Both wild type and mutant synaptotagmin I-GFP were delivered almost exclusively to the plasma membrane, as judged by both GFP staining and immunolocalization, using an antibody to the C2A domain. Receptor internalization in control cells was not significantly affected by either wild type (31±3%, n=8) or mutant (26±5%, n=8) synaptotagmin. However, in cells expressing dominant-negative arrestin-2 internalization was significantly increased by co-transfection with wild type (32±4%, n=8, $P<0.01$) but not with mutant (18±2%, n=4) synaptotagmin. Receptor internalization in cells expressing dominant-negative dynamin was also significantly increased by wild type (19±6%, n=8, $P<0.01$) but not by mutant (1±6%, n=4) synaptotagmin.

We conclude that in tsA-201 cells, agonist-stimulated internalization of M_4 muscarinic acetylcholine receptors requires arrestin-2 and dynamin. Synaptotagmin, through its ability to interact with AP-2, offers an alternative internalization mechanism when the normal protein machinery is compromised. The potential involvement in endocytosis of an endogenous synaptotagmin isoform remains to be tested.

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48P M_3 MUSCARINIC RECEPTOR / Gq UNCOUPLING IS ENHANCED BY OVEREXPRESSION OF GRK6 BUT NOT GRK3

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G protein-coupled receptor kinases (GRKs) are known to phosphorylate M_3 muscarinic (M_3 mACh) receptors (DebBurman *et al.*, 1995), raising the possibility that GRKs may regulate M_3 mACh receptor signalling. However, the recent report that GRKs 2 and 3 not only sequester free Gβγ but also bind activated $G\alpha_q$ (through an RGS domain; Carman *et al.*, 1999), further complicates assessment of the initial stages of receptor desensitisation directly related to GRK-mediated receptor phosphorylation. We have attempted to dissect phosphorylation-dependent and -independent GRK-mediated M_3 mACh receptor desensitization, via assessment of methacholine (MCh)-induced M_3 mACh/$G\alpha_{q/11}$ uncoupling (Bundey and Nahorski, 2000).

Stably transfected human SH-SY5Y neuroblastoma cells, expressing either pcDNA3 (control) or GRKs 3 or 6 (in pcDNA3) and matched for receptor expression, were pretreated with varying concentrations of MCh (Table 1) for 3 min at 37 °C. Cells were then washed thoroughly, transformed into membranes (50 µg per tube), and challenged again with 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 determined by addition of excess cold GTPγS (10 µM). $G\alpha_{q/11}$ was immunoprecipitated (Akam *et al.*, 2001) and bound [^{35}S]-GTPγS quantified by scintillation counting. The degree of receptor uncoupling was determined as the percentage reduction of [^{35}S]-GTPγS binding after pretreatment, when compared to the maximal [^{35}S]-GTPγS binding in the vehicle-pretreated control.

Overexpression of GRK6 (~25 fold over endogenous levels) enhanced M_3 mACh/$G\alpha_{q/11}$ uncoupling both at high (100 µM) and low (3 µM) MCh pretreatment concentrations when compared to plasmid control cells (Table 1). In contrast, GRK3 overexpression (~25 fold) failed to enhance MCh-induced M_3 mACh/$G\alpha_{q/11}$ uncoupling. However, when compared to control MCh-stimulated (100 µM) total [^{3}H]-inositol phosphate accumulation, after 3 min, was inhibited by 58 ± 4 %* and 29 ± 3 %* in GRK3 and GRK6 overexpressing cells, respectively (data means ± s.e.mean (n = 3), *p< 0.05 Student's t-test).

Table 1. Effects of GRK3 or 6 overexpression on MCh-induced M_3 mACh receptor/$G\alpha_{q/11}$ uncoupling in SH-SY5Y cells. Data are expressed as the percentage increased uncoupling when compared to vehicle-pretreated controls. Values are means ± s.e.mean of 3 separate experiments. (*p<0.01; repeated measures ANOVA with Bonferroni post-test).

Pretreatment conc. (µM)	Vector control	GRK3	GRK6
0.3	2.1 ± 2.1	4.6 ± 2.7	18.9 ± 8.4
3	6.1 ± 3.1	8.7 ± 3.4	40.3 ± 5.0*
100	38.3 ± 4.0	42.0 ± 2.1	64.5 ± 1.0*

GRK6 appears to increase uncoupling of the M_3 mCAh receptor and $G\alpha_{q/11}$, upstream of phospholipase C. Conversely, GRK3 may inhibit M_3 mACh receptor signalling independently of receptor/$G\alpha_{q/11}$ uncoupling, possibly via Gβγ binding, and/or direct $G\alpha_q$ binding via its RGS domain to inhibit phospholipase C activity.

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49P DIRECT VISUALIZATION OF LIGAND-PROTEIN INTERACTIONS USING ATOMIC FORCE MICROSCOPY

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We are developing methods for imaging molecular interactions using atomic force microscopy (AFM; see for example, Ellis *et al.,* 1999). As a model ligand-protein interaction, we have investigated the binding of biotin to streptavidin. Streptavidin is a 66-kDa tetramer which binds four molecules of biotin with extremely high affinity ($K_A \sim 10^{14}M^{-1}$). In order to visualize the binding of biotin to streptavidin, we tagged the ligand with a short (152 bp; 55 nm) DNA rod. We then incubated the DNA/biotin with streptavidin, and imaged the complexes by AFM.

Biotinylated DNA rods were produced by PCR amplification using *Taq* polymerase. Streptavidin (Sigma) was incubated with DNA/biotin at various molar ratios at 22°C for 1h. Samples were adsorbed onto freshly cleaved, poly-L-lysine-coated mica and air-dried. Imaging was performed with a multimode atomic force microscope (Digital Instruments, Santa Barbara, CA). Samples were imaged in air, using tapping mode. The molecular volume of the protein particles was determined from particle dimensions, as described previously (Schneider *et al.,* 1995). To assess the thermal stability of streptavidin, the protein was incubated at various temperatures, and then analysed by SDS-polyacrylamide gel electrophoresis. Data are means ± s.e.m. The statistical significance of differences between means was assessed using Student's *t*-test for unpaired data.

It is known that biotin binding causes a conformational change in streptavidin that confers an increased thermal stability (Gonzalez *et al.,* 1997). This conformational change was apparent in images of the molecule: the molecular volume of streptavidin increased from 105±3 nm^3 (n=237) in the free state, to 141±11 nm^3 (n=130, $P<0.01$) when complexed with untagged biotin, and to 133±2 nm^3 (n=210, $P<0.001$) when complexed with DNA/biotin. Images of streptavidin with one, two, three and four molecules of DNA/biotin bound were obtained. When two ligands were bound, the angle between the DNA rods was either acute or obtuse, as expected from the relative orientations of the biotin binding sites (Weber *et al.,* 1989). The ratio of obtuse to acute angles was 3:1, greater than the value of 2:1 expected for equivalent sites. This result indicates a degree of steric hindrance, presumably as a consequence of the relatively large size of the ligand. A similar conclusion can be drawn from the fact that the frequency of tri- and tetra-liganding was lower than predicted.

We conclude that the ability to image directly the binding of a ligand to its protein target by AFM provides useful information about the nature of the interaction, and about the effect of complex formation on the structure of the protein. It should now be possible to apply a similar approach to the study of molecular interactions which are less well characterized, for instance the binding of ligands to multi-subunit receptors.

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50P PHARMACOLOGY AND DIRECT VISUALIZATION OF THE FLUORESCENT β_2-ADRENOCEPTOR LIGAND BODIPY-CGP 12177 IN CHO CELLS TRANSFECTED WITH THE HUMAN β_2-ADRENOCEPTOR

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The high affinity, hydrophilic β_1/β_2-adrenoceptor antagonist CGP 12177 is an important probe for cell surface β_2-adrenoceptors (Bylund et al., 1994). Here, we show that a fluorescent analogue (BODIPY-CGP, Molecular Probes) has partial agonist activity on cyclic AMP accumulation (cAMP) and cyclic AMP response element (CRE) – mediated gene transcription responses and the binding of this ligand can be directly visualized in living cells using confocal microscopy.

CHO-K1 cells expressing the human β_2-adrenoceptor at 300fmol/mg protein and a secreted placental alkaline phosphate (SPAP) reporter gene under the trancriptional control of six CREs (McDonnell et al., 1998) were used. Measurements of ^{3}H-cyclic AMP accumulation and SPAP secretion were made as described previously (McDonnell et al., 1998). A Zeiss LSM510 laser microscope (Zeiss 40x1.3NA oil immersion lens, 543nm HeNe laser) was used to monitor the binding of BODIPY-CGP to CHO cells.

BODIPY-CGP had partial agonist activity in both assays however the response was much greater for CRE-mediated SPAP secretion (EC_{50} 80.45 ± 10.58nM n=3; 5.17 ± 0.43% of the response to 10μM isoprenaline for cAMP accumulation; EC_{50} 22.5 ± 3.3nM n=16; 50.8 ± 2.63% of isoprenaline E_{MAX} for SPAP secretion). The SPAP response was inhibited by ICI 118551 (K_D 0.85 ± 0.24nM n=6) consistent with a β_2-adrenoceptor mediated action. 100nM BODIPY-CGP antagonized the responses to isoprenaline in a partial agonist manner with a K_D of 3.12 ± 0.70nM (n=7) for SPAP secretion and as a competitive antagonist K_D 2.46 ± 0.70nM (n=3) for cAMP accumulation.

