165P ADRENERGIC MODULATION OF CALCIUM-ACTIVATED POTASSIUM CURRENT IN RABBIT PIGMENTED CILIARY EPITHELIAL CELLS

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The ciliary epithelium of the eye is a secreting epithelium comprised of two different epithelial cell layers: a pigmented epithelial (PE) cell layer whose basal membrane faces the stromal side and a non-pigmented epithelial cell layer whose basal membrane faces the posterior and vitreal spaces of the eye. These two cell layers are coupled via gap junctions at their apical membranes. Recently, it has been demonstrated that α_1-adrenergic receptors are present on PE cells and their activation is associated with increases in $[Ca^{2+}]_{in}$ the epithelium as well as alterations in solute secretion (Krupin et al., 1991; Schütte et al., 1996). We used whole-cell and nystatin perforated patch recording techniques to investigate adrenergic receptor-coupled signaling pathways modulating ionic currents in isolated cultured rabbit PE cells. Whole-cell patch-clamp recordings in PE cells revealed a TTX-sensitive inward Na^+ current and at least two outward K^+ currents, a slowly-inactivating TEA- and ibeirotoxin (IbTX)-sensitive Ca^{2+}-activated K^+ current and a delayed rectifier K^+ current. External application of 10^{-5}M epinephrine resulted in an increase in outward K^+ current (I_K), measured at +60 mV, by 116 ± 8% (n=3) in 3/5 cells tested). When PE cells were exposed to the α_1-selective agonist phenylephrine, an increase in I_K of 46.06 ± 16% at +50 mV was observed in 4/11 cells tested. Adrenergic-induced increases in I_K were also blocked by pretreating cells with IbTX (5 nM; n=4). Our results suggest that α_1-adrenergic receptor activation may increase Ca^{2+}-activated K^+ current in PE cells via a signaling pathway that requires release of intracellular Ca^{2+}.

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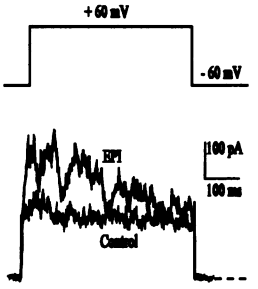

Figure 1. Outward current elicited in a representative PE cell in Na^+-Asp (external) and K^+ -Asp (internal) solutions. Outward current, measured at +60 mV is enhanced by external application of 10^{-5} M EPI.

166P POSSIBLE INTERACTIONS OF L-2-CHLOROPROPIONIC ACID WITH VOLTAGE-SENSITIVE CALCIUM CHANNELS IN THE RAT CEREBELLUM

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When administered orally in a single dose, L-2-chloropropionic acid (L-CPA) results in the selective destruction of cerebellar granule cells via a mechanism which involves activation of N-methyl-D-aspartate receptors (Widdowson et al., 1996). We explored the possibility that voltage-sensitive sodium and calcium channels may play a role in the development of the L-CPA-induced neurotoxicity. Male AP rats (200-250g) were orally dosed with L-CPA diluted in water (750 mg/kg, pH 7.0). Rats received two i.p injections of saline, phenytoin (50 mg/kg), rilazole (4 and 25 mg/kg), lifarzine (0.5 mg/kg) (±)verapamil (5 mg/kg) or nifedipine (5 mg/kg) 30 min prior to and 1 h following L-CPA dosing. Rats were killed by CO_2 anaesthesia 48 h after dosing. Cerebellar neurotoxicity was assessed by measuring the development of cerebellar oedema and the loss in cerebellar glutamate and aspartate concentrations, as described previously (Widdowson et al., 1996). Phenytoin, rilazole, and lifarazine were not able to significantly alter the L-CPA-induced neurotoxicity. However, nifedipine and verapamil significantly potentiated the L-CPA-induced cerebellar neurotoxicity, oedema and reductions in cerebellar aspartate concentrations (controls = 18.8 ±2.5 µmol/g dry weight, L-CPA = 9.3 ±2.5*, L-CPA and verapamil = 5.7 ±0.5**, L-CPA and nifedipine = 5.6 ±1.5**, * P<0.05 vs. controls, ** P<0.05 vs. L-CPA treated, mean ± S.E.M. for n= 5 rats.) None of the channel blockers changed cerebellar water, sodium or aspartate concentrations when injected alone. We explored whether L-CPA may alter calcium channels in the cerebellum to modulate neurotransmitter release. Release of endogenous aspartate, glutamate and [^{3}H]GABA was examined in cerebellar and cerebral cortex slices (0.3 x 0.3 mm) prepared from Halothane killed male 220 g AP rats. Slices were perfused with warm, gassed Krebs buffer at 0.3 ml/min, for 30 min then 3 min fractions were collected for 30 min. After 12 min of collecting fractions, slices were electrically stimulated (10 mA, 8 Hz) for 90 s. When slices were incubated with 2 mM neutralised L-CPA during the pre-loading stage and was present in the superfusion medium, the electrically stimulated release of amino acids was inhibited. (electrically stimulated release, minus basal release of [^{3}H]GABA from cerebellum = 12.9 ±4.5, release in calcium free = -0.2 ±0.2*, release with L-CPA -1.9 ±0.3 *; * P<0.05 vs. controls, mean ± S.E.M. for n = 18). Stimulated, but not basal release of aspartate, glutamate and [^{3}H]GABA was dependent on the presence of calcium. Basal and stimulated release of [^{3}H]GABA from cerebral cortex slices was not affected by L-CPA. In conclusion, L-CPA neurotoxicity is potentiated by verapamil and nifedipine and L-CPA inhibited the stimulated release of aspartate, glutamate and GABA from the cerebellum suggesting that L-CPA may modulate voltage-dependent calcium channels.

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167P LARGE CONDUCTANCE BUT NOT APAMIN-SENSITIVE K^+ CHANNELS IN SMOOTH MUSCLE CELLS ISOLATED FROM THE RABBIT MESENTERIC ARTERY

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Vascular endothelial cells release a variety of substances which affect the membrane potential and tone of vascular smooth muscle. These substances include nitric oxide (NO) and an endothelium-derived hyperpolarizing factor (EDHF), the identity of EDHF is unknown. In rabbit mesenteric artery, the mechanism of hyperpolarization by EDHF has recently been shown to involve apamin-sensitive K^+ channels (Murphy and Brayden, 1995). Reports of apamin-sensitive K^+ channels in smooth muscle cells are rare, although, a 68 pS apamin-sensitive K^+ channel has been described in smooth muscle cells from rat kidney (Gebremedhin, Kaldunski, Jacobs, Harder and Roman, 1996). Also, in the endothelium of intact rat aorta, a much smaller (9 pS) apamin-sensitive K^+ channel has also been described (Marchenko and Sage, 1996). We have addressed the possible existence of apamin-sensitive K^+ channels in single smooth muscle cells isolated from the rabbit mesenteric artery.

Single smooth muscles cells were isolated from the second and third order branches of rabbit mesenteric arteries taken from New Zealand female white rabbits. Cells were isolated using a combination of collagenase/pronase and conventional patch-clamp techniques employed (Hamill, Marty, Neher, Sakmann and Sigworth, 1981). Patch pipettes contained 130 mM KCl, 1 mM EGTA and 2 mM Na_2ATP.

Voltage-activated K^+ currents were evoked by depolarizing steps from either -60 or -40 mV for a duration of 200 ms. 300 nM apamin had no effect on the amplitude of the outward K^+ currents evoked by voltage steps. In 12 cells, the amplitude of the current was 101.7 ± 2.0 % (mean ± s.e.) of the control (100 %) value measured at +40 mV. The Ca^{2+}-activated K^+ channel blocker tetraethylammonium (TEA) at 1 mM could significantly inhibit the outward K^+ current especially at depolarized potentials (72.1 ± 6.9 % of control, n = 6, $P \leq 0.05$). Outside-out patches isolated from mesenteric artery smooth muscle cells contained large conductance channels. The unitary current amplitude at 0 mV was 7.4 ± 0.55 pA (n = 13), this increased in size to 10.84 ± 0.98 pA (n = 10) at +40 mV. The slope conductance calculated at 0 mV was 93 ± 4 pS. These channels had an extrapolated reversal potential close to E_K (-82 mV). The opening probability (P_o) of the channel was voltage dependent (increasing with depolarization). For example, in one patch, P_o at -20 mV was 0.001, whilst at a patch potential of +40 mV, this increased to 0.0458. The pharmacological properties of this channel included reversible inhibition by 1 mM TEA (n = 6). 100 nM charybdotoxin significantly decreased P_o of the channel in 3 patches ($P \leq 0.05$). However, apamin at 200 nM, however, had no effect on P_o (n = 3).

In summary, we have been unable to detect any apamin-sensitive K^+ currents at the whole-cell level. All cells, however, were sensitive to TEA. Outside-out patches contained large conductance K^+ channels, which were reversibly blocked by either TEA or charybdotoxin but were insensitive to apamin. The channel shows characteristics described for the maxi Ca^{2+}-activated K^+ channel in these cells (Langton, Nelson, Huang and Standen, 1991).

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168P SCIATIC NERVE CONSTRICTION IN RATS INDUCES UP-REGULATION OF Na^+ CURRENTS IN IPSILATERAL L_{4-5} DORSAL ROOT GANGLION (DRG) NEURONAL SOMATA

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Type III Na^+ channel mRNA, which is normally undetectable in adult rat lumbar L_{4-6} DRG neurones, is expressed after sciatic nerve axotomy (Waxman *et al.*, 1994). Furthermore, Jakeman *et al.* (1995) found an increased number of [^{3}H]-saxitoxin binding sites in L_{4-5} DRGs in rats in which the sciatic nerve had been constricted. In the present study we have examined whether sciatic nerve injury in rats induces a functional change in Na^+ current expression in L_{4-5} DRG neurones.

Whole-cell Na^+ currents were recorded (at 19-22°C) from neurones dissociated from DRGs excised from the right L_{4-5} region of adult male rats subjected 4-10 days previously to constriction of the right sciatic nerve (*ligated group*; $n = 9$ rats; Bennett, 1993) or to sham operation (*control group*; $n = 10$). Placement of rats in shallow ice-cold water on the day of DRG dissociation confirmed a 'cold allodynia' response (Bennett, 1993) in the right hindlimbs of the *ligated group*. To maintain the currents within the compliance of the amplifier, the Na^+ concentration in the external solution was reduced to 10 mM (supplemented with 135 mM choline-Cl). Na^+ current *I/V* curves were generated by 30 ms depolarizations from a holding potential of -100 mV.

In agreement with previous reports (e.g., Van den Berg *et al.*, 1995), most of the neurones studied (27/32 cells) expressed two-component Na^+ currents: a fast tetrodotoxin-sensitive (TTX-S) component and a slower TTX-resistant (TTX-R) component. The remaining cells (3/16 in the *control group*; 2/16 in the *ligated group*) expressed only TTX-S currents. In cells expressing both TTX-S and TTX-R currents, the TTX-R component was separated from the composite current by addition of 0.3 µM TTX. The TTX-S component was then estimated by subtraction of the TTX-R component from the composite current. Currents are expressed as conductances normalized by cell capacitance to compensate for differences in cell size. Cell capacitances and diameters were not different between the two groups (*control group*: 25 ± 2 pF, 19.2 ± 0.1 µm; *ligated group*: 23 ± 0.1 pF, 18.9 ± 0.1 µm).