Confocal microscopy confirmed that concentrations of 30nM BODIPY-CGP and above were needed to achieve clear membrane localized fluorescence (n<30) that remained unchanged for at least 30 minutes at room temperature (n=5). Higher concentrations (300nM) caused a diffuse cytoplasmic fluorescence in addition to the membrane localized fluorescence (n=4). Pre-incubation with 1-10nM ICI 118551 was able to inhibit the binding of BODIPY-CGP to the cell membrane (n=5) but not the diffuse cytoplasmic fluorescence, as was 1nM CGP 12177 (n=4) and 1-10μM isoprenaline (n=4).

This study showed that the partial agonist actions of BODIPY-CGP were more clearly seen in the amplified CRE-mediated SPAP signal transduction pathway than ^{3}H-cyclic AMP accumulation. Confocal imaging showed that this binding can be visualized and inhibited in living cells at similar concentrations to those suggested by the functional assays. The 10-40 fold difference in affinity between the agonist and antagonist abilities of BODIPY-CGP suggested that isoprenaline induced a different receptor state capable of this higher affinity BODIPY-CGP binding.

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51P STIMULATION OFINSULIN SECRETION *IN VIVO* BY THE IMIDAZOLINE ANALOGUE KU14R

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There is evidence from both electrophysiogical and functional endocrine studies *in vitro* that an atypical imidazoline binding site (I_3) exists on K^+_{ATP} channels of pancreatic β cells which can stimulate insulin release (Chan *et al.*, 1994) and a number of imidazolines have been shown to act as insulin secretagogues. KU14R (2-(2-ethyl-2,3-dihydro-2-benzofuranyl) 1H-imidazole) an analogue of the I_3 ligand efaroxan, has been proposed to act as an antagonist at this site (Chan *et al.*, 1997), although KU14R has an insulinotropic effect in BRIN-BD11 cells (Ball *et al.*, 2000). Since KU14R has not been studied *in vivo* we have investigated its acute effects on blood glucose (BGL) and plasma insulin levels, and also the interaction between KU14R and the I_3 agonist efaroxan.

Adult male CBA/Ca mice were given drugs or saline by i.p. injection. Pre- and post-drug blood samples, obtained by venepuncture of the tail vein under ether anaesthesia, were assayed for glucose using a Glucochek II as described previously (Williams *et al.* 2000). Plasma insulin was measured by a standard radioimmunoassay (Linco Research). Differences between groups, shown as means ± s.e.mean of (n), were analysed by Student's t test and the time course of drug effects by ANOVA.

Preliminary studies with KU14R (4 - 25 mg/kg) showed a dose-dependent fall in BGL which was maximal at around 120 min, returning to control by 240-300 min. In subsequent experiments we used 16 or 20 mg/kg KU14R; the latter dose was equipotent with efaroxan at 5mg/kg (Table 1), although efaroxan had a longer time course of action. The fall in BGL following KU14R coincided with a rise in insulin (Table 1). When KU14R was injected 60 min after efaroxan, the fall in BGL was significantly increased ($F_{1,35} = 5.56$, $P < 0.01$) and prolonged ($F_{6,35} = 8.24$, $P < 0.05$).

Similar results were obtained when KU14R was injected 60 min prior to efaroxan; the fall in BGL was increased and prolonged.

Treatment group	Blood glucose (mmol/L) Pre-drug	Blood glucose (mmol/L) 120 min	Plasma insulin (μg/L) Pre-drug	Plasma insulin (μg/L) 120 min
Control saline	7.57±0.41 (3)	8.20±0.32 (3)	11.7±0.78 (3)	13.0±1.6 (3)
KU14R (20mg.kg^{-1})	8.43±0.54 (6)	5.25±0.78 (6)*	12.4±2.4 (6)	26.7±3.7 (6)**
Efaroxan (5mg.kg^{-1})	7.6±0.39 (6)	5.85±0.63 (6)*	-	-

* $P<0.05$; **$P<0.01$ paired t-test

Our results indicate that the effects of KU14R and efaroxan, in terms of BGL, are least additive and that there is no functional antagonism between the drugs. This suggests that they have similar effects if they are acting at the same site.

We conclude that KU14R does not block K^+_{ATP} I_3 site-mediated insulin release *in vivo*, and is not behaving as an I_3 antagonist. It may act at an additional alternative site *in vivo* to evoke insulin secretion.

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52P CHANGES IN RAT MELANIN CONCENTRATING HORMONE AND MCH RECEPTOR (SLC-1) mRNA INDUCED BY DIETARY OBESITY

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Melanin concentrating hormone (MCH) and its receptor (SLC-1) are known to influence body weight in mammals (Qu *et al.*, 1996). However, the precise role of the MCH system in regulating energy stores and metabolism is presently unclear. We have therefore examined hypothalamic MCH and SLC-1 mRNA concentrations in two physiological states of altered energy balance – dietary induced obesity (DIO) and food restriction – to understand the mechanisms regulating this system.

Male Wistar rats (~ 200g) were either restricted to 65% of their normal daily intake for 10 days (n=8) or made obese by feeding a highly palatable diet, rich in fat, for 8 weeks (n=18). Control animals (n=8 or 9 respectively) were allowed free access to standard laboratory chow. Rats were killed by CO_2 inhalation and blood removed from the heart for RIA of plasma leptin and insulin concentrations. The hypothalami were dissected and snap frozen in N_2. mRNA was measured in hypothalamic blocks by Taqman PCR using FAM labelled fluorogenic probes specific for the two transcripts. Amplification data were collected by an ABI Prism 7700 Sequence Detection System (PE Biosystems) and normalised against rat acidic ribosomal protein (36B4) expression.

Neither MCH nor SLC-1 mRNA levels were significantly altered in food restricted rats relative to controls (0.75±0.07 vs 0.62±0.04 and 0.003±0.0005 vs 0.004±0.0001 arbitrary units) despite reduced plasma leptin (3.98±0.24 vs 1.18±0.11 ng/ml; $P<0.001$; ANOVA and Bonferonni modified t-test) and insulin concentrations (13.79±0.79 vs 5.88±0.70 μU/ml; $P<0.0001$). However, MCH (0.548±0.037 vs 0.386±0.037 arbitrary units; $P<0.023$) and SLC-1 (0.001±0.000 vs 0.003±0.000 arbitrary units; $P<0.0001$) mRNA were both elevated in DIO rats as were plasma leptin (8.31±0.22 vs 4.69±0.14 ng/ml; $P<0.0001$) and insulin concentrations (26.67±1.06 vs 20.30±0.89 μU/ml; $P<0.0001$). The unchanged MCH mRNA levels in food restricted rats contrasts with the transient increase previously reported in animals fasted for 48h (Hervé *et al.*, 1997). This may be a consequence of the extended treatment period, with any changes being restored to original levels over 10 days.

The observed increase of orexigenic MCH synthesis in DIO animals is inappropriate and consequently could be driving the hyperphagia, which leads to the development of obesity in this model (Harrold *et al.*, 2000). As insulin has been reported to stimulate MCH synthesis (Bahjaoui-Bouhaddi *et al.*, 1994), this increase could arise as a result of elevated insulin concentrations in the DIO rats. However, it has been suggested that leptin regulates SLC-1 mRNA levels in the brain, with reduced concentrations leading to increased SLC-1 expression (Kokkotou *et al.*, 2001). Whilst the lack of change observed in the hypoleptinaemic food restricted state could again be attributable to study duration, elevated SLC-1 mRNA levels in the hyperleptinaemic DIO animals may be explained by the development of leptin resistance.

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53P ENHANCED BIOEFFICACY OF A NOVEL ORALLY ACTIVE CALCITONIN ANALOG

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Calcitonin is widely used for the treatment and prevention of endocrine disorders such as post-menopausal osteoporosis and Paget's disease. It is currently available in injectable and intranasal forms, which have the disadvantages of inconvenience, discomfort, and also of low bioavailability with the latter, leading to high inter- and intrapatient variability. Our objective was, therefore, to develop an oral form of calcitonin that was superior in these respects.

Starting with recombinant salmon calcitonin (sCT), we synthesized a series of analogues containing amphiphilic oligomers covalently conjugated to basic residues. One of these, CT-025, was selected for further evaluation, based on its *in vitro* and *in vivo* performance. Intrinsic activity was measured in cultured T47D cells, a mammary tumour line that expresses the human calcitonin receptor (Disa *et al.*, 1998). Increases in cellular metabolic activity in response to drugs were quantified using a Cytosensor™ microphysiometer, which measures real-time rates of extracellular acidification. CT-025 was found to have virtually identical activity to sCT, as determined by the EC_{50} values (0.43 and 0.41 nM respectively) and the maximal response (97% of sCT, n = 4-6).

Proteolytic stability was determined by HPLC analysis after incubating sCT or CT-025 (0.4 mg/ml) with 1 U/ml trypsin in 10 mM phosphate buffer, pH 7.4. After 2 h, 74±3% of the original CT-025 remained intact, compared to only 32±10% of the sCT (means ± SEM of 3 experiments).

Hypocalcaemia in conscious mice was used as a biomarker of calcitonin activity *in vivo* (Kapurniotu *et al.*, 1999). Groups of male CF-1 mice (20-25g, five per data point), fasted overnight, were administered single oral or subcutaneous doses of CT-025 or sCT, and a single serum sample was obtained after the appropriate time interval for analysis of total calcium using a Vitros DT60 II analyser. Oral doses were administered, at 10 ml/kg, in a proprietary phosphate buffered formulation. Subcutaneous administration (10 ml/kg) was in 113 mM Na acetate buffer, pH 4.0, containing 1% gelatine. Dose response curves were constructed using a single time point 60 min after drug administration, this having been found to be the time of peak effect for both compounds. Both sCT and CT-025 reduced serum Ca from a baseline of 9.5-10.0 mg/dl to an average minimum of 7.0-7.5 mg/dl. After oral administration of CT-025, significant hypocalcaemia was seen at doses as low as 0.2 µg/kg, with an average ED_{50} of 0.55 µg/kg (n = 2). CT-025 had similar potency to sCT after subcutaneous administration (ED_{50} values of 0.15 and 0.11 µg/kg respectively, n= 2). The duration of action of both CT-025 and sCT by either route of administration was between 3 and 4 h.

In conclusion, conjugation of sCT with an amphiphilic oligomer confers proteolytic stability without compromising activity. CT-025 thus provides an effective means of delivering calcitonin activity orally in mice. The oral bioefficacy relative to the subcutaneous form is about 27%.

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54P FUNCTIONAL EVIDENCE FOR A 5-HT_{2B} RECEPTOR CONTROLLING MOTILITY IN HUMAN ISOLATED COLON

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5-HT has been proposed to play a key role in the aetiology of irritable bowel syndrome, although the receptor involved has not previously been established. In animal studies, the excitatory effects of 5-HT on intestinal motility appear to be mediated by 5-HT_3 and/or 5-HT_4 receptors (Read & Gwee, 1994). In the present study we have investigated the possible role of 5-HT_{2B} receptors in 5-HT-induced excitatory effects in human isolated colon.

The expression, localisation and function of 5-HT_{2B} receptors were investigated in human colon. For gene expression studies, after extraction of total RNA, the level of expression of mRNA for the 5-HT_{2B} receptor was determined by quantitative reverse transcription-polymerase chain reaction using a sequence detection system (ABI PRISM™ 7700; see Bowen *et al.* (2000) for methods). By immuno-cytochemistry, a 5-HT_{2B} receptor-selective antibody (Pharmingen) was used to localise the receptor within fresh frozen (10 µm) and formalin-fixed paraffin-embedded (5-7 µm) sections of human colon. For pharmacology studies, strips of human colon longitudinal smooth muscle were set up in organ baths containing gassed (95% O_2:5% CO_2) Krebs solution maintained at 37°C, for measurement of changes in tension. Strips were stimulated electrically, via platinum wire electrodes placed at either side of the muscle strip, and the effects of 5-HT (in the absence and presence of a range of 5-HT antagonists) were tested against the resulting contractile responses. All samples of colon were obtained through medically qualified intermediaries with the informed consent of the donor or next of kin, and with approval of the local ethics committee.

In human colon, mRNA for 5-HT_{2B} receptors was highly expressed and immunocytochemistry studies showed that these receptors are localised on both smooth muscle and myenteric nerves in human colon. When mounted in organ baths, longitudinal muscle strips of human colon showed little or no direct response to exogenous 5-HT. Electrical stimulation of muscle strips resulted in a transient contractile response, which was inhibited by both atropine (1-10µM) and tetrodotoxin (3µM), implicating the involvement of cholinergic nerves (n>4 for each treatment). Application of 5-HT to electrically-stimulated muscle strips resulted in significant potentiation of the neurally-mediated contractile response, with a pEC_{50} of 8.2±0.1 (n=49 donors). Addition of the potent and selective 5-HT_{2B} receptor antagonists RS-127445 (Bonhaus *et al.*, 1999), SB-206553 (Forbes *et al.*, 1995a) and SB-204741 (Forbes *et al.*, 1995b) caused rightward displacements of concentration-effect curves to 5-HT, with no change in the response maxima, yielding pK_B estimates of 9.4±0.4, 8.5±0.1 and 6.8±0.2 respectively (n≥3 donors each). These data are consistent with the response being mediated by 5-HT_{2B} receptors.

In summary, we have investigated the role of 5-HT_{2B} receptors in human colon. The mRNA for these receptors is highly expressed in human colon, and the receptor is localised to both nerves and smooth muscle. We have shown that stimulation of these 5-HT_{2B} receptors causes potentiation of neurally-mediated responses, with little or no direct contractile effect on the smooth muscle itself. The mechanism of this 5-HT_{2B} receptor-mediated activity is unclear. These data provide the first evidence that 5-HT_{2B} receptors may play a pivotal role in controlling motility in human colon.

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55P LIPOXINS INDUCE REORGANISATION OF THE ACTIN CYTOSKELETON IN MONOCYTE-DERIVED MACROPHAGES: THE ROLE OF RhoA

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Lipoxins (LX) are endogenously produced eicosanoids that inhibit neutrophil trafficking. We have recently reported that LXs stimulate non-phlogistic phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages (Mϕ) (Godson *et al.*, 2000).

The aim of this study is to assess the effect of LXs on reorganisation of the actin cytoskeleton in Mϕ and the potential role of the monomeric GTPase RhoA in this process. Human Mϕ were stimulated with the native LXs (LXA_4, LXB_4, 10 nM), the stable synthetic analogues 15-(*R/S*)-methyl LXA_4 and 16 phenoxy-LXA_4 (10^{-11} M), the LX precursor 15-(*S*)-HETE (10 nM) or with vehicle (0.1% ethanol) for 15 mins at 37° C. After this period, cells were fixed and F-actin stained with Oregon Green phalloidin and images were obtained by immunofluorescence microscopy. All the LXs tested, but not the 15-(*S*)-HETE, induce actin reorganisation in Mϕ leading to the formation of cortical actin structures, lamellipodia and filopodia (n=5). A cAMP analogue, the 8-Bromo-cAMP (2 mM, 15 mins, 37° C) inhibited the formation of actin extensions induced by LXA_4. In contrast, inhibition of protein kinase A by Rp-cAMP (100 μM, 15 mins, 37° C) mimicked the effect of LXA_4. The Rho family of small GTPases (Rac, Rho and Cdc42) are known to be involved in cytoskeleton rearrangement (Hall, 1998) and phagocytosis. Distinct signalling mechanisms are involved in Fcγ receptor and complement receptor C3 mediated phagocytosis. In particular, the complement-mediated phagocytosis that is not associated with release of pro-inflammatory mediators requires Rho (Caron & Hall, 1998).

To investigate the role of RhoA in LX-induced actin reorganisation, we measured the changes in membrane-associated-Rho by Western blotting with a monoclonal antibody against RhoA. The monocytic THP-1 cells were differentiated into a Mϕ like phenotype by incubation with phorbol 12, myristate 13, acetate (PMA, 10 nM, 48 hours). Differentiated THP-1 cells stimulated with LXs, but not with 15-(S)-HETE showed an approximate doubling in membrane translocation of RhoA, compared to vehicle (n=3). This effect of LXs on Rho activation was inhibited by 8-Bromo-cAMP and mimicked by Rp-cAMP. To directly examine the ability of LXs to activate Rho we use a GTP-Rho "pull down" assay by Rhotekin Rho binding domain to measure GTP-loaded (active) Rho (Ren *et al.*,1999). In differentiated THP-1 cells stimulated with LXs a 2-fold increase in Rho activity was detected (n=3).

In conclusion, LXs trigger the reorganisation of actin in Mϕ concomitant with RhoA activation. These processes are sensitive to modulation by intracellular cAMP.

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56P THE PHARMACOLOGICAL IMPLICATIONS OF THE ASSOCIATION AND ACTIVITY OF MAST CELLS AND MYOFIBROBLASTS IN PERI-IMPLANT FIBROSIS.

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In many branches of surgery, the formation and contraction of fibrous capsules around subcutaneously implanted devices remains a common problem of unknown aetiology (Becker & Springer, 1999). There are no non-operative treatment options, indicating our lack of basic knowledge of the factors that result in this process. A similar proliferation of fibrous tissue also occurs in hypertrophic scars/keloids, lung and liver fibrosis where these conditions are associated with mast cell hyperplasia (Metcalfe *et al.*, 1997). In these conditions, myofibroblasts have also been observed (Desmouliere and Gabbiani, 1996) but few studies have attempted to determine if there exists a relationship between these two cell types in fibroproliferative disease. This study investigates the kinetic relationship of mast cells and myofibroblasts in the development of peri-implant fibrosis.

One silicone implant was sited in the sub-pannicular fascia in each of 28 male Hooded-Lister rats (weight 200-250 grams). Animals were randomly sacrificed (n=4) on days 1, 3, 5, 7, 14, 30 and 60. Implants were dissected *en bloc* with surrounding tissues. Control tissue was obtained from the unwounded contralateral side. Specimens were analysed by conventional histology for collagen (Herovici trichrome) and mast cells (toluidine blue). Immunohistochemistry for alpha-smooth muscle actin was used to demonstrate myofibroblasts. Mean mast cell counts (number/mm^2) were determined using a standard method.