In TTX-free medium, composite peak Na^+ conductances were larger in the *ligated group* compared to *control group*: 8.7 ± 0.9 nS/pF and 6.1 ± 0.7 nS/pF, respectively ($P < 0.05$; Mann-Whitney *U* test; 16 cells per group). In the presence of 0.3 µM TTX, the TTX-R component was not significantly different between the two groups (*control group*: 3.4 ± 0.7 nS/pF, $n = 13$; *ligated group*: 3.2 ± 0.6 nS/pF, $n = 14$). Although the TTX-S component tended towards higher values in the *ligated group*, this difference was not statistically significant (*control group*: 5.7 ± 0.8 nS/pF, $n = 13$; *ligated group*: 7.9 ± 0.8 nS/pF, $n = 14$).

This study shows (1) that sciatic nerve injury induces potentiation of Na^+ currents in L_{4-5} DRG neurones and (2) that this up-regulation may be confined to TTX-S channels. It is conceivable that the increased composite current in the ligated group arises from an elevated expression of type III Na^+ channels, as these channels are known to be sensitive to nanomolar concentrations of TTX (Suzuki *et al.*, 1988).

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169P ACTIONS OF TERIKALANT ON THE KINETICS OF SHORTENING OF ACTION POTENTIAL DURATION FOLLOWING A RAPID AND SUSTAINED INCREASE IN PACING RATE

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Cardiac action potential duration (APD) shortens with an increase in heart rate. This shortening occurs in two phases, an immediate shortening followed by a slower accommodation to steady state. The specific ion currents which contribute to the accommodation process are unclear. Terikalant selectively blocks the inward rectifying K^+ current (I_{K1}) in guinea pig cardiac cells (Escande *et al.*, 1992). Aims: To compare the slow accommodation of APD, after a rapid and sustained increase in pacing rate, in isolated guinea pig ventricular myocytes and Langendorff-perfused whole-heart preparations, and to assess the effect of terikalant on this process. Methods: Myocytes were isolated from guinea pig ventricle and superfused with a HEPES buffered extracellular solution (36°C). The whole-cell and perforated patch (amphotericin) configurations of the patch-clamp technique were used. Langendorff-perfused whole-hearts were maintained in standard oxygenated Krebs solution at 37°C. The heart was stimulated using silver-wire epicardial electrodes and monophasic action potentials were recorded using a contact epicardial probe. Results: For myocytes, the steady state APD at 90% repolarization (APD_{90}) was 283±7ms at 500ms cycle length (CL) and 211±5ms at 300ms CL (n=4). The slow accommodation of APD_{90} was best fit with a single exponential, having a time constant of 34±4s for a step from 500ms to 300ms CL (n=6) and 34±1s for a step from 550ms to 350ms CL (n=8). For whole-heart, the slow accommodation could also be fit with a single exponential, although a biexponential gave a better fit to the initial 10-15s of the accommodation curve. In whole hearts, the time constant was 31±2s (n=3) for a step from 500ms to 300ms CL. For a biexponential fit, the slow time constant was not significantly different (29±5s, n=3). Terikalant (1μM-30μM) increased APD_{90}, as previously reported (Escande *et al.*, 1992). Up to 10μM terikalant had no significant affect on the resting membrane potential (77±3mV in control, 77±2mV in 3μM terikalant, n=3 cells). In cells subjected to a CL change from 500ms to 300ms, both the time constant, τ, and the magnitude of the slow shortening, A_o, was decreased; τ=34±4s, A_o=21.8±0.7ms (n=6) in control, and τ=18±6s, A_o=15±3ms (n=4) in the presence of 10μM terikalant. Above 30μM terikalant, no exponential fits could be obtained since at 300ms CL, either there was no slow accommodation or the action potentials were longer than 300ms, producing alternans (n=3). A similar effect was observed in the whole-heart; in 3μM terikalant, τ was decreased by 23%. Conclusions: These observations suggest that I_{K1} contributes to the accommodation process since terikalant decreased the time constant and reduced the magnitude of APD shortening following a sustained increase in pacing rate in myocytes and whole heart.

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170P ON THE NATURE OF THE CALCIUM CHANNELS CONTROLLING NORADRENALINE RELEASE IN THE RAT ISOLATED ANOCOCCYGEUS MUSCLE: AN ELECTROPHARMACOLOGICAL STUDY

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We have recently reported a substantial component of action potential-evoked ATP release in the guinea-pig vas deferens which is resistant to the irreversible N - type calcium channel blocker ω - conotoxin GVIA (ω - CTX GVIA) (Smith and Cunnane, 1996). In the present study we have investigated whether multiple calcium channels control noradrenaline release in the rat anococcygeus muscle.

Conventional intracellular recording techniques were used to monitor changes in membrane potential of individual smooth muscle cells from male rats (150 - 200 g) as a measure of neurotransmitter release. Trains of stimuli at 5 - 50 Hz were delivered through Ag/AgCl electrodes positioned close to the point of insertion of the pelvic nerves supplying the anococcygeus muscle.

The mean amplitudes of excitatory junction potentials (EJPs) evoked by trains of 5 stimuli at 5 Hz and 3 stimuli at 10 Hz were 14.8 ± 0.9 mV and 12.5 ± 0.5 mV (mean ± s.e.m., n = 6) respectively. In single cell experiments the amplitude of evoked EJPs gradually decreased after the application of ω - CTX GVIA (50 nM) and EJPs were abolished after 20 - 30 minutes. However, when the stimulation frequency and/or train length was increased, trains of stimuli evoked EJPs even in the presence of 1 μM ω - CTX GVIA. This ω - CTX GVIA-resistant neurotransmitter release has been termed 'residual release'.

In the presence of ω - CTX GVIA, EJP amplitudes after trains of 20 stimuli at 10, 20 and 50 Hz were 6.6 ± 1.7 mV, 17.3 ± 1.9 mV and 26.4 ± 2.3 mV (n = 6) respectively. 'Residual release' was inhibited by 63.4 ± 5.2 % (n = 3) by the P - type calcium channel blocker ω - Aga IVA (100 nM) and abolished by the Q - type calcium channel blocker ω - conotoxin MVIIC (100 nM) (n = 3) and by the non-selective calcium channel blocker ω - grammotoxin SIA (100 nM) (n = 3).

Therefore, it would appear that a heterogeneous population of calcium channels control action potential-evoked noradrenaline release in sympathetic nerve terminals. This population of calcium channels appears to be different to the population controlling ATP release in the guinea-pig vas deferens (Smith and Cunnane, 1996).

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171P MULTIPLE CALCIUM CHANNEL SUB-TYPES ARE INVOLVED IN GLUTAMATE RELEASE FROM RODENT AND PRIMATE STRIATAL SYNAPTOSOMES: MODULATION BY κ-OPIOID RECEPTOR ACTIVATION

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Recently we have demonstrated that κ-opioid agonists inhibit depolarisation-induced glutamate release from rat and marmoset striatal synaptosomes (Hill and Brotchie, 1995). The pre-synaptic release of glutamate is dependent on the influx of calcium through voltage-gated calcium channels. In the present study, we have investigated whether the κ -opioid agonist, enadoline, mediates its effects on glutamate release through actions on calcium flux through voltage-gated calcium channels. In addition, we have attempted to determine the specific calcium channels involved. 4Amino-pyridine (4-AP, 2mM)-induced glutamate release from rat or marmoset striatal synaptosomes was measured continuously using an enzyme-linked fluorometric assay (Nicholls and Sihra, 1986). Intracellular calcium concentration was monitored using the fluorescent calcium indicator fura-2. In rat and marmoset synaptosomes, 4-APinduced increases in intracellular calcium concentration were inhibited by enadoline in a concentration-dependent manner.

Maximum inhibition was seen at 100 μM enadoline and the IC50's were 8.75 μM and 9.38 μM in rat and marmoset respectively. The effect of enadoline was inhibited by the selective κ -opioid receptor antagonist nor-binaltorphimine (5 μM). In the rat, the selective calcium channel antagonists ω-agatoxin-IVA, ω-conotoxin-MVIIC and ω-conotoxin GVIA reduced glutamate release in a concentrationdependent manner with IC_{50} values of 8.07nM, 86.29nM and 160.20nM, respectively. In the marmoset, glutamate release was significantly (ANOVA followed by Tukey-Kramer multiple comparisons test) inhibited by ω-agatoxin-IVA (30nM), ω-conotoxin-MVIIC (300nM) and ω-conotoxin GVIA (1μM).

These data suggest the involvement of N, P and Q-type calcium channels in 4-AP-induced glutamate release from rat and marmoset striatal synaptosomes. In addition, experiments using combinations of calcium channel antagonists and enadoline suggest that enadolineinduced inhibition of glutamate release occurs primarily via blockade of P-type calcium channels in the rat but via blockade of N-type calcium channels in the marmoset. This suggests a species difference in the coupling of κ-opioid receptors to calcium channels.

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172P EFFECTS OF THE VECURONIUM ANALOGUE, ORG-9643, ON THE QUANTAL RELEASE OF ACETYLCHOLINE FROM RAT MOTOR NERVE TERMINALS

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Certain 17C-ester analogues of the muscle relaxant vecuronium attenuate phenylephrine and KCl-evoked contractions of rat aortic rings (Prior and Fiddes, 1994). This is thought to be due to inhibition of verapamil-sensitive voltage-operated Ca^{2+}-channels (VOCCs). VOCCs are critical in the evoked release of acetylcholine from motor nerve terminals; although these channels are not verapamil-sensitive. If the effect of the vecuronium analogues on VOCCs was not sub-type specific then it is possible that a component of their neuromuscular blocking activity could be due to a decrease in acetylcholine (ACh) release following inhibition of nerve terminal VOCCs. To determine if the analogues might possess VOCC-blocking activity at the neuromuscular junction, we have measured the effect of one of them (Org-9643) on the quantal release of acetylcholine from rat motor nerve terminals.

Org-9643, the 17β-pivalate derivative of vecuronium, was chosen for study since it was one of the most post potent inhibitory compound in rat aortic rings (Prior and Fiddes, 1994). Experiments were performed at room temperature (18 - 20°C) on rat isolated hemidiaphragm muscle/phrenic nerve preparations. Miniature end-plate potentials (m.e.p.ps) and end-plate potentials (e.p.ps, evoked at 0.5 Hz) were recorded using a conventional intracellular microelectrode technique. Preparations were paralysed with an excess of extracellular Mg^{2+}. The amplitudes of all m.e.p.ps and e.p.ps were scaled to a fixed resting membrane potential of -90 mV and e.p.p. quantal content was calculated as the ratio of the amplitudes of simultaneously recorded e.p.ps and m.e.p.ps. In each muscle fibre, all measures were expressed as a percentage of their respective control (pre-Org-9643) value.