A marked decline in mast cell numbers from control (89/mm^2) occurred on days 1 (36/mm^2)* and 3 (41/mm^2)*. Normal mast cell numbers were restored by day 7 (88/mm^2), coinciding with the first appearance of capsular organization and myofibroblast expression. Capsular development, myofibroblast expression and mast cell numbers increased progressively until day 30 (186/mm^2)*. Capsular architecture, myofibroblast expression and mast cell counts remained unchanged between days 30 and 60.

In this animal model, fibrosis occurs around the subcutaneously implanted silicone device, mimicking the post-operative process in human surgical cases. During capsular development, mast cell degranulation occurs followed by hyperplasia. There is a coincident persistence of mast cell hyperplasia and myofibroblast expression. This suggests that mast cell degranulation may be one of the initiating factors in peri-implant fibrosis. Mast cells may stimulate myofibroblast activity, thereby increasing the potential contractility of the capsule. Pharmacological suppression of mast cell activity may be one therapeutic approach to help prevent excessive fibrosis occurring in the processes of capsular contracture and other fibroproliferative disorders. Further studies using human capsular tissues are indicated to determine if a similar mast cell-myofibroblast association exists.

(* $p<0.025$ vs. control - Mann Whitney U Test)

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57P AN *IN VITRO* INTESTINAL CELL CULTURE MODEL OF M-CELLS DISPLAYS A TH1 TYPE CYTOKINE PROFILE

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The follicular associated epithelium (FAE) of the intestine contains specialised antigen sampling cells (M-cells) that play a pivotal role in antigen sampling and processing. M cells represent an opportunity to initiate and/or modulate mucosal immune responses and are of particular interest as a portal or entry site for mucosal vaccines (Bye *et al.*, 1984). The aim of this work was to study the translocation of liposome nanoparticles across an *in vitro* intestinal cell culture model of human M-cells. A secondary aim was to examine the polarized expression of a selection of cytokines in the M cell model in an attempt to identify soluble mediators involved in M-cell formation and lympho-epithelial communication.A human B-lymphocyte cell line was co-cultured with human intestinal epithelial Caco-2 cells grown on permeable supports. Corresponding mono-cultures of Caco-2 cells on matched filter supports were used as controls Transepithelial electrical resistances (TEER) was measured to assess the monolayer integrity. Adherence and translocation of rhodamine-labelled liposome particles (0.2μm) at 37°C and measured by FACScan. as described previously (Gullberg *et al.*, 2000). Cytokine expression was measured by ELISA and was compared using a two-tailed unpaired Student's t-test. As a control for B cells, Raji B cell-conditioned medium was used to rule out secreted factors.

Translocation of the 0.2μm-sized particles was significantly increased in the co-cultures (186 ± 28 x 10^3 particles versus 72 ± 10 x 10^3 particles in controls; n=4). Targeting of the rhodamine particles to CTB and UEA receptors did not enhance transport across the cellular compartment. Examination of the affect of co-culturing the Caco-2 monolayers with B-lymphocytes on adherence of rhodamine labelled orasome particles was also unaffected (861 ± 44 x 10^3 particles and 822 ± 57 x 10^3 particles for control and co-culture respectively; n=4). No difference in adherence was seen for rhodamine particles targeted to UEA and CTB receptors. A significant decrease in the otherwise stable TEER occurred in Caco-2 monolayers co-cultured with Raji B lymphoid cells (427 ± 30 Ω.cm^2) compared to control mono-cultures (579 ± 40 Ω.cm^2; n=43). A detailed summary of the cytokines and their polarized secretion is presented below in Table 1.

Table 1. Summary of the polarized secretion of cytokines from Caco-2 monolayers co-cultured in the absence/presence of Raji B-cells (pg.ml^{-1})

	Apical Control	Apical Coculture	Basolat. Control	Basolat. Coculture	Raji Cond. Medium
[IL-4]	0.00	0.00	6 ± 4	2 ± 1	0.00
[IL-5]	0.00	0.00	0.00	0.00	0.00
[IL-6]	115 ± 72	125 ± 69	51 ± 29	53 ± 31	0.00
[IL-8]	13± 2	17 ± 4	10 ± 7	34 ± 15	2 ± 1
[IL-10]	22 ± 3	47 ± 7*	6 ± 7	225 ± 25***	207 ± 25
[IFNγ]	8 ± 4	57 ± 11*	7 ± 4	45 ± 9	0.00
[TNFα]	7 ± 4	11 ± 7	10 ± 6	8 ± 4	0.00

Control and co-culture supernatants (n=8) of respective sides were statistically compared by two-tailed unpaired Student's t-test. *p<0.05, ***p<0.001

The results obtained here support existing evidence that co-culture with B cells can alter particle-sampling properties of intestinal epithelial monolayers (Kerneis et al., 1997). Targeting of the rhodamine orasome particles to CTB and UEA receptors did not enhance transport. It remains to be determined whether the co-culture model can be successfully used to assess targeted particle formulations or whether particle translocation through the monolayer is dominated by a high capacity non-specific endocytotic adsorption process. Taken together the pattern of cytokine expression indicate that the model is polarized towards a Th1 signature. The IL-10 secretion would appear to represent a protective function.

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58P SIGNALLING PATHWAYS INVOLVED IN THE REGULATION OF MMP-9 (GELATINASE B) AND TIMP-1 BY RENAL PROXIMAL TUBULAR CELLS IN RESPONSE TO TNFα AND IL-1β.

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Inflammatory cytokines may play a key role in the development of renal disease. Disruption of tubular basement membrane and interstitial extracellular matrix (ECM) via cytokine induced alterations in matrix metalloproteinases (MMPs), and their inhibitors tissue inhibitors of metalloproteinases (TIMPs) may be an important mechanism in this process. Our aim was to investigate mechanisms by which TNFα and IL-1β could affect these processes in renal proximal tubular cells.

The human proximal tubular cell line (HK_2) was cultured in hormonally defined medium. Cells at 90% confluency were exposed to TNFα (0.1-100 ng/ml) or IL-1β (0.1-100 ng/ml) or a combination of both at 10 ng/ml for periods ranging from 0-72 h. Activity and expression of MMP-9 was examined by gelatin zymography and Western blotting. TIMP-1 expression was examined by Western blotting. m-RNA levels of fibronectin and collagen type IV were measured by RT-PCR.

TNFα (0.1-100 ng/ml) showed a time and dose-dependent increase in MMP-9 ranging from 5-fold to 19-fold over control. In contrast, TIMP-1 production as evaluated by immunoblotting was decreased by TNFα in a dose dependent manner, for example to 60% of control by 10 ng/ml TNFα. IL-1β (0.1-100 ng/ml) had no significant effect on the expression of MMP-9 or TIMP-1. IL-1β (10 ng/ml), however, abolished the TNFα-stimulated induction of MMP-9. In marked contrast, IL-1β (10 ng/ml) augmented the inhibition of TIMP-1 by TNFα (10 ng/ml) to 30% of control. mRNA levels of both fibronectin and collagen type IV remained unaffected by cytokine treatment. The protein kinase C inhibitor GF109203X (10μM) completely abolished the cytokine mediated changes in TIMP-1 whereas alterations in MMP-9 were unaffected. Blockade of the extracellular regulated kinases (ERK 1,2) with 10μM PD98059 produced a 3-fold reduction in TNFα-induced MMP-9 whereas TIMP-1 suppression was unchanged. Inhibition of the p38 MAPK pathway with SB203580 (10μM) reduced the TNFα-induction of MMP-9 by 80% and prevented the MMP-9 inhibition following co-treatment with IL-1β allowing an 8-fold increase over control. A TNF receptor blocking antibody (10μg/ml) abolished TNFα-induced MMP-9 induction while suppression of TIMP-1 in the presence of the 2 cytokines was reduced to 60% control. Blockade of the IL-1 receptor 1 with the antagonist (10μg/ml) reduced the synergistic suppression of TIMP-1 to 60% control and also completely prevented the inhibitory effect of IL-1β on TNFα stimulated MMP-9.

These findings suggest that TNFα modulate ECM degradation in proximal tubular cells by the suppression of TIMP-1 in addition to acceleration of MMP-9 production. In contrast IL-1β suppresses the TNFα induction of MMP-9 but augments its effects on TIMP-1. Here the differential effects of TNFα and IL-1β are mediated through the TNFR1, the IL-1R1 and the different signalling pathways of PKC, ERK 1,2 and P38 MAPK kinase pathways.

59P SIGNALLING MECHANISMS INVOLVED IN ALTERATION OF EPITHELIAL BARRIER FUNCTION BY CYCLOSPORINE IN RENAL MDCK CELLS

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The use of the immunosuppressive drug cyclosporine A (CsA) is limited by nephrotoxicity and is associated with renal magnesium (Mg) wasting (Andoh *et al.*, 1996). The mechanism of the renal Mg wasting induced by CsA is not understood. Alterations in renal epithelial cell-cell contact and paracellular transport may be involved. Thus, the aim of this work was to investigate the effects of CsA on the tight junction and paracellular permeability of the MDCK renal epithelial cell line. The possible involvement of intracellular signalling pathways was investigated by probing the role of the mitogen activated protein kinase (MAPK) ERK 1/2 and p38 pathways.

MDCK cells were cultured in DMEM on Transwell filters and when confluent, transepithelial resistance (TER) was measured as an index of the cell-cell contact and paracellular transport. All results are expressed as Δ TER ± S.E.M. of 3 independent experiments each carried out in triplicate. The expression and localisation of the junctional protein occludin (Furuse *et al.*, 1993) was determined by Western blotting and immuno-fluorescence.

CsA (4.2μM) increased the TER of MDCK monolayers. This increase was significant ($p<0.01$) at 48 and 72h with increases of 44.6 ± 7.5 $\Omega.cm^2$ and 86.1 ± 4.6 $\Omega.cm^2$ respectively. Baseline TER was 120 $\Omega.cm^2$. 120 CsA (4.2μM) increased the expression of occludin 2-fold over basal at 72h. Occludin was localised to the cell-cell borders in control and treated cells. CsA (4.2μM) increased the phosphorylation of ERK 1/2 and this was prevented by the ERK 1/2 inhibitor, PD98059 (10μM). CsA had no effect on p38 phosphorylation. The whole cell expression of ERK 1/2 and p38 was unaltered by CsA treatment. PD98059 (10μM) significantly decreased the TER by 19.2 ± 3.0 $\Omega.cm^2$ at 48hrs ($p<0.05$) and by 27.3 ± 3.1 $\Omega.cm^2$ at 72hrs ($p<0.01$). Concurrent treatment with CsA (4.2μM) and PD98059 (10μM) significantly ($p<0.001$) reduced the CsA-induced increase in TER at 24hrs (79.1 ± 6.4 v 46.2 ± 4.3 $\Omega.cm^2$), at 48hrs (55.9 ± 8.1 v 15.5 ± 4.3 $\Omega.cm^2$) and at 72hrs (73.8 ± 6.1 v 9.0 ± 3.9 $\Omega.cm^2$). The p38 inhibitor SB203580 (10μM) alone had no effect on the TER. However, SB203580 (10μM) did significantly ($p<0.001$) augmented the CsA-induced increase in TER at 24hrs (79.6 ± 5.9 v 137.1 ± 6.3 $\Omega.cm^2$) and 48hrs (55.7 ± 7.1 v 93.0 ± 7.6 $\Omega.cm^2$).

These results indicate that CsA can alter renal epithelial cell-cell contacts. Increased expression of occludin may be involved. Activation of the ERK 1/2 pathway appeared to be critical. Additional studies from our laboratory on other renal cell models also indicate that CsA can activate the ERK 1/2 pathway. This is distinct from the classical view that all CsA actions involve immunophilins and inhibition of calcineurin. These studies provide new insights into the effects of CsA on renal epithelial cell-cell junctions and junctional proteins and may provide a model to understand mechanisms of CsA nephrotoxicity and renal Mg wasting.

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60P PATHWAYS INVOLVED IN CISPLATIN-INDUCED APOPTOSIS IN RENAL TUBULAR EPITHELIAL LLC-PK_1 CELLS

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CIS-PLATINUM (II) diamine dichloride (cisplatin-Cp) is a chemotherapeutic agent used in the treatment cancer. However, its clinical use is limited by acute and chronic nephrotoxicity. Although it has been shown that cisplatin can induce apoptosis in proximal tubular epithelial cells, the mechanisms are not fully understood (Lieberthal *et al.*, 1996). The apoptotic death-receptor-mediated pathway involves caspase-8-like proteases, whereas the mitochondrial-mediated pathway is thought to involve caspase-9-like proteases (Budihardjo et al., 1999). Our aim was to investigate the possible involvement of these two pathways in cisplatin-induced apoptosis, in addition to the possible role of the tumour suppressor protein p53 in cisplatin-induced renal epithelial cell death.

Renal proximal tubular epithelial LLC-PK_1 cells were grown to confluency in DMEM. Cells were exposed to Cp 50μM or vehicle DMSO for periods of 6, 12, 18 or 24 hours. Cells were exposed to Cp either in the absence or presence of the caspase-8-like inhibitor (40μM Z-IETD-fmk) or the caspase-9 like inhibitor (40μM Z-LEHD-fmk) or a combination of both inhibitors. Apoptosis was assessed by i) morphological characteristics using phase contrast microscopy, ii) nuclear condensation using DAPI staining, iii) Poly-ADP-ribose polymerase (PARP) cleavage using Western blotting and iv) caspase-3 and -7 activation using Western blotting with anti-caspase-3 and anti-caspase-7 antibodies. Possible alterations in p53 were examined at the protein level using Western blotting with a p53 antibody and at the mRNA level by RT-PCR.

Cp 50 μM induced a time-dependent induction of apoptosis as evidenced by (i) detachment of cells from the monolayer, (ii) nuclear chromatin condensation with DAPI staining, (iii) PARP cleavage with detection of lower molecular weight fragments by Western blotting, (iv) cleaved fragments of caspase 3 and caspase 7. The addition of 40μM Z-LETD-fmk or 40μM Z-LEHD-fmk or a combination of both inhibitors together did not prevent the Cp-induced apoptosis as assessed by all of the criteria indicated above. Expression of p53 protein as detected by Western blotting was increased by Cp as follows: 1.9-fold (6 h), 1.8-fold (12 h), 2.3-fold (18 h) and 1.5-fold (24 h). However, no changes in p53 mRNA were observed at the 6 and 12 h time points examined.

These results indicate that the death-receptor and mitochondrial mediated pathways did not appear to be involved in Cp-induced apoptosis in LLC-PK_1 cells. Alterations in p53 protein involving stabilisation rather than alterations in transcription may have played a role.

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61P NIFLUMIC ACID (NFA) INHIBITS UTP-STIMULATED INCREASES IN SHORT CIRCUIT CURRENT (ISC) IN HUMAN BRONCHIAL EPITHELIAL CELLS (HBECs)

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Uridine-triphosphate (UTP) both stimulates anion secretion and inhibits amiloride-sensitive Na^+ absorbtion in cultures of human airway epithelial cells (Devor & Pilewski, 1999). In the course of studying the mechanism of UTP-stimulated anion secretion in HBECs we have utilised NFA, a chloride channel blocker (Kidd & Thorn, 2000), and have observed that its inhibitory activity against the UTP-stimulated ISC increases is more complex than a simple inhibition of an apical chloride conductance.

HBECs (Clonetics) were cultured on Snapwell permeable supports for 21 days, the final 14 days at an apical air interface. These conditions provided a differentiated transporting epithelial structure. Epithelia were placed in Ussing chambers bathed in Ringer solution containing (mM): 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose (37°C, 5%CO_2:O_2) and voltage clamped at 0mV. The effect of NFA (3-300μM) on UTP (30μM) and forskolin (FSK,0.6μM) stimulated ISC changes were recorded in the presence of amiloride (10μM). The effect of NFA on the basal ISC was also studied in intact epithelia and in epithelia that had been previously treated with amphotericin B (10μM) to selectively permeablise the apical membrane. In the permeablisation studies, NaCl in the apical media was replaced with equimolar K-gluconate to establish an apical to basolateral K-gradient to enable the study of the basolateral K^+ conductance. All compounds were added to the apical membrane except FSK, which was added to both membranes. Data are expressed as absolute changes (mean±s.e.mean) or mean % inhibition (±s.e.mean) when compared with vehicle control and significance assumed when $P<0.05$ (Student t-test).

In HBECs hyperpolarised with amiloride, UTP stimulated a transient increase in ISC of 7.5±0.5μAcm^{-2} (n=22) that was inhibited by NFA (0.3-1000μM) to a maximum of 81.3±3.5% ($P<0.05$,n=6) with an estimated IC_{50} apparent of 100μM. In separate studies FSK stimulated a sustained increase in ISC of 17.1±0.3μAcm^{-2} (n=6) that was reduced by the cumulative addition of NFA (3-1000μM) to a maximum decrease of 25.9±1.7μAcm^{-2} (IC_{50}=240±39μM,n=6). The inhibition of ISC with NFA was greater than the FSK-stimulated ISC indicating that a current in addition to that stimulated by FSK was NFA-sensitive. In separate studies, when administered to unstimulated HBECs under basal conditions, NFA (300μM) attenuated the spontaneous ISC by 34.4±3.4% ($P<0.05$,n=6). Under an established apical to basolateral K^+ gradient, permeablisation of the apical membrane with amphotericin B induced a sustained increase in ISC of 58.2±3.3μAcm^{-2} (n=9) that was due to the conductance of the basolateral membrane to K^+ (G_K). NFA (300μM) caused a 34.9±4.4% decrease in G_K ($P<0.05$,n=5).

The mechanism underlying the inhibitory activity of NFA on the UTP-induced increase in ISC is clearly complex and not necessarily simply due to a blockade of the apical Cl^- conductance. To our knowledge this is the first report of the ability of NFA to inhibit K^+ channels and adds to its pharmacological repertoire as a Cl^- channel blocker and BK-channel opener (Ottolia & Toro, 1994).

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62P MUCUS SECRETION FROM DIFFERENTIATED HUMAN BRONCHIAL EPITHELIAL CELLS: REGULATION THROUGH THE PURINORECEPTOR $P2Y_2$

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The mechanisms of regulation of mucus secretion from the goblet cells of humans are not clearly defined. Recently the culture conditions permitting differentiation of human bronchial epithelial cells (HBECs) into a mucociliary epithelium exhibiting functional goblet cells and ciliated cells were established (Gray *et al.*, 1996). A model of agonist induced mucus secretion in differentiated mucociliary human bronchial epithelial cells (HBECs) has subsequently been established. Agonist potency profiles were undertaken using ATP, UTP, ADP, UDP and 2MeSATP to determine which receptor mediates mucus secretion whilst kinase inhibitors were used to study elements of the signal transduction pathway linking receptor activation to mucus secretion.