In all preparations (n = 25), average control values were: 0.790 ± 0.033 mV for m.e.p.p. amplitude; 3.26 ± 0.29 mV for e.p.p. amplitude and 4.26 ± 0.40 for e.p.p. quantal content. A five minute exposure to Org-9643 (5 - 40 μM) had no effect on m.e.p.p. amplitude (Table 1), indicating a lack of an effect on postsynaptic ACh receptors over this range of concentrations. However, this range of concentrations of Org-9643 produced a concentration-dependent decrease in e.p.p. amplitude (Table 1). Consequently, there was a marked concentration-dependent depression in the e.p.p. quantal content (Table 1).

Table 1: Effects of Org-9643 on m.e.p.p. and e.p.p. amplitudes and e.p.p. quantal content recorded in Mg^{2+}-paralysed rat hemidiaphragm muscles.

Org-9643 (μM)	*n*	M.e.p.p. amplitude	E.p.p. amplitude	E.p.p. quantal content
5	4	94 ± 10	63.0 ± 9.3	67.4 ± 8.6
10	6	98.7 ± 8.9	57.4 ± 5.7	59.0 ± 5.7
20	10	88.6 ± 6.8	47.7 ± 4.1	56.8 ± 6.1
40	5	99 ± 12	34.3 ± 4.8	36.4 ± 6.5

Values in table are mean and standard error of the mean of data, expressed as % control, from the number of fibres indicated in *n*.

Thus it appears that Org-9643 does possess an ability to depress quantal ACh release from rat motor nerve endings by a mechanism with the characteristics of a Mg^{2+}-like inhibition of nerve terminal VOCCs. This would suggest that the ability of the vecuronium analogues to inhibit VOCCs is not sub-type specific. However, the extent to which this putative presynaptic effects of Org-9643 contributes to its muscle relaxant activity is uncertain. These present studies were performed at room temperature in Mg^{2+} paralysed preparations. However, in tension studies performed at 32°C and in normal extracellular Mg^{2+}, we observed changes in the profile of twitches (at 0.1 and 2 Hz) and tetani (at 50 Hz) with 50 - 150 μM Org-9643 that resemble the effects of a classical non-depolarising muscle relaxant that acts solely through an inhibition of ACh receptors, without any effect on presynaptic VOCCs. Clearly, the electrophysiological study indicates that a component of action of Org-9643 on presynaptic VOCCs cannot, at present, be completely rejected. However more comprehensive studies need to be performed before a definitive profile of action of the compound can be stated.

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173P STIMULATION FREQUENCY-DEPENDENT EFFECTS OF (-)-VESAMICOL ON QUANTAL RELEASE PARAMETERS AT A RAT NEUROMUSCULAR JUNCTION

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(-)-Vesamicol, an inhibitor of the vesicular acetylcholine transporter (AChT), enhances the rundown of end-plate current amplitudes during short trains of high frequency (50 Hz) motor nerve stimulation (Pemberton *et al.*, 1992). This action of the compound has been attributed to an effect of (-)-vesamicol on transmitter mobilisation; as has been extensively described for nicotinic antagonists such as tubocurarine (see e.g. Prior *et al.*, 1995). To further study this phenomenon, we have performed a detailed frequency-dependent analysis of the effects of (-)-vesamicol on quantal acetylcholine release from rat motor nerve terminals. Included in this study has been a full statistical analysis of the binomial parameters of acetylcholine release.

In each cut rat hemidiaphragm preparation, about 20 - 40 miniature endplate currents (m.e.p.cs) and 60 - 100 endplate currents (e.p.cs) elicited at nine different stimulation frequencies ranging from 0.5 - 150 Hz (each block seperated by a 10 s interval) were recorded. In each fibre studied, e.p.cs and m.e.p.cs were recorded, using a two microelectrode voltage clamp technique, at -50mV and 32°C both in the absence and presence of 1 μM (-)-vesamicol. All the release parameters were calculated from the mean amplitude of m.e.p.cs and the amplitude of e.p.cs in plateau portion of each block nerve stimulation. Binomial statistical analysis of e.p.c. amplitudes (Miyamoto, 1975) was used to determine the changes in quantal release parameters (*n*, size of the immediately available pool; *p*, probability of release and *m*, quantal content) throughout nine stimulation frequencies.

Under the experimental conditions utilised here, 1 μM (-)-vesamicol had no effect on the amplitude of m.e.p.cs (control, 2.01 ± 0.08 nA; vesamicol: 1.93 ± 0.05 nA; $P > 0.05$, paired Student's *t* test), confirming a lack of postjunctional activity of the compound. However, there was a frequency-dependent decrease in the amplitude of e.p.cs in the presence of 1 μM (-)-vesamicol. Binomial statistical analysis of e.p.c. amplitudes showed that e.p.c. quantal content was not changed by vesamicol up to a stimulation frequency of 5 Hz, and only at high stimulation frequencies at or beyond 10 Hz, was the decrease in e.p.c. quantal content produced by (-)-vesamicol statistically significant (Figure 1).

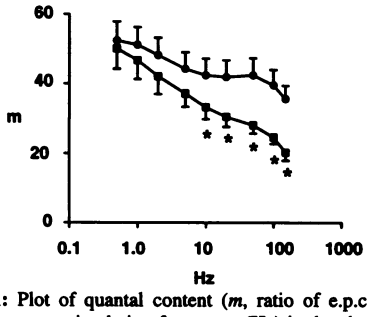

FIGURE 1: Plot of quantal content (*m*, ratio of e.p.c and m.e.p.c amplitudes) versus stimulation frequency (Hz) in the absence (●) and presence (■) of 1 μM (-)-vesamicol. Data are mean and s.e.mean of values from the same eight individual impalements. * $P < 0.05$, control versus 1 μM (-)-vesamicol, paired Student's *t* test.

Binomial statistical analysis revealed that the frequency-dependent reduction in e.p.c. quantal content produced by vesamicol was entirely due to a decline in the size of the immediately available pool (*n*). The probability of release of an individual quantum (*p*) remained unchanged throughout the nine stimulation frequencies studied. These results suggest that, by blocking the transport of ACh into synaptic vesicles, vesamicol inhibits the supply of vesicles to the release sites, thus decreasing transmitter mobilisation and release. Further, the stimulation frequency-dependent nature of this phenomenon suggests that the process that is inhibited by the presence of the (-)-vesamicol is a rate limiting step in transmitter mobilisation.

Work supported by project grants from the Wellcome Trust.

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174P TETRODOTOXIN-SENSITIVITY OF NICOTINE-EVOKED DOPAMINE RELEASE FROM RAT STRIATUM

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Presynaptic nicotinic acetylcholine receptors (AChRs) are thought to play an important role in mediating the central actions of nicotine. Presynaptic nicotinic AChRs have been considered to operate in a tetrodotoxin (TTX)-insensitive manner (Wonnacott *et al.*, 1990) and thus TTX sensitivity has been taken to distinguish presynaptic nicotinic AChRs from those located elsewhere. However, we recently showed that anatoxin-a-evoked [^{3}H]dopamine release from striatal synaptosome preparations is sensitive to TTX (Soliakov *et al.*, 1995). In an attempt to clarify this issue, we have compared the TTX sensitivity of nicotine-evoked dopamine release from rat striatum using superfused synaptosomes, superfused slices and *in vivo* microdialysis.

P2 and Percoll-gradient purified synaptosomes, prepared as previously described (Soliakov *et al.*, 1995; Soliakov and Wonnacott, 1996) and slices (0.3mm) from rat striata were superfused in a Brandel apparatus with Krebs bicarbonate buffer. After 15 min washing with normal buffer, followed by a further 15 min with normal buffer or buffer containing 1.5 μM TTX or 20 μM mecamylamine, 10 μM nicotine, 10 μM veratridine or 25 mM KCl were applied for 40 s. Two min fractions were collected and counted for radioactivity. *In vivo* microdialysis experiments were performed as previously described, with all drugs delivered directly to the terminal regions via the dialysis probe (Marshall *et al.*, 1995). Student's paired t-test and one way ANOVA were used to compare control responses to the appropriate controls.

In P2 and Percoll-purified synaptosomes TTX partially inhibited nicotine-evoked [^{3}H]dopamine release by 54±7% (n=3, $p<0.05$) and 37±9% (n=5, $p<0.05$), respectively, whereas mecamylamine (20μM) inhibited nicotine-evoked [^{3}H]dopamine release by 77±2% (n=3, $p<0.05$) and 71±8% (n=5, $p<0.01$), respectively. In slices, nicotine-evoked [^{3}H]dopamine release was inhibited 71±4% by mecamylamine (n=3, $p<0.05$) and 71±5% by TTX (n=3, $p<0.05$). In all three *in vitro* preparations veratridine-evoked [^{3}H]dopamine release was almost totally blocked by TTX ($p<0.05$), whilst KCl-evoked [^{3}H]dopamine release was unaffected (n=3-4). In dialysis experiments, $3x10^{-3}$M nicotine increased dialysate dopamine levels to 329±100% of basal (n=8). This was completely blocked by the prior addition of 100μM mecamylamine (n=3) or 10μM TTX (n=4) to the artificial CSF ($p<0.01$).

Thus, TTX partially blocked mecamylamine-sensitive nicotine-evoked [^{3}H]dopamine release from synaptosomes and totally inhibited release from more intact preparations. These results suggest that caution should be exercised in the interpreatation of TTX sensitivity of nicotine evoked response with regard to the localisation of nicotinic AChRs.

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175P SUBSTANCE P FRAGMENTS, SP1-9 AND SP6-11, MODULATE ENDOGENOUS DOPAMINE OUTFLOW IN RAT STRIATUM

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Substance P (SP) is metabolised by peptidases in the extracellular fluid within rat striatum and the fragments SP6-11 and SP1-9 have been shown to be generated in significant concentrations (Andrén and Caprioli 1995). We have shown previously that SP1-7, SP5-11 and the complementary fragments SP1-4 and SP8-11 locally modulate endogenous dopamine (DA) outflow in rat striatal slices. Only the effects of C-terminal fragments were reversed by the NK1 antagonist WIN51708 (Khan *et al.*,1995a & b). The aim of this study was to investigate the effects of SP1-9 and SP6-11 in the same model.

Striatal slices (400-500 μm) were obtained from adult male Wistar rats (200-250 g). After 60 min equilibration in oxygenated Krebs-Ringer buffer at 34°C, 2 slices were sequentially transferred every 5 min, in tissue holders, between incubation tubes containing 1ml buffer. Basal outflow was monitored in the first 10 min followed by 10 min exposure to peptide in the absence or presence of WIN51708 (2.5×10^{-9}M), maintained throughout. Samples were collected for a further 20 min. DA was quantified by HPLC-ECD. Changes in DA were expressed as percent of basal outflow representing area under the curve.

Mean basal DA outflow (± s.e.mean, n=72) was 6.8 ± 0.7 pmol/ml/mg protein. One way ANOVA showed significant increases in DA outflow at 10^{-10} and 10^{-9}M ($F(6,65) = 8.98, 16.1$, $p < 0.05$ for SP1-9 and SP6-11 respectively) (Figure 1). Two way ANOVA showed that WIN51708 fully reversed the effect of SP6-11, failed to antagonise the effect of SP1-9 ($F(1,44) = 31$, $p < 0.05$, 0.02 n.s, respectively) and was devoid of any intrinsic effect on striatal DA outflow (Figure 2).

This study demonstrates that both SP1-9 and SP6-11 locally modulate endogenous DA outflow in rat striatum. All the data reported to date indicate that several N- and C-terminal SP fragments can exert similar neuromodulatory effects in this model.

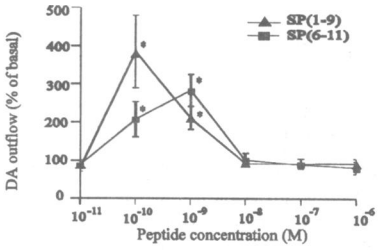

Figure 1. Effects of SP(1-9) and SP(6-11) on DA outflow from striatal slices. Means ± s.e.mean of 6 determinations.
*P<0.05, Bonferroni's test versus respective non-treated controls.

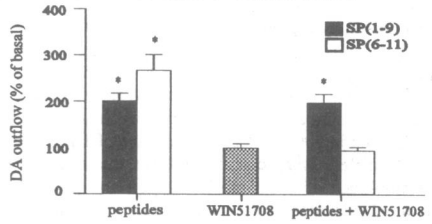

Figure 2. Effect of WIN51708 (2.5×10^{-9} M) on peptide (10^{-9} M) stimulated DA outflow from striatal slices. Means ± s.e.mean of 6 determinations.
*P<0.05, Bonferroni's test versus respective non-treated controls.

The receptor mechanisms underlying these effects remain to be fully elucidated.

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176P BIPHASIC INHIBITION OF STIMULATED DOPAMINE RELEASE BY SELECTIVE D_3 RECEPTOR AGONISTS IN SLICES OF RAT CAUDATE PUTAMEN AND NUCLEUS ACCUMBENS

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Studies using *in vivo* microdialysis (e.g. Gobert *et al.*, 1995) have suggested that both D3 and D2 receptors control the synthesis and release of dopamine (DA) in the rat caudate putamen (CPu) and nucleus accumbens (NAc). However, whether D3 autoreceptors are located on DA cell bodies and / or on DA axon terminals is unclear. We have previously shown that 7-OH-DPAT (a putative selective D3 receptor agonist) inhibits stimulated DA release in slices of rat NAc by acting at two different sites (Patel *et al.*, 1995). The results were consistent with activation of functional D3 and D2 autoreceptors. The aim of this study was to extend these findings using the selective D3 agonist (+)-PD-128,907 (Pugsley *et al.*, 1995) and compare data obtained between the CPu and NAc.

Experiments were conducted in rat coronal brain slices (350 μm thick), prepared from male Wistar rats (230 to 300 g). Dopamine release was evoked by single pulse (1P) stimulation (20 V, 0.1 ms) applied every 2 minutes and measured by fast cyclic voltammetry (see Patel *et al.*, 1995). Agonists were added to the perfusing fluid at increasing concentrations either in the absence or presence of haloperidol. Concentration-response data (% inhibition of peak DA release) were analysed by a least-squares iterative curve fitting method (slopes constrained to 1) and EC50, Emax and pKB values calculated. Student's t-test was used for statistical comparisons.

In both the CPu and NAc, 7-OH-DPAT and (+)-PD-128,907 (0.01 nM -3 μM) inhibited 1P stimulated DA release in a concentration-dependent manner. Analysis of the curves showed that they were best fitted to a two site model (F-test: $P < 0.05$). The relative EC50 and Emax values for each component are shown in Table 1. In contrast, concentration-response curves for the non-selective D2 / D3 agonist apomorphine (0.1 nM - 10 μM) were monophasic (Table 1).

Table 1. EC50 values (geometric means with 95 % confidence intervals) and Emax values (arithmetic mean ± s.e.mean).

	CPu	NAc
7-OH-DPAT (n = 7)		
EC50 High (nM)	0.023 (0.015 - 0.035)	0.015 (0.007 - 0.032)
EC50 Low (nM)	8.61 (6.8 - 10.9)	8.4 (6.3 - 11.1)
Emax High (%)	31.7 ± 2.6	18.0 ± 2.1**
Emax Low (%)	64.2 ± 3.6	77.5 ± 2.8*
Emax Total (%)	95.9 ± 1.5	95.5 ± 0.6
(+)-PD-128,907 (n = 5)		
EC50 High (nM)	0.013 (0.0033 - 0.054)	0.017 (0.0045 - 0.065)
EC50 Low (nM)	48.23 (27.0 - 87.0)	24.6 (19.0 - 31.8)*
Emax High (%)	32.62 ± 4.25	23.98 ± 4.70
Emax Low (%)	64.30 ± 4.73	76.00 ± 5.53
Emax Total (%)	96.92 ± 1.76	99.98 ± 1.61
apomorphine (n = 5)		
EC50 (nM)	45.7 (27.4 -79.2)	82.05 (49.5 - 135.6)
Emax Total (%)	91.48 ± 1.06	93.06 ± 1.28

*$P < 0.05$, **$P < 0.01$ significant difference between regions. Data for 7-OH-DPAT in the NAc are from Patel *et al.*, (1995).

The selective D2 antagonist haloperidol (30 nM) displaced the low affinity (pKB values: CPu 9.40 ± 0.19; NAc 9.45 ± 0.06; $P > 0.05$; $n = 5$) components of concentration-response curves for 7-OH-DPAT whilst the high affinity components were essentially unaffected (pKB values: CPu 7.84 ± 0.30; NAc 7.89 ± 0.26; $P > 0.05$; $n = 5$).

These findings give further support to the concept that both D2 and D3 receptors may act as functional release-regulating autoreceptors at DA axon terminals. It appears that the D3 receptor may play a greater role in the presynaptic regulation of DA release in the CPu than in the NAc.

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177P EFFECTS OF LOCAL APPLICATION OF NITRIC OXIDE ON DOPAMINE RELEASE IN SUB-REGIONS OF THE RAT CAUDATE PUTAMEN BRAIN SLICES MEASURED USING FAST CYCLIC VOLTAMMETRY

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Several studies have reported a nitric oxide (NO)-mediated release of dopamine (DA) from the nigrostriatal nerve terminals in the rat caudate putamen (CPu) both invitro and invivo. In most of these investigations the source of NO was an NO-donor. We have already shown that NO is electroactive and can be measured in real-time using fast cyclic voltammetry (FCV) (Iravani et al., 1993; Iravani et al., 1994). The aim of this study was to examine the direct effects of local application of dissolved NO gas by pressure ejection through a micropipette into subregions of rat CPu. In this study we have used a novel dual-channel voltammeter to detect DA and NO simultaneously.

350 µM coronal striatal slices were prepared from 250 - 300 g male Wistar rats, set up in a brain bath and superfused with 95% O_2 5% CO_2 gassed aCSF at a rate of 1 $ml.min^{-1}$ at 32° C. NO was dissolved in de-gassed 0.01 M PBS at room temperature and stored in a stoppered container, shortly prior to experimentation. Based on its solubility data in water (Merck Index), it was assumed that saturated NO solution was 2 mM. FCV was effected by a carbon fibre electrode (CFE) produced by a sinusoidal waveform (Iravani et al., 1994) using a dual-channel voltammeter. Sample and hold signals from channels 1 and 2 were set at +600 mV and +1200 mV, the oxidation potentials of DA and NO respectively. The sample and hold outputs were displayed on a dual channel chart recorder. All concentrations were estimated by calibration of the CFE with DA and NO ranging from 0.1 µM to 1 µM and 20 µM to 200 µM respectively in deoxygenated PBS. NO was pressure ejected (10 psi) from a micropipette (tip-diameter: 5 µm) placed 20 µm away from the CFE.

Pressure ejection of NO into either a dorsomedial or dorsolateral site (corresponding to either "hotspot" or "coldspot" DA release sites; Iravani and Kruk, 1995), resulted in generation of signals with redox characteristics indistinguishable from exogenous NO and DA mixtures invitro. Based on the calibration curves for DA and NO, concentrations of pressure ejected NO were estimated and compared with the resulting concentrations of DA generated as a result of NO pressure ejection. NO produced concentration dependent release of DA in the CPu, with more DA released in the dorsolateral sites ("coldspots"). These results are summarised in the table 1.

Table 1 *The effect of NO pressure ejection on DA release in the rat CPu. *P<0.05, unpaired t-test.*

Dorsomedial CPu (Hotspot)			Dorsolateral CPu (Coldspot)		
NO (µM)	DA (µM)	n	NO (µM)	DA (µM)	n
17 ± 4	0.05 ± 0.02	3	16 ± 3	0.061	2
48 ± 7	0.14 ± 0.02	6	51 ± 5	0.3 ± 0.1*	5
109 ± 4	0.65 ± 0.18	5	138 ± 19	2.0 ± 0.2*	5

The results of this experiment show that direct applications of NO release DA in a concentration dependent fashion. The effect of NO on DA release was site dependent. We have previously shown that by comparing electrically-evoked DA release evoked by trains of pulses with DA released by a single pulse, two distinct sites of high or low ratio DA release in the dorsolateral and dorsomedial can be identified (Iravani & Kruk, 1995). NO released more DA in the dorsolateral sites than in the dorsomedial sites. The anatomical basis for this difference needs to be further investigated.

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178P SIMULTANEOUS REAL-TIME DETECTION OF DOPAMINE AND A NITRIC OXIDE-LIKE SIGNAL IN RAT CAUDATE PUTAMEN SLICES FOLLOWING LOCAL ELECTRICAL STIMULATION: THE EFFECTS OF 7-NITRO INDAZOLE

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We have previously used fast cyclic voltammetry (FCV) to detect nitric oxide (NO) in aqueous solution (Iravani et al., 1993) and demonstrated that it is possible to detect a large, NO synthase sensitive, NO-like signal following pressure ejection of NMDA in rat caudate putamen (CPu) slices (Iravani et al., 1994). We now report the release and detection of an NO and dopamine-like signal following electrical stimulation at dorsolateral and dorsomedial sites in the rat CPu, using a dual channel Millar voltammeter, and show the effects of a selective brain-type NO synthase inhibitor, 7-nitroindazole (7-NI) (Moore et al., 1993) on the dopamine and NO-like signals.

FCV was carried out in 350 µm rat striatal slices using a sinusoidal driving waveform (Iravani et al., 1994) using a dual channel voltammeter. Channels 1 and 2 were set to sample at +600 mV and +1200mV, the oxidation potentials of DA and NO respectively. Sample and hold outputs were displayed on a dual channel chart recorder. All concentrations were estimated by calibration of the electrodes with 1 µM DA or 2-10 µM NO in deoxygenated PBS.