HBECs (Clonetics) were cultured according to the method of Gray *et al.*, (1996) in media supplemented with BEGM SingleQuots and retinoic acid on collagen I coated inserts (Becton Dickinson). All experiments were carried out at 14-17 days after the establishment of an air-liquid interface. Cells were prepared for the stimulation of mucus secretion by:- 1) removing supplements from the media 48 hours before the experiment, 2) removing pre-secreted mucus from the apical surface by a series of 4, one hour incubations with supplement free media starting 4 hours before the experiment. Immediately before the stimulation of mucus secretion medium was aspirated from the apical surface of the cells. Nucleotide agonists (1-1000μM) were added to the apical surface of the cells and incubated for 30 minutes (37°C, 5% CO_2:O_2) following which the supernatant was removed and assayed for mucus content using an enzyme linked lectin assay (ELLA). In all other experiments cells were pretreated with test compounds or vehicle (0.1% DMSO in supplement free medium) for 30 minutes prior to challenge with ATP-γS (500μM) or vehicle. Cells were incubated for a further 30 minutes following addition of ATP-γS to the apical surface of the cells. The supernatants were removed and assayed for mucus content. Baseline mucus secretion (vehicle control) was nominally set as 100% and data are expressed as mean % baseline (±s.e.mean). or mean % inhibition (±s.e.mean).Significance was assumed when $P<0.05$ (One Way ANOVA with Dunnets).

ATP and ADP (1-1000μM) concentration dependently increased mucus secretion to a maximum of 755±180% ($P<0.05$,n=4) and 355±90% ($P<0.05$,n=4) respectively. UTP and UDP (1-1000μM) also concentration dependently increased mucus secretion to a maximum of 714±157% ($P<0.05$,n=4) and 400±56% ($P<0.05$,n=4) respectively and were equipotent with their respective adenine tri- and di-phosphate counterparts. In contrast 2MeSATP did not dose dependently elevate mucus secretion. These data are consistent with activation of the P2Y2 receptor. Suramin, an antagonist known to block activation of P2Y2 receptor inhibited ATP-γS induced mucus secretion in a concentration dependent manner inhibiting secretion by 66±5% at 500μM ($P<0.05$,n=6). Similarly the PKC inhibitor, Calphostin C and the PI3-kinase inhibitor, LY294002 inhibited ATP-γS induced mucus secretion in a concentration dependent manner. Secretion was inhibited by 94±3% at 5nM ($P<0.05$,n=6) and 59±4% at 500μM ($P<0.05$,n=6) respectively. All compounds tested were without effect on baseline secretion at the highest concentration.

The agonist potency profile ATP=UTP>ADP=UDP>>2MeSATP is consistent with $P2Y_2$ activation dependent mucus secretion. Furthermore the inhibition of mucus secretion by suramin, Calphostin C and LY294002 also constant with known signalling pathways associated with $P2Y_2$R mediated mucus secretion.

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63P ATTENUATION OF PULMONARY HYPERTENSION IN 5-HT_{1B} RECEPTOR KNOCKOUT MICE

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The 5-HT_{1B} receptor is thought to be important in mediating pulmonary vasoconstriction and may contribute significantly to the increased 5-HT-induced vasoconstriction observed in pulmonary arterial hypertension (PAH) (MacLean *et al.*, 2000). Here we investigate the development of PAH in 5-HT_{1B} receptor knockout mice (SV/129, -/-, male, 25-40g). These mice and their wild-type controls (SV/129, +/+) were exposed to hypoxic/hypobaric (10% O_2) conditions for 10 days. Age matched controls were subjected to normal atmosphere. After sacrifice, the heart and lungs were excised and pulmonary resistance arteries (PRAs, ~200μm i.d.) dissected from the lungs. The right ventricle (RV) of the heart was weighed as was the left ventricle plus septum. Lungs were sectioned (4μm sections) and stained with Elastin Van Giesen stain. Using light microscopy, pulmonary arterioles of ~50μm i.d. were identified and those exhibiting vascular remodelling with a double elastic lamina and thickened media were counted.

Table 1. Effect of 10 days hypoxia on RV hypertrophy and small vessel remodelling in wild type and 5-HT_{1B} receptor knockout mice.

Group	RV/TV ratio	n	% Remodelled vessels	n
Control (+/+)	0.194 ± 0.01	15	3.0 ± 1.1	5
Hypoxic (+/+)	0.270 ± 0.01**	15	19.6 ± 1.7**	5
Control (-/-)	0.197 ± 0.01	15	1.9 ± 1.1	5
Hypoxic (-/-)	0.227 ± 0.01^f	15	13.9 ± 1.4^f	5

(-/-): knockout mice; (+/+); wild type mice; RV/TV: right ventricular/total ventricular weight. Statistical comparisons using ANOVA: compared with appropriate control *P<0.05, **P<0.001; compared with hypoxic (+/+) ^{f}P <0.05.

PRAs were set up on a wire myograph under tension equivalent to 15mmHg or 35mmHg (PAH mice) and bubbled with 16%O_2/6%CO_2 balance N_2. Cumulative concentration response curves (CCRCs) were constructed for 5-carboxamidotryptamine (5-CT) to examine functional changes. Results are summarised in Table 1 and Table 2. In the wild-type mice exposed to hypoxia, there was significant remodelling of vessels with RV hypertrophy and increased sensitivity to 5-CT. The (-/-) mice exhibited significantly less vascular remodelling and RV hypertrophy. This demonstrates a marked attenuation in the development of PAH in 5-HT_{1B} receptor knockout mice. Absence of the 5-HT_{1B} receptor had no effect on normal responses to 5-CT in control mice. This study suggests that the 5-HT_{1B} receptor may play a role in PAH and may be a potential therapeutic target in the pulmonary hypertensive state.

Table 2. Effect of 10 days hypoxia on 5-CT-induced contractions in wild type and 5-HT_{1B} receptor knockout mice.

Group	5-CT pEC_{50}	5-CT Emax	n
Control (+/+)	5.73 ± 0.14	111 ± 8	7
Hypoxic (+/+)	6.17 ± 0.06*	167 ± 19	9
Control (-/-)	5.45 ± 0.11	88 ± 12	9
Hypoxic (-/-)	5.57 ± 0.11^f	114 ± 21	7

(-/-): knockout mice; (+/+); wild type mice; Emax: maximum response expressed as % response to 50mM KCl. Statistical comparisons using ANOVA: compared with appropriate control *P<0.05; compared with hypoxic (+/+) ^{f}P <0.05.

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64P THE EFFECT OF 5-$HT_{1B/1D}$ RECEPTOR ANTAGONTIST TREATMENT ON INDICES OF CHRONIC HYPOXIA-INDUCED PULMONARY HYPERTENSION IN RATS

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The 5-HT_{1B} receptor has been implicated in the pathogenesis of pulmonary hypertension secondary to chronic hypoxia (MacLean,1999). Here we examined the effects of GR127935 (5-$HT_{1B/1D}$ antagonist) pretreatment on responses to 5-HT in pulmonary resistance arteries from chronic hypoxic pulmonary hypertensive (CHPHT) rats. The effects of treatment on right ventricular hypertrophy and pulmonary vascular remodelling were also assessed. Male Wistar rats (35 days old, 180-200g) were subjected to 2 weeks of chronic hypoxia (10% O_2) and were treated with either GR127935 (3mg^{-1} kg^{-1} day^{-1}) or vehicle (distilled H_2O), starting 2 days before hypoxic exposure. Age matched normoxic controls (AMCs) were treated with either GR127935 or vehicle for the same time period. Upon cessation of hypoxia, rats were killed and the heart and lungs were removed. Pulmonary resistance arteries (~200 μm i.d.) from each group were mounted on wire myographs under tension equivalent to 15mmHg or 35mmHg (hypoxic rats) and bubbled with 16%O_2/6%CO_2 balance N_2 for the construction of cumulative concentration response curves to 5-HT. Lungs were sectioned (4μm sections) and stained with Elastin Van Giesen stain. Pulmonary arterioles of ~50μm i.d. were microscopically assessed those exhibiting vascular remodelling with a double elastic lamina and thickened media were counted. Statistical comparisons were made by one way analysis of variance. When significance was attained (P<0.05) differences were established using the Newman-Keuls multiple comparison test Maximum contractions (Emax; % response to 50 mM KCl ± SEM) to 5-HT were increased in pulmonary resistance arteries from vehicle treated CHPHT rats (Emax=84.9 ± 5.6% n=8) compared with both GR127935 (Emax=27.6 ± 5.9% n=11; P<0.001) and vehicle treated AMCs (Emax=38.6 ± 5.4 % n=9; P<0.001). Pretreatment of CHPHT rats with GR127935 partially prevented the enhanced response to 5-HT such that Emax (55.4 ± 8.2% n=10) was reduced (P<0.01) compared to vehicle treated CHPHT levels. Hypoxia caused an almost 60% increase in the right ventricle to total ventricular + septum (RV/TV+S) weight ratio in vehicle treated CHPHT rats (0.316 ± 0.08 n=15) compared to vehicle treated AMCs (0.197 ± 0.01 n=15; P<0.001). GR127935 treatment had no effect on the RV/TV+S weight ratio in AMC rats (0.197 ± 0.01 n=15) The development of the right ventricular hypertrophy was partly offset by GR127935 pretreatment (0.274 ± 0.01 n=15; P<0.01 cf. Vehicle treated CHPHT rats). Lung cross sections from vehicle treated CHPHT rats showed an almost tenfold increase in the percentage of vessels showing double elastic lamina in comparison to vehicle treated AMCs (23.6 ± 1.5% n=5 cf. 2.5 ± 0.2% n=4 P<0.001). GR127935 treatment did not alter the percentage of remodelled vessels in AMC rats (2.2 ± 0.9% n=4). CHPHT rats treated with GR127935 showed a highly significant decrease in the percentage of remodelled vessels (14.7 ± 1.9% n=6; P<0.01). Thus, this study indicates that the 5-HT_{1B} receptor is a potential target for the treatment of pulmonary hypertension.