Electrical stimulation of either the dorsomedial or dorsolateral CPu with trains of 100 pulses at 50 Hz, 0.2 ms pulse width resulted in a rapid release of dopamine with peak oxidation potential at +600 mV; this signal had a brief half-life. On the declining phase of the DA signal, a more slowly developing and longer lasting faradaic signal appeared at +1200 mV. Examination of the signals suggested a mixture of electrochemical faradaic currents similar to signals generated by NO and DA mixtures *invitro*.

In the dorsomedial sites DA and NO-like signals were estimated to represent maxima of 404 ±143 nM DA and 690 ± 118 nM NO; in the dorsolateral sites110 ± 3 nM DA and 700 ± 195 nM NO (mean ± s.e.m. n=5). Addition of 10µM 7-NI to the superfusion fluid led to a rapid and sustained rise in the signal on channel 1 (DA). This rise equilibrated within 30 min. and was equivalent to 1-3 µM DA. The NO-like signal was significantly reduced in the presence of 7-NI (peak control signal: 545 ± 44.5 nM; peak signal after 30 min. exposure to 10µM 7-NI: 215 ± 22 nM; n=5, P<0.05, paired t-test).

Following intense electrical stimulation of the CPu, an electrochemically active substance with oxidation and reduction characteristics similar to NO is released together with DA. Inhibition of this signal by 7-NI suggests that part of this signal at +1200 mV is due to NO or a closely-related NO species. 7-NI elevated the basal DA signal and this accords with suggestions that NO may modulate DA transmission (Silva et al., 1995).

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179P L-ARGININE-INDUCED CHANGES IN DOPAC, HVCA AND HYDROXYL RADICAL FORMATION ARE NO-INDEPENDENT IN THE RAT STRIATUM *IN VIVO*

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High concentrations of L-arginine (L-ARG) produce a nitric oxide (NO)-independent biphasic response on dopamine (DA) efflux *in vivo*. D-arginine (D-ARG) (100mM) and L-citrulline (L-CIT) (100mM) had no effect on DA efflux (Silva *et al.*, 1996). We now report the effects of high concentrations of L-ARG, D-ARG and L-CIT on striatal 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) efflux and hydroxyl radical ($OH^{\cdot}$) formation *in vivo* and the effects of NO synthase (NOS) inhibition.

Male Wistar rats (280-320g) were anaesthetised with chloral hydrate (500mg/kg i.p.). DOPAC, HVA and 2,3-dihydroxybenzoic acid (2,3-DHBA) (formed following salicylic acid trapping of $OH^{\cdot}$) efflux was measured in the striatum by *in vivo* microdialysis as previously described (Silva *et al.*, 1996, Rose *et al.*, 1995). Probes were continuously perfused with artificial CSF (aCSF) at a rate of 1-2µl min^{-1}. At least 1 h following implantation, 10-20 min fractions of dialysate (20µl) were collected into 0.25M acetic acid (25µl). Following a 1h period to determine basal DOPAC & HVA, L-ARG (10-100mM), D-arginine (100mM) or L-citrulline (100mM), were included in the aCSF for a period of 150min. Control animals received only aCSF. In a further series of experiments probes were perfused with the NOS inhibitors N^{G}-nitro-L-arginine methyl ester (L-NAME;1mM) or 7-nitroindazole sodium salt (7-NINA;1mM) for 1h prior to and for 120min following perfusion with L-ARG (50mM). Dialysate samples were analysed for DOPAC and HVA as above. In a further series of experiments following a 1h period to determine basal 2,3-DHBA, DOPAC and HVA efflux, either L-ARG (100mM), L-NAME (100mM) or L-ARG (100mM) plus L-NAME (100mM), were included in the aCSF for a period of 120min. Control animals received only aCSF.

Treatment	HVA	DOPAC
Control	-9.6±13.3	-6.9±12.3
L-ARG (10mM)	-74.9±6.5	-63.9±6.2
L-ARG (50mM)	-121.5±9.8*	-132.0±16.7*
L-ARG (100mM)	-112.9±21.5*	-94.2±24.7
D-ARG (100mM)	-106.2±22.3*	-134.4±40.5*
L-CIT (100mM)	-100.1±13.1*	-31.0±28.8

Table. Effect of L-ARG (10-100mM), D-ARG (100mM) and L-CIT (100mM) on striatal HVA and DOPAC efflux in the anaesthetised rat. Values expressed as mean ± SEM for total efflux over basal (pmol 150$mins^{-1}$). *$p<0.05$ compared to control (Dunnett's test).

D-ARG (100mM) and L-CIT (100mM) produced similar reductions in HVA & DOPAC to L-ARG (50-100mM)(Table). L-ARG (50mM or 100mM) induced decreases in DOPAC and HVA were not affected by NOS inhibition with L-NAME (1mM or 100mM) or 7-NINA (1mM). Neither L-ARG (100mM) or L-NAME (100mM) produced any changes in 2,3-DHBA levels compared to basal.

These data provide further evidence that high concentrations of L-ARG exert their effects independently of NO via a mechanism not involving the increased production of $OH^{\cdot}$, and that the use of these concentrations to investigate the role of NO in the striatum *in vivo* is inappropriate.

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180P REGULATION OF HIPPOCAMPAL DOPAMINE RELEASE BY NITRIC OXIDE IN THE RAT

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Activation of N-methyl-D-aspartate (NMDA) receptors has previously been found to cause a concentration-dependent decrease in dopamine (DA) release in the hippocampus of freely moving rats (Whitton et al., 1994). NMDA receptor activation is linked to generation of nitric oxide (NO) and it has been recently suggested that NO plays an important role in mediating regulation of DA release following activation of NMDA receptors in the nucleus accumbens of rats (Ohmo et.al., 1995). Additionally, it has been shown that the 7-nitroindazole monosodium salt, which is believed to be a specific neuronal nitric oxide synthase (NOS) inhibitor, caused an increase in DA, suggesting that endogenous NO decreases DA release in striatum of intact animal. (Silva et al., 1995)

In the present study we have investigated whether NO has a regulatory role over DA release in ventral hippocampus and also the effect of inhibiting NO production on NMDA induced changes in extracelluar DA. In order to do this we have used in vivo microdialysis to investigate the effects of the NOS inhibitor L-nitro-arginine methyl esther (L-NAME) on basal DA release and NMDA evoked changes in extracelluar DA in rat ventral hippocampus. We have also studied the effect of the NO donor S-nitroso-acetyl-penicillamine (SNAP) on DA release in this structure.

Male Wistar rats (280-320g) were anaesthetised with chloral hydrate (400mg/kg) and implanted with concentric dialysis probes into the ventral hippocampus. The following day all rats were dialysed (0.5µl /min) with artificial cerebrospinal fluid (composition in mM; KCl 2.5; NaCl 125; $MgCl_2$; 1.18; $CaCl_2$ 1.26.). After a one hour equilibration period, four 30 mins samples were collected to establish basal release of DA. Various drugs were infused via the dialysis probe and the experiment continued for up to 420 mins. All dialysates were analysed for DA content using HPLC with electrochemical detection. Basal levels of DA were found to be 61± 8 fmol/10µl (mean ±s.e mean, n=32). All data were represented as mean s.e mean, n=6-8. For statistical analysis, drug treated and control animals were compared for significant differences at each point using a Mann-Whitney U-test.

Infusion of NMDA (100µM) caused a decrease in DA release of 64±8% ($p<0.05$) below basal. When L-NAME (100µM) was perfused via the dialysis probe for 60 mins prior to co-infusion with NMDA (100µM) for 30 mins the effect of NMDA was abolished, whereas infusion of 1mM L-NAME for 60 mins prior to co-infusion with NMDA(100µM) led to massive rise in DA release, of up to 900 ± 150 % ($p<0.05$). Thus L-NAME infusion was found to have profound effects on NMDA -evoked release of DA. When 100µM L-NAME alone was infused throughout the entire experiment (360 min.) no change in extracelluar DA was observed, however, 1.0mM L-NAME increased DA release by up to 300 ± 25% ($p<0.05$) but this returned to basal values within 180 mins after comencing L-NAME infusion. Since L-NAME increased DA release we investigated the effects of the NO donor SNAP on extracelluar DA. SNAP exerted what appears to be a biphasic effect over extracellular DA. Infusion of SNAP (500µM) for 30 mins increased dialysate DA up to 310 ± 25% ($p<0.05$) while 1.0mM SNAP did not significantly alter hippocampal dialysate DA. In contrast SNAP infusion at 5mM decreased DA release by up to 80±10% ($p<0.05$) below basal values and this was maintained for the duration of the experiment (420 mins).

These data suggest that NO plays a regulatory role over DA release in the ventral hippocampus. There also appears to be a complex interaction between NMDA receptor regulation of DA release and the prevailing NO concentration. This is illustrated by the fact that NOS inhibition markedly altered the effect of NMDA infusion on extracelluar DA. Furthermore the data with SNAP may imply that NO controls dopamine release in hippocampus in a biphasic manner. We have previously observed that NO appears to regulate glutamate release in biphasic manner suggesting that these phenomena may be connected.

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181P PHOSPHODIESTERASE INHIBITORS SUPPRESS α_2 RECEPTOR-STIMULATED 5-HT RELEASE FROM NEUROEPITHELIAL CELLS OF TRACHEAE OF NEWBORN RABBITS

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Neuroepithelial cells (NECs) are distributed throughout the airway mucosa of vertebrates and are rich in 5-HT (see Scheuermann *et al.*, 1989). Recently, we demonstrated that 5-HT release from isolated rabbit tracheae reflects 5-HT secretion of NECs (Freitag *et al.*, 1995). Moreover, *in vitro* release of 5-HT from tracheae of newborn rabbits can be stimulated via activation of α_2-adrenoceptors whereas activation of ß-adrenoceptors or direct activation of adenylyl cyclase by forskolin mediated strong inhibitory effects (Freitag *et al.*, 1996a,b). In the present experiments the effects of different phosphodiesterase (PDE) inhibitors were studied in order to characterize further the role of cyclic nucleotides in the control of 5-HT secretion from NECs.

Isolated tracheae of newborn mongrel rabbits of either sex were incubated in modified Krebs-HEPES medium (Freitag *et al.*., 1995). The overflow of 5-HT into the incubation media, which were changed every 10 min, was determined by HPLC with electrochemical detection.

The spontaneous overflow of 5-HT determined between 40 and 50 min of incubation in the absence of test drugs was 21.4±1.0 pmol/g/10 min (mean±s.e.m., n=92). It declined by 33±4 % within the next 20 min (n=7). Tissue 5-HT determined at the end of incubation amounted to 746±98 pmol/g. Phenylephrine (10 µM) and clonidine (1 µM) enhanced 5-HT overflow by 351±21 % (n=51) and 300±40 % (n=5), respectively. These effects of phenylephrine and clonidine were blocked by 1 µM rauwolscine. The non-selective PDE inhibitor IBMX completely inhibited phenylephrine-evoked 5-HT release at 100 µM, with an IC_{50} of 3 µM. Benzafentrine (AH 21-132), an inhibitor preferentially of the PDE III and IV isoenzymes, inhibited the phenylephrine-evoked 5-HT release with a similar potency (IC_{50} 1 µM), whereas rolipram, a potent inhibitor selectively of PDE IV, had no significant effect at a concentration of 10 µM.

In conclusion, inhibition of PDE, most likely of the PDE III isoenzyme, causes strong inhibitory effects on α_2-receptor stimulated 5-HT release from NECs of rabbit tracheae, further pointing to the role of cyclic nucleotides as inhibitory intracellular messenger in NECs. Inhibition of 5-HT release by PDE inhibitors may contribute to their anti-obstructive effect, as 5-HT appears to play a role as broncho-obstructive mediator (see Cazzola *et al.*, 1995).

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182P N-METHYL-D-ASPARTATE RECEPTORS REGULATE 5-HT RELEASE IN THE RAPHE NUCLEI AND TERMINAL 5-HT RELEASE IN FRONTAL CORTEX

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Current evidence supports a role for N-methyl-D-aspartate (NMDA) receptors in regulation of 5-hydroxytryptamine (5-HT) release in brain structures such as hippocampus and striatum *in vivo* (Whitton et al., 1994). The cell bodies from which ascending serotonergic pathways originate are located in the raphe nuclei and activation of 5-HT_{1A} somatodendritic autoreceptors leads to decreased firing rate in the dorsal raphe nucleus (De Montigny et al., 1984) as well as decreased 5-HT release in terminal structures (Hutson et al., 1989). *In vitro*, using primary raphe cultures, application of NMDA increases release of 5-HT (Bequet et al., 1993). This suggests that *in vivo* NMDA receptors may play a role in regulating 5-HT release in the raphe nuclei, which in turn may alter terminal release of 5-HT.

In the present study we have used *in vivo* microdialysis to investigate the effect of NMDA infusion, via dialysis probes, into the raphe nuclei on release of 5-HT in this structure and also terminal 5-HT release in the frontal cortex of freely moving rats.

Male Wistar rats (280-330g) were anaesthetised with chloral hydrate (400mg/kg) and concentric dialysis probes implanted into the raphe nuclei and frontal cortex of the same rats, using the procedure of Adell and Artigas (1991). The following day rats were dialysed with artificial cerebrospinal fluid (composition in mM; KCl 2.5; NaCl 125; $MgCl_2$ 1.18; $CaCl_2$ 1.26). After four 30 min samples had been collected, NMDA (25 or 100µM) was infused for 30 min. Dialysate 5-HT and 5-HIAA were determined using HPLC with electrochemical detection. Basal dialysate concentrations of 5-HT and 5-HIAA were 207.3 ± 32.7 fmol/10µl and 34 ± 6.4 pmol/10µl respectively in raphe nuclei and 89.6 ± 13.5 fmol/10µl and 12.1 ± 3.2 pmol/10µl respectively in frontal cortex (mean ± s.e. mean, n = 22). All data were represented as mean ±s.e mean, n=6. For statistical analysis, drug treated and control animals were compared for significant differences at each point using a Mann-Whitney U-test, where $p<0.05$ was significant.

Infusion of 25µM NMDA into the raphe nuclei led to a relatively small increase in dialysate 5-HT (34 ± 4 % above basal, $P < 0.05$ v controls) which was maintained for 30 min. Thereafter raphe dialysate 5-HT progressively declined to a minimum of 53 ± 12 % of basal values ($p < 0.05$) 180 min after NMDA infusion. During the same period 5-HT release in the frontal cortex increased by up to 239 ± 34 % above basal values ($p < 0.05$ v controls) at 120 min after NMDA infusion into the raphe and remained elevated for a further 90 min. 100µM NMDA infusion into the raphe caused a progressive increase in dialysate 5-HT by up to 502.6 ± 95.3 % ($p < 0.05$), 120 min after NMDA infusion, returning to basal values after 210 min. In the same rats dialysate 5-HT in the frontal cortex fell to 19 ± 9 % 0f basal values after 120 min ($p < 0.05$ v controls). Thereafter 5-HT fell below the limit of detection until 240 min post NMDA infusion, at which point 5-HT 'rose' to 14 ± 5 % of basal values. Neither concentration of NMDA significantly altered extracellular 5-HIAA in either of the brain structures studied.

In conclusion, NMDA receptor activation has a biphasic effect on 5-HT release in the raphe nuclei and a consequent inverse effect on 5-HT release from 5-HT terminals in the frontal cortex.

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183P EFFECT OF THE SELECTIVE 5-HT$_{1B/1D}$ RECEPTOR ANTAGONIST, GR127935, IN COMBINATION WITH FLUOXETINE ON RAT BRAIN 5-HYDROXYTRYPTOPHAN LEVELS

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Selective 5-hydroxytryptamine (5-HT) reuptake inhibitors (SSRIs) are widely used in the treatment of depression. Acute SSRI administration decreases 5-HT turnover due to activation of both terminal autoreceptors and a decrease in cell firing mediated by 5-HT$_{1A}$ cell body autoreceptors (Fuller, 1992, Rigdon & Wang, 1991). Acute blockade of terminal autoreceptors might, therefore, prevent this decrease in 5-HT turnover, thereby enhancing 5-HT function. These experiments have tested this hypothesis by examining the effect of acute administration of fluoxetine on 5-hydroxytryptophan (5-HTP) levels in rat brain, both alone and in combination with the selective 5-HT$_{1B/1D}$ antagonist, GR 127935 (Skingle *et al.*,1993).

Male CD rats (140-160g) received saline (2 ml/kg), (doses in mg/kg i.p.) fluoxetine (Flu, 10), or GR 127935 (1 or 10) either alone, or in combination, 30 min before the administration of m-hydroxybenzylhydrazine dihydrochloride, (100, aromatic acid decarboxylase inhibitor). 30 min later, the frontal cortex and hippocampus were removed and frozen for subsequent analysis. Brain areas were homogenised in 5 vols (w/v) 0.1 M perchloric acid, 0.4 mM sodium metabisulphite, then centrifuged (2 x 15,000 g, 5 min, 4°C) prior to injection (50 µl) onto the HPLC system for analysis of 5-HTP content. 5-HTP was detected electrochemically. Log transformed levels were analysed by two-way ANOVA followed by Dunnett's test for comparisons with control, or multiple t-test to analyse interactions between treatment groups (Table 1).

In both brain areas, Flu (10) alone produced a significant decrease in 5-HTP levels. GR 127935 (1) had no significant effect while GR 127935 (10) caused a significant increase in 5-HTP in both brain areas. When combined, the effects of both Flu (10) + GR 127935 (1) or Flu (10) + GR 127935 (10) were not significantly different from the effects of Flu (10) alone ($P>0.05$, multiple t-test). We conclude that the blockade of terminal autoreceptors by GR 127935 is not sufficient to reverse the effect of Flu on 5-HT turnover. This implies that the acute inhibition of firing caused by Flu, via indirect activation of 5-HT$_{1A}$ cell body autoreceptors, has a greater influence over 5-HT turnover than its indirect action at 5-HT$_{1B/1D}$ autoreceptors.

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Table 1 5-HTP levels (ng/g wet weight, mean ± s.e.mean, n = 17-18) in rat brain areas following the administration of saline, fluoxetine, and GR 127935 both alone or in combination (** $P<0.01$, Dunnett's test).

	Frontal Cortex	Hippocampus		Frontal Cortex	Hippocampus
Saline	98.6 ± 4.4	124.4 ± 5.7	**Saline**	103.3 ± 6.7	115.2 ± 4.2
Flu (10)	64.0 ± 3.3 **	89.4 ± 5.4 **	**Flu (10)**	54.7 ± 4.2 **	63.9 ± 4.7 **
GR 127935 (1)	100.6 ± 6.7	111.3 ± 7.5	**GR 127935 (10)**	140.1 ± 9.4 **	148.9 ± 5.9 **
Flu (10) + GR 127935 (1)	74.2 ± 5.7 **	86.0 ± 6.9 **	**Flu (10) + GR 127935 (10)**	66.3 ± 5.7 **	90.1 ± 4.0 **

184P CHRONIC PAROXETINE: EFFECTS ON RAPHÉ SEROTONIN TURNOVER AND *EX VIVO* RELEASE IN THE RAT

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Long-term treatment with serotonin selective re-uptake inhibitors (SSRIs) is reported to result in desensitisation of the 5-HT1A somatodendritic autoreceptor leading to loss of autoinhibition and a consequent increase in terminal 5-HT release (Bel & Artigas, 1993). Social isolation from weaning results in decreased pre-synaptic serotonergic function (Bickerdike *et al.*, 1993); an effect similar to the reported serotonin dysfunction in human depression. The present study compared the effects of the SSRI paroxetine on serotonin turnover and *ex vivo* release in the midbrain raphé nuclei.

Adult male Lister hooded rats (190 - 280g, n=24) were housed from weaning either four per cage or singly under conventional laboratory conditions with food and water freely available. One week later, both group and singly housed rats were divided to receive one of two treatments; either saline (0.154M, 2ml kg^{-1} n=6) or paroxetine (10mg kg^{-1}, i.p. n=6) once daily for 21 days. The rats were killed 24h after the final injection and the midbrain raphé nuclei was dissected out by taking a 3mm sagital, 5mm coronal brainstem section which was bordered at the rostral end by the aqueduct of Sylvius and extended 1.5mm lateral to the midline on both sides, leaving a central portion containing the dorsal and median raphé nuclei. *Ex vivo* release of endogenous 5-HT was determined by a modification of the method described by Ebstein *et al.*, (1982) for noradrenaline, using 10 min incubation at 37 °C in Krebs containing 1.3mM $CaCl_2$ and 10^{-6}M pargyline and paroxetine in Bio-rad Poly-Prep chromatography columns, but without Sephadex. Both tissue and released 5-HT were measured using HPLC with electrochemical detection (Bickerdike *et al.*, 1993).

The brain dissection described allows rapid and reliable removal of a rostral brainstem region containing the dorsal and median raphé nuclei as tissue levels of 5-HT and 5-HIAA in the central portion were more than 50% greater than those in the adjacent dorsal and lateral sections. Social isolation significantly ($P<0.005$, Fisher test following ANOVA) increased 5-HT (1.40 ± 0.09 ng mg^{-1} wet weight, mean ± s.e. mean) and 5-HIAA (1.35 ± 0.10) compared with group reared rats (1.10 ± 0.05 and 1.13 ± 0.05, respectively). Paroxetine treatment produced a small but significant ($P<0.05$) increase in 5-HIAA in the group but not the isolation reared rats, though the 5-HT:5-HIAA ratio was significantly ($P<0.05$) increased in both. Potassium stimulation (30mM for 5min) increased basal 5-HT release from raphé slices in both group (by 442% above control) and isolation (543%) reared rats. In the paroxetine treated rats this increase was enhanced in the group (617%) but not the isolation reared rats (306%) resulting in a significant difference ($P<0.02$, Mann-Whitney following Kruskal-Wallis ANOVA) between the group and isolation reared rats.