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65P EFFECTS OF ROLIPRAM AND DEXAMETHASONE ON AIRWAY FUNCTION, CELL INFLUX AND LUNG HISTOLOGY OF GUINEA-PIGS CHRONICALLY EXPOSED TO LPS

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Chronic obstructive pulmonary disease (COPD) is associated with airways hyperreactivity (AHR), neutrophil influx and a reduced airway calibre (increased wall thickening, oedema, and mucus producing goblet cells: GC), causing a reduced airflow (FEV_1) (Postma *et al.*, 1998). Lipopolysaccharide (LPS) inhalation also causes AHR and neutrophil influx (Toward & Broadley, 2000). Corticosteroids have little clinical benefit in COPD (Burge *et al.*, 1999). Evidence infers that phosphodiesterase-4 (PDE4) inhibition may ameliorate COPD progression (Torphy, *et al.*, 1999). We therefore characterised the steroid- and PDE4 inhibitor-sensitivity of airway function, cell influx, reactivity and GC induction, in conscious guinea-pigs exposed chronically to LPS, as a model of COPD.

Specific airways conductance (sGaw) was measured in groups (n=6) of conscious Dunkin-Hartley (male, 300-350g) guinea-pigs by whole-body plethysmography (Griffiths-Johnson *et al*, 1998). Baseline (BL) sGaw values were obtained and 30min later they received a nose-only exposure to a threshold dose of nebulised ($0.2ml.min^{-1}$) histamine (hist: 1mM, 20s) and sGaw was recorded at 0, 5 and 10min afterwards. 24h later, animals were box-exposed (1h) to either LPS ($30\mu g.ml^{-1}$) or vehicle (saline) and airway reactivity (AR) to hist was re-assessed at 1, or 24h post exposure. For chronic exposures, animals received LPS or saline (1h, 48h apart) 9 times. AR was re-assessed 24h after the 8th exposure. In other animals, the corti-costeroid, dexamethasone (dex:20mg. kg^{-1}), or PDE4 inhibitor, rolipram (roli:$1mg.kg^{-1}$) were dosed (i.p.) 24 and 0.5h prior to exposure and in chronic studies, daily afterwards. Roli dosing ceased at 24h after the 7th exposure, due to a persistent bronch-odilation that would prevent accurate AR measurement. In dex and roli treated animals, AR was therefore examined 1h after the 1st, or 24h after respective 8th and 9th exposures. Animals were terminated (pentobarbitone sodium: $0.6mg.100g^{-1}$, i.p.) 24h after exposure, and bronchoalveolar lavage fluid (BALF: saline, $1ml.100g^{-1}$ twice) cell content determined. Paraffin wax embedded large-bronchi were stained for general morphology (H&E) and GC (Alcian Blue-Periodic Acid Schiff). GC were expressed per mm of epithelium (*ImageAcquisition:V22,Leica*).

Initial LPS exposures caused persistent bronchodilation (+27.4 ± 10.7 peak % change from BL sGaw), whereas later exposures caused progressively persistent bronchoconstriction (BC: -23.1 ± 4.97%). Dex exacerbated (-28.7±4.03%), whereas roli reversed (+25.1 ± 0.47%), the chronic LPS-induced BC. At 1h after a single LPS exposure hist caused an increased (-26.3 ±7.9%, $P<0.02$) BC compared to before LPS (+16.1±5.9%). This AHR was absent 24h later or after saline exposure. AHR (-17.4±5.6%, $P<0.02$) was extended to 24 h, after the 8th LPS exposure (pre-exposure: +12.8±6.5%). Dex and roli inhibited the single and chronic LPS-induced AHR. BALF macrophages, eosinophils and neutrophils were raised after single LPS (5, 3 and 127-fold) and further raised after chronic LPS (42, 54 and 5651-fold) 24h, compared to single and chronic saline. Compared to naïve animals or chronic saline, chronic LPS also caused oedema and increased GC (20±9, 32±6 and 110±9 $GC.mm^{-1}$, respectively). Dex and roli attenuated the chronic LPS-induced macrophage (21and 45% reduction), eosinophil (91 and 87%) and neutrophil (89 and 64%) influx and GC by 89 and 71%, respectively.

In conclusion, as with COPD, chronic LPS inhalation caused prolonged BC, AHR, airway leukocyte influx, oedema and a GC hyperplasia. The AHR, cell influx and GC were dex- and roli-sensitive. Dex exacerbated, whereas roli reversed airflow obstruction.

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66P PHARMACOLOGICAL CHARACTERISATION OF THE β-ADRENOCEPTOR EXPRESSED BY HUMAN LUNG MAST CELLS

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Bronchodilator β_2-adrenoceptor (β_2-AR) agonists continue to be used widely in the treatment of asthma. The primary action of these drugs is to relax airway smooth muscle but additional effects may include the inhibition of human lung mast cell (HLMC) activity. *In vitro* functional studies suggest that the receptor mediating the inhibition of HLMC activity is the β_2-AR (Butchers *et al.*, 1980). However, no direct evidence to demonstrate whether β_2-AR are expressed by HLMC has been provided. Hence the aim of the present study was to characterise the sub-type(s) of β-AR expressed by HLMC using radioligand binding techniques.

Human lung tissue was obtained from surgical resections. The tissue was physically and enzymatically dispersed to generate a single cell suspension of which about 5% were mast cells. HLMC were purified (>90% purity) using immunomagnetic bead separations by methods that have been described (Weston *et al.*, 1997). Membranes were prepared by homogenisation. In order to determine β-AR subtypes in HLMC membranes, competition binding assays were performed using the radioligand [^{125}I]-cyanopindolol ([^{125}I]-CYP; 0.05 nM) in the presence of several antagonists. Competition curves were analysed using GraphPad Prism software (version 2).

Binding of [^{125}I]-CYP to HLMC membranes was antagonised by the non-selective antagonist, propranolol, in a concentration-dependent manner. The high affinity (pK_i; 9.0±0.1, mean±s.e., n=4) obtained for propranolol indicates the presence of β_1-AR and/or β_2-AR in these membranes and that neither β_3-AR nor atypical-AR are present. Data from studies employing the β_2-selective antagonist, ICI118551, in HLMC membranes show that nanomolar concentrations of ICI118551 (pK_i; 8.9±0.1, n=4) displaced radioligand from a single binding site (Hill slope of unity) indicating that β_2-AR alone are present in HLMC. Studies with the β_1-selective antagonist, CGP20712A, also suggest that β_2-AR are present in HLMC membranes as low concentrations ($<10^{-8}$ M) of the antagonist did not displace the radioligand, indicating an absence of β_1-AR, whereas higher concentrations (pK_i; 6.0±0.03, n=4) did displace the radioligand from a single binding site (Hill slope of unity) showing the presence of β_2-AR. In order to confirm that our methods could discriminate effectively between β-AR sub-types, membranes from human lung tissue (known to contain a mixture of β_2-AR and β_1-AR) were prepared and subjected to competition radioligand binding by ICI118551 (n=4) and CGP20712A (n=4). These experiments indicate that lung tissue membranes contain both β_2-AR and β_1-AR (4:1) which is consistent with values reported in the literature (eg. Nishikawa *et al.*, 1996).

These data indicate that the HLMC expresses a homogeneous population of β_2-AR.

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67P CHARACTERISATION OF PHOSPHODIESTERASES IN HUMAN LUNG MAST CELLS AND BASOPHILS

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We have previously reported that the cAMP-specific phosphodiesterase (PDE), PDE4, is the predominant isoform regulating the responses of human basophils (Weston *et al.*, 1997). In contrast, PDE4 does not appear to regulate the responses of human lung mast cells (HLMC). These findings are based on data showing that, of a variety of isoform-selective inhibitors, the PDE4-selective inhibitor rolipram, attenuated the IgE-mediated release of histamine from human basophils whereas none of these compounds (rolipram included) had any effect on HLMC activity. Further studies showed that rolipram inhibited the cAMP hydrolytic activity present in basophil extracts but rolipram had little effect on the hydrolytic activity in HLMC extracts (Weston *et al.*, 1997).