Previous results have demonstrated diminished presynaptic serotonergic function in isolation reared rats (Bickerdike *et al.*, 1993) and the present results indicate that at the level of the raphé nuclei this effect of isolation is not fully reversed by 21 days administration of the antidepressant paroxetine.

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185P EFFECTS OF ω-AGATOXIN AND ω-CONOTOXIN GVIA ON ISCHAEMIA-INDUCED DOPAMINE RELEASE *IN VITRO*

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Massive release of dopamine (DA) occurring during ischaemia has been implicated in resulting striatal neuronal damage (Buisson et al 1992). We have previously found that non-specific blockade of voltage gated calcium channels (VGCC) attenuates ischaemia induced dopamine release *in vitro*. Selective blockade of L - type channels had no effect on DA release (Toner and Stamford 1995). In this study, we have examined the effects of antagonism at T-, N- and P-type VGCCs in the same model.

Striatal slices from male Wistar rats (300-400g) were superfused with gassed (95% O_2/5% CO_2) artificial CSF (Toner & Stamford, 1996) and subjected to periods of "global ischaemia" by substitution of N_2 for O_2 and a reduction in (+)-glucose from 4 to 2 mM. DA release was measured by fast cyclic voltammetry at carbon fibre microelectrodes (Stamford 1990). Three parameters were measured:- *a)* the time to onset of DA release (T_{on}), *b)* the time from onset to peak DA release (T_{pk}) and *c)* the rate of DA release (DA_{rte}).

Statistical comparisons were made by paired t - test and data expressed as means ± s.e.m.

TABLE 1	T_{on} (s)	T_{pk} (s)	DA_{rte} (μM/s)
Control	156 ± 7	28 ± 2	2.9 ± 0.9
ω-conotoxin GVIA (100nM)	210 ± 17*	54 ± 17	2.9 ± 0.3

$P < 0.05$ vs control, $n = 9$

Table 1 shows that selective blockade of N- type VGCCs with ω-conotoxin GVIA (100nM) significantly delays DA release. Table 2 shows that selective blockade of P- type VGCCs with ω-agatoxin IVA delays and slows DA release.

Neomycin (500μM), a putative N- and P- type VGCC antagonist, showed a similar pattern of changes to those occurring in the presence of ω - agatoxin IVA (data not shown). Blockade of T - type channels with Ni^{2+} (200μM) had no significant effect on DA release (not shown).

TABLE 2	T_{on} (s)	T_{pk} (s)	DA_{rte} (μM/s)
Control	162 ± 9	21 ± 2	4.1 ± 1.0
ω-Aga IVA (200nM)	198 ± 8*	53 ± 13*	1.5 ± 0.4*

$P < 0.05$ vs control, $n = 8$

We have found that L - and T - type VGCC's do not make an independent contribution to neurotransmitter release in this model. The results of the present study extend our previous findings that calcium entry mediates DA release during ischaemia (Toner and Stamford 1995) and furthermore show that N- and P-type VGCCs are both significant mediators of ischaemia induced DA release *in vitro*.

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186P THE EFFECTS OF THE PEPTIDES, ALA-VAL-PRO-GLY-VAL-LEU-ARG-PHE-AMIDE (AF3) AND GLY-ASP-VAL-PRO-GLY-VAL-LEU-ARG-PHE-AMIDE (AF4) ON THE MUSCLE OF *ASCARIS SUUM*

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Several anthelmintics paralyse nematodes by actions at the neuromuscular junction. Here, the actions of two *Ascaris* peptides AF3 and AF4 (Cowden *et al.*, 1995) are compared to acetylcholine (ACh), the neuromuscular transmitter.

Ascaris were obtained from pigs at a local abattoir. Experiments were performed on dorsal muscle strips in artificial perienteric fluid (APF; composition in mM NaCl 67, CH_3COONa 67, $MgCl_2$ 15.7, $CaCl_2$ 3, KCl 3, Tris base 5, glucose 3, pH 7.6). For organ bath experiments, the muscle was mounted in an organ bath at 37°C and attached to an isometric transducer. For the electrophysiological experiments, two-electrode intracellular recordings were made from muscle cells at 34°C. All drug additions were noncumulative. Data are expressed as mean ± s.e.mean. Statistical significance ($P<0.05$) was determined using the Student's two-tailed paired t-test.

ACh, AF3 and AF4 contracted muscle with EC_{50}s of 13±1 μM, 24±6 nM and 37±2 nM respectively (n=6). The maximal increase in tension for AF3 and AF4 was 48±4 % and 36±9 % of the maximum response to ACh. The muscle cells were depolarized by ACh (3μM, 5.2 ± 0.4 mV, n=42), AF3 (1 μM 2.6 ± 0.3 mV, n=19) and AF4 (3.3 ± 0.4 mV, n=19). The depolarization to 3 μM ACh was abolished by the nicotinic receptor antagonist mecamylamine (10 μM; n=5) but the responses to the peptides were 111 ± 7 % and 108 ± 17 % with respect to control (n=5). The ionic bases of the depolarizations to ACh, AF3 and AF4 were determined in ion substitution experiments (Table 1).

Table 1. In paired experiments, the effect of low Na^+ APF (NaCl was substituted with glucosamineHCl) and $CoCl_2$ was determined first on ACh and AF3 depolarizations, and then on ACh and AF4 depolarizations. The data are the % of the control response (n) and P values compared to the response in unmodified APF.

APF	3 μM ACh	1 μM AF3	3 μM ACh	1μM AF4
low Na^+	34±9 (6)	101±13 (6)	35±7 (5)	92±5 (5)
	P<0.001	P=0.97	P<0.02	P= 0.20
1mM cobalt	75±8 (6)	14±9 (6)	84±21 (5)	91±13 (5)
	P< 0.05	P< 0.001	P= 0.47	P= 0.51
10mM cobalt	27±9 (5)	0 (5)	60±3 (5)	2±2 (5)
	P< 0.01	P< 0.001	P< 0.001	P<0.001

In conclusion, AF3 and AF4 are more potent than, but elicit a smaller maximal effect than, ACh on *Ascaris* muscle. Their action is not blocked by mecamylamine and has a different ionic mechanism to ACh, therefore the peptides are unlikely to act through the *Ascaris* muscle nicotinic receptor.

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187P PHARMACOKINETICS AND METABOLISM OF A NEW CARTILAGE PROTECTIVE AGENT

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Cartilage Protective Agent, CPA, Ro 32-3555, 3(R)-(Cyclopentylmethyl)-2(R)-[(3,4,4-trimethyl-2,5-dioxo-1-imidazolidinyl)methyl]-4-oxo-4--piperidinobutyrohydroxamic acid is a novel, orally active inhibitor of collagenase. CPA is a potent selective inhibitor of collagenase *in vitro* and oral administration inhibits cartilage degradation *in vivo* in models of arthritis (Lewis et al.).

Single dose pharmacokinetic studies have been performed in male rats and marmosets at 25 mg/kg po and 10 mg/kg iv. Drug levels in plasma samples were measured by a specific HPLC assay with mass spectral detection after extraction of CPA using solid phase extraction. CPA is rapidly absorbed in both species. Although clearance is high CPA has an oral bioavailability of 26% in the rat and 42% in the marmoset. Other pharmacokinetic parameters are given below.

	$t_{1/2}\beta$ (h)	$t_{1/2}\alpha$ (h)	T_{max} (h)	C_{max} (µg/mL)	Cl (L/h/kg)	V_{ss} (L/kg)	$AUC_{0\text{-}inf}$ (mg.h/L)
rat iv	3.0	0.4			3.6	1.9	2.8
rat po			0.25	1.5			2.0
marmoset iv	2.0	0.3			2.9	1.8	3.6
marmoset po			0.3	3.6			3.7

The single dose kinetic results suggest the compound should have good bioavailability in man and show dose related exposure in the ascending dose studies.

Multiple dose, oral gavage, studies of 1 and 4 weeks duration were conducted in rats and marmosets to determine the potential for adverse events in man and to satisfy international regulatory requirements. Doses ranged from 10 - 2000 mg/kg/day in the rat and 25 - 2000 mg/kg/day in the marmoset. As shown in Table 2 there was a dose-related increase in $AUC_{(0\text{-}24h)}$ in these studies.

Dose	50 mg/kg/day	225 mg/kg/day	1g/kg/day	2g/kg/day
Rat (mg.h/l)	1.4	10	67	190
Marmoset (mg.h/l)	46	132	581	no result

The *ex vivo* metabolites of CPA produced by the rat and marmoset were compared to metabolites produced in liver slices of the toxicology species and man to provide evidence of exposure to human metabolites in the toxicology studies. The metabolic profiles of CPA in rat bile was very similar to that seen in rat liver slices *in vitro* and the profile in marmoset urine was similar to that in marmoset liver slices *in vitro*. CPA was metabolised in human liver slices in a similar way to marmoset slices.

The rat and marmoset are appropriate species for the toxicological assessment of CPA. Exposure to CPA is high and dose related in both species. The metabolites produced by man will probably be similar to those produced by rat and marmoset.

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Efficacy of the Cartilage Protective Agent Ro 32-3555. E J Lewis et al. P108 BPS Bath 9-12 July 1996.

188P EFFICACY OF THE CARTILAGE PROTECTIVE AGENT Ro32-3555 IN *IN VITRO* AND *IN VIVO* MODELS OF CARTILAGE DEGRADATION

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Collagenases are key enzymes in the degradation of cartilage in both rheumatoid arthritis and osteoarthritis (Krane *et al.*, 1988). They cleave fibrillar type II collagen at a single locus within the triple helical region, which is an irreversible step in the destruction of the principal structural component of articular cartilage. Cartilage Protective Agent (CPA or Ro32-3555) is a potent inhibitor of human collagenase 1 with an Ki value of 2.95±0.07nM (n=2), which shows 50 and 20 fold selectivity for collagenase over human gelatinases A and B respectively, and 175 fold selectivity for collagenase over stromelysin 1.

CPA inhibits hydroxyproline loss (IC_{50} 60nM) from bovine nasal cartilage explants cultured in DMEM medium containing 10^5U/ml human recombinantinterleukin II (Nixon *et al.*,1991).

Granuloma induced cartilage degradation was studied the sponge cartilage model using male, AHH/r strain, rats with a weight range of 180-210g (Bishop *et al.*, 1993). CPA protected against the loss of hydroxyproline which occurs in cylinders of bovine nasal cartilage (20±2mg) implanted subcutaneously into the backs of rats. The ED_{50} for CPA was 10mg/kg when dosed orally, twice daily for 14 days (group size was 10 animals/dose). Levels of hydroxyproline were 40.7±4.9 nmol/mg of cartilage in vehicle-dosed animals and 74.7±5.3 nmol/mg in non-implanted cartilages.