In further attempts to characterise PDEs present in basophils and HLMC, we have performed RT-PCR in order to establish whether mRNA for cAMP-specific PDEs is expressed. In addition, the potential presence of PDE isoforms in cell preparations was investigated by Western blotting.

Basophils and HLMC were purified (>98% purity) by immunomagnetic bead separations. Mononuclear cells (MNC) were also isolated and used as comparative controls. RNA was extracted from the cells using Trizol. Reverse transcription was performed using the Moloney Murine Leukaemia Virus reverse transcriptase. PCR reactions were performed, using primers and conditions (Giembycz *et al.*, 1996; Seybold *et al.*, 1998) designed to identify cDNAs for PDE3, PDE4, PDE7 and for GAPDH which was included as an inter-preparation control. Moreover, Western blots were performed on purified preparations of cells using antibodies to PDE4A, 4B, 4D and 7A with recombinant proteins used as positive controls in the blots.

RT-PCR experiments indicate that, relative to MNC (n=4), basophils (n=4) express comparable levels of message for PDE4A, PDE4D, PDE7A with some modest expression of PDE3B. In contrast, HLMC (n=3) express relatively little if any PDE4A, PDE4D and PDE3B but do express message for PDE7A.

Immunoblotting studies indicate that basophils (n=4) express PDE4A and PDE4D whereas neither of these isoforms is present in HLMC. Neither basophils (n=4) nor HLMC (n=4) contain PDE7A despite both cells expressing message for this isoform.

These data reinforce findings generated from previous functional studies showing that regulation of PDE4 modulates basophil but not HLMC activity. The nature of the cAMP hydrolytic activity present in HLMC remains uncertain.

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68P EFFECT OF A NOVEL PDE4 INHIBITOR, NVP-ABE171, A 1,7-NAPHTHYRIDINE DERIVATIVE IN MODELS OF LUNG INFLAMMATION IN MICE AND RATS

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The cyclic nucleotide phosphodiesterases (PDEs) are a family of enzymes that play an important role in regulating intracellular levels of cyclic nucleotides. Among them, PDE4 plays a major role in modulating the activity of inflammatory cells. Inhibitors of this enzyme family have been shown to be anti-inflammatory in experimental animal models and in patients. In this report we investigated the pharmacological profile of a novel PDE4 inhibitor, NVP-ABE171, 4-(8-Benzo[1,2,5]oxadiazol-5-yl-[1,7]naphthyridin-6-yl)-benzoic acid, in animal models of lung inflammation. Ariflo® (SB207499), a PDE4 inhibitor in advanced clinical development, was used as a comparitor compound.

Female BALB/c mice (20 g, 6-8 animals per group) were challenged intranasally, under Halothane/oxygen/nitrous oxide anaesthesia, with 50 µl of a solution of lypopolysaccharide (LPS, *Salmonella Typhosa*, 0.3 mg kg^{-1}) or with phosphate buffered saline (PBS). Three h after the provocation, terminal anaesthesia was induced with pentobarbital (60 mg kg^{-1}, i.p.). Compounds were given orally in PBS containing 2% dimethyl sulfoxide (DMSO) 1 h before the challenge. Male Brown Norway rats (200 g, 6-10 animals per group) were immunised, on day 0, 15 and 21, with ovalbumin (OA)/Alum® (20/20 µg ml^{-1}, s.c.) and *B. pertussis* vaccine (0.25 ml, i.p.). On day 28, animals were exposed (1 h) to an aerosol of OA (3.2 mg ml^{-1}) and sacrificed 48 h later with pentobarbital (250 mg kg^{-1}, i.p.). Compounds were dosed orally in PBS 2% DMSO 1 h before and 24 h after the challenge.

Statistical significance ($P < 0.05$) were determined using an ANOVA. Data are expressed as mean ± s.e.mean.

NVP-ABE171 and Ariflo® were potent inhibitors of purified human PDE4 isotypes with respective IC50 values (nM) of 602 ± 25, 398 ± 7 (PDE4A); 34 ± 0.5, 288 ± 7 (PDE4B); 1230 ± 39, 813± 13 (PDE4C) and 1.5 ± 0.1, 63 ± 2 (PDE4D) (n = 3-6). Both compounds were inactive against PDE1, 2, 3, 5 and 7.

At 3 h post-challenge, LPS induced an increase in neutrophil numbers and in TNF-α levels in the bronchoalveolar lavage (BAL) fluid obtained from BALB/c mice. Neutrophils were inhibited, in a dose-dependent manner by NVP-ABE171 (dose in mg kg^{-1}, % of LPS challenge): 0.05, 78 ± 6; 0.1, 40 ± 3; 1, 26 ± 4; 10, 4 ± 0.6) as was TNF-α (dose in mg kg^{-1}, % of LPS challenge: 0.05, 88 ± 9; 0.1, 66 ± 11; 1, 64 ± 8; 10, 55 ± 7). Ariflo® was inactive at the highest dose tested, 10 mg kg^{-1}. Forty eight h after the OA challenge, sensitised rats show an increase in eosinophil number and eosinophil peroxidase (EPO) activity in the BAL which were inhibited, in a dose dependent manner by NVP-ABE171; Eosinophils (dose in mg kg^{-1}, % of OA challenge): 0.01, 113 ± 32; 0.1, 55 ± 13; 1, 9 ± 0.8). EPO activity (dose in mg kg^{-1}, % of OA challenge: 0.01, 67 ± 22; 0.1, 42 ± 7; 6 ± 3). Ariflo®, at a dose of 10 mg kg^{-1}, produced a significant inhibition of both parameters (in % of OA challenge: eosinophils, 44 ± 7; EPO activity, 59 ± 5).

These data demonstrated that despite similar potency on the purified PDE4 enzymes in vitro, NVP-ABE171 is a more potent compound than Ariflo® in in vivo models of lung inflammation. In conclusion, NVP-ABE171 is a promising drug for the treatment of inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease.

69P KCO912: A POTENT AND SELECTIVE OPENER OF ATP-DEPENDENT POTASSIUM (K_{ATP}) CHANNELS WITH SELECTIVITY FOR THE AIRWAYS

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Airways hyperreactivity (AHR) is a defining feature of asthma which persists despite successful anti-inflammatory treatment (Brusacco et al., 1998). K_{ATP} channel openers can obviate experimental AHR and have shown therapeutic benefit in asthma; however, the clinical potential of such compounds is compromised by cardiovascular side-effects (Fozard & Manley, 2000). We report here the pharmacological properties of (3*S*, 4*R*)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-piperidinyl)-*N*-phenyl-2H-1-benzopyran-6-sulfonamide (KCO912), a K_{ATP} channel opener which, when given locally to the airways, suppresses AHR at doses devoid of cardiovascular effects.

Binding of ^{3}H-P1075 and ^{3}H-glibenclamide to endothelium denuded rat aortic strips and efflux of $^{86}Rb^{+}$ from aortic rings with intact endothelium were measured as previously described (Quast et al., 1993). Guinea pigs (400-600g) were made hyperreactive to the bronchoconstrictor effects of histamine and methacholine by acute i.v. injection of preformed immune complexes or exposure to ozone (3 ppm, 30 min), respectively and set up for measuring airway resistance, blood pressure (BP) and heart rate (HR) (Buchheit & Hoffmann, 1996). Rhesus monkeys, pre-selected for the high sensitivity of their airways to methacholine, were anaesthetised and set up for measuring airway resistance, BP and HR as described in detail by Fozard & Buescher (2001).

KCO912 inhibited specific binding of ^{3}H-P1075 and ^{3}H-glibenclamide to rat aortic strips to 100% with pKi values of 8.28 ± 0.03 and 7.96 ± 0.04 (mean ± s. e. mean, n=4), respectively. The efflux of $^{86}Rb^{+}$ from rat aortic rings was increased concentration-dependently by KCO912 (pEC50, 7.51 ± 0.11, n=4); the effect of 50 nM KCO912 was inhibited (>90%) by glibenclamide, 1 μM.

Following intratracheal (i.t.) administration of KCO912 (0.01-10 μg kg^{-1}), AHR induced by ozone or immune complexes in guinea pigs was rapidly (<5 min) reversed (ED_{50} values derived from mean dose-response curves 1 and 0.03 μg kg^{-1} n = 4-5, respectively); changes in BP were seen only at doses 100 μg kg^{-1} yielding 'therapeutic ratios' of 100 and 3333, respectively. At doses which suppressed AHR, KCO912 had no anti-bronchoconstrictor effects in normoreactive guinea pigs. The effect of KCO912 (10 μg kg^{-1} i.t).on immune complex-induced AHR was fully inhibited by glibenclamide (30 mg kg^{-1} i.v.). In spontaneously hyperreactive rhesus monkeys, KCO912, given by inhalation, inhibited methacholine-induced bronchoconstriction at doses between 0.35 and 109 μg kg^{-1} (ED_{50} value derived from mean dose response curve 1.2 μg kg^{-1}, n = 5) but was devoid of effects on BP or HR at all doses tested ('therapeutic ratio' >100).

The present data show that when given locally to the airways in both guinea pigs and monkeys, KCO912 has a significantly better 'therapeutic window' than representative earlier generation K_{ATP} channel openers defined in the same models (Buchheit & Fozard, 1999; Fozard & Manley, 2000). Given the pivotal role of AHR in the pathophysiology of asthma, KCO912 appears to be a suitable candidate for clinical evaluation.

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