The protective effects of CPA on articular cartilage were measured in the rat *P. acnes*-induced monoarthritis model (Trimble *et al.*, 1987). The area of the articular cartilage of the lateral femoral condyle of female, AHH/r strain rats (weight range 140-175g) was measured from coronal histological sections by using image analysis techniques. Animals injected with *P.acnes* and dosed with drug vehicle exhibited pannus-driven destruction of the cartilage in a 14 day period. CPA administered orally at 50 mg/kg once per day showed statistically significant protection of the articular cartilage ($p<0.05$; by Student's 't' test, unpaired where group size was 10 animals per/dose).

These studies show that CPA is a potent selective inhibitor of collagenase *in vitro* and that oral administration of CPA can protect cartilage *in vivo* in models of cartilage degradation and arthritis. CPA is a novel, orally-active, drug which has potential for therapeutic benefit by inhibiting the cartilage destruction characteristic of both rheumatoid arthritis and osteoarthritis.

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189P INDUCTION OF ADHESION MOLECULE EXPRESSION ON HUMAN LUNG MICROVASCULAR ENDOTHELIAL CELLS BY CYTOKINES OR LIPOPOLYSACCHARIDE

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Increases in cell adhesion molecule (CAM) expression on lung microvascular endothelium may mediate neutrophil-induced lung injury seen in sepsis. Thus we have investigated the effects of tumour necrosis factorα (TNFα), interleukin-1β (IL-1β), interferonγ (IFNγ), or lipopolysaccharide (LPS) on induction of intercellular adhesion molecule-1 (ICAM-1), E-selectin or vascular cell adhesion molecule (VCAM-1) on normal human lung microvascular endothelial cells (HLMVEC) in culture. Functional consequences of CAM expression were assessed by measuring human neutrophil adhesion to HLMVEC monolayers.

HLMVEC (Clonetics, San Diego, USA) were maintained in endothelial cell growth medium supplemented with foetal calf serum (5%), epidermal growth factor (10ng ml^{-1}) & antibiotics. Confluent monolayers of HLMVEC grown on 96-well plates were incubated for 0-24h with TNFα, IL-1β, IFNγ, or LPS. A specific enzyme-linked immunosorbant assay was used to measure CAM expression (Piggot *et al*, 1991) and results were expressed as mean optical density (OD_{405}) ± s.e. mean of 5 experiments (table 1).

Human neutrophils, isolated from peripheral blood and labelled with a fluorescent dye (Calcein-AM, 10μM; (Akeson & Woods, 1993) were incubated for 30 min with HLMVEC monolayers. Results were expressed as a percent of adherent cells over total cells (1.25×10^5) added per well, as determined by fluorescence.

Table 1 shows the effects of cytokines/LPS on CAM expression. Adhesion of unstimulated neutrophils to unactivated HLMVEC was 9.4 ± 3.2% (mean ± s.d. of 4 replicates from one of three similar experiments) and adhesion was significantly ($P<0.05$) increased following activation of HLMVEC for 6h with TNFα (16.2±1.4%), IL-1β (33.3±1.6%), LPS (18.0±2.1%) but not IFNγ (6.0±1.5%). An understanding of the profile of cytokine/LPS-induced CAM on HLMVEC may allow targeting of specific cytokines or adhesion molecules in sepsis.

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Table 1: CAM expression on HLMVEC. Statistical tests were performed using ANOVA followed by Tukey-Kramer multiple comparisons.

	ICAM-1 (OD_{405})[1]			E-SELECTIN (OD_{405})[2]			VCAM-1 (OD_{405})[2]		
	6h	16h	24h	6h	16h	24h	6h	16h	24h
TNFα 10ngml^{-1}	1.17±0.04	1.50±0.04	1.70±0.05*	1.18±0.01	0.60±0.03*	0.40±0.02*	0.50±0.05	0.90±0.06*	0.90±0.01*
IL-1b 10ngml^{-1}	0.97±0.06	1.13±0.03	1.26±0.03*	1.13±0.02	0.59±0.03*	0.34±0.02*	0.32±0.02	0.44±0.04	0.30±0.03
IFNγ 40ngml^{-1}	0.37±0.04	0.51±0.04	0.59±0.04*	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.02±.008	0.03±0.01
LPS 10μgml^{-1}	0.58±0.02	0.70±0.03	0.87±0.02*	0.78±0.05	0.20±0.02	0.11±0.02	0.24±0.03	0.22±0.03	0.15±0.04

[1] Basal ICAM-1, 0.2±0.001 OD_{405}; [2] Basal E-selectin or VCAM-1 were not detected; * $P<0.01$, denotes a significant difference from CAM expression at 6h

190P HYPOXIA INCREASES ENDOTHELIN-1 RELEASE FROM AN ISOLATED PERFUSED RAT LUNG MODEL

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Increased endothelin-1 (ET-1) levels have been found in plasma and lungs from rats exposed to hypoxia (Oparil *et al.*, 1995) and ET receptor antagonists can prevent the development of pulmonary hypertension induced by hypoxia (DiCarlo *et al.*, 1994). We have provided evidence for endothelin (ET) involvement in the responses to systemic hypoxia in the isolated rat lung by utilising the ET receptor antagonists BQ123 and bosentan, and the endothelin converting enzyme inhibitor phosphoramidon (Smith *et al.*, 1995a).In the present study we have measured the perfusate levels of ET-1 from isolated lungs perfused in a recirculating manner under both normoxic and hypoxic conditions, and in the presence of three compounds (phalloidin, colchicine and cycloheximide) that interfere with cellular secretion and protein synthesis. All of these agents have been shown to attenuate the pressor response and the increase in lung weight seen in response to systemic hypoxia in this isolated rat lung model (Smith *et al.*, 1995b).

Lungs were isolated, ventilated with room air and perfused *via* the pulmonary artery with Krebs solution gassed with 20%O_2/5%CO_2 /75%N_2 and allowed to stabilise for 15 min prior to recirculation (50 ml, 5 ml min^{-1}) for 15 min prior to the onset of hypoxia (95%N_2/5%CO_2). In studies involving drug treatment, the lungs were perfused with the relevant agent for 15 min prior to the onset of hypoxia and for the duration of the hypoxic period (90 min). At the end of the experiment ETs were isolated from the perfusate and concentrated prior to measurement using an ELISA. Results are expressed as pg ET-1 ml^{-1} perfusate.

Systemic hypoxia caused an increase in the level of ET-1 in the perfusate, when compared to normoxic lungs ($p<0.001$, see Figure 1). The hypoxia-induced increase in ET-1 was significantly attenuated by the f-actin stabiliser phalloidin, the microtubule disrupting agent colchicine and the protein synthesis inhibitor cycloheximide ($p<0.005$, n=4-9), all of which also attenuated the increases in lung weight and perfusion pressure seen in the isolated lungs.

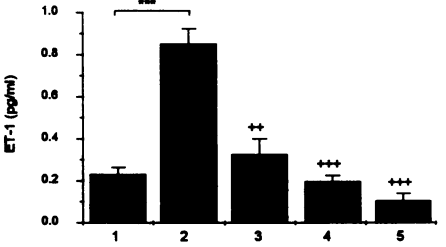

Figure 1. Measurement of ET-1 in perfusate samples (25ml) from recirculated (90min) rat lungs (mean±SEM, n=4-9). 1 Normoxic; 2 Hypoxic; 3 Hypoxia+phalloidin (50 nM);4 Hypoxia+colchicine (100 nM); 5 Hypoxia+cycloheximide (5μM). *** $p<0.001$ vs Normoxic; ++ $p<0.005$ vs Hypoxic; +++ $p<0.001$ vs Hypoxic.

These data show that ET-1 is released in response to systemic hypoxia in this model. Attenuation of the hypoxia-induced increase in ET-1 by cycloheximide suggests that the ET-1 is derived from *de novo* synthesis and not from release of preformed ET-1. Agents which block secretion also lower ET-1 levels in this model, suggesting that cytoskeletal components play an important role in the release of ET-1 following the onset of hypoxia.

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191P EFFECT OF R15.7, AN ANTI CD18 ANTIBODY, ON THE CELLULAR RECRUITMENT, THE LATE AIRWAY RESPONSE (LAR) AND AIRWAY HYPERRESPONSIVENESS (AHR) IN AN ALLERGIC RABBIT MODEL

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Chronic airway inflammation is a characteristic feature of asthma (Jeffery *et al.*, 1992) and there is increasing evidence for up regulation of leukocyte-endothelial cell molecules in asthmatic airways (Montefort *et al.*, 1994). In this study, we have investigated the role of β_2 integrin molecules (CD18), a sub family of adhesion molecules expressed on leukocytes which are crucial in leukocyte-endothelial interaction, on allergen induced LAR, cellular recruitment and AHR in a well characterized allergic rabbit model (Herd *et al.*, 1995).

Litter-matched New Zealand White rabbits were immunized within 24h of birth and until 12 weeks of age with *Alternaria tenuis* antigen (Ag) in $Al(OH)_3$ gel (i.p.). On day 1, rabbits were pre-medicated with diazepam (5 mg kg^{-1}, i.p.) followed 15 min later by hypnorm (0.4 mg kg^{-1} i.m.). Rabbits were then challenged with *Alternaria tenuis* aerosol (20,000 PNU ml^{-1} in saline for 20 min). Lung function was monitored for 6h and all rabbits that demonstrated a late airway response as assessed by changes in lung function were included in the study. On day 14 airway responsiveness to aerosolised histamine was determined as a measure of lung function. Cumulative dose-response curves were performed to establish the provocative concentration that produces a 50% increase in airway resistance (R_l ; PC_{50}) and 35% decrease in dynamic compliance (C_{dyn}; PC_{35}). Bronchoalveolar lavage (BAL) was then performed by administration of 5 ml sterile saline directly into the airways. On day 15, rabbits were pre-treated with a mouse IgG_1 monoclonal antibody directed at CD18 molecules or the control antibody (a non-binding mouse IgG_1) both administered at 1 mg Kg^{-1} (i.v.) and 1h later rabbits were challenged with Ag as on day 1 and lung function was monitored for 6 h. On day 16, airway responsiveness to histamine and BAL were performed as on day 14.

Mean ± s.e.mean values are shown. The significance of differences between means was assessed by paired or unpaired t-tests as appropriate.
Antigen challenge of rabbits on day 1 resulted in development of LAR, since the C_{dyn} was significantly reduced ($P<0.05$) at the 0-6 h period. On day 15, rabbits pre-treated with the control antibody developed a LAR of similar magnitude, as there was no significant difference in the area under the curve (AUC) between rabbits challenged on day 1 and day 15. However, in the rabbits pre-treated with anti-CD18 antibody (n=8), the AUC was significantly reduced ($P<0.05$) on day 15 as compared to day 1. Antigen challenge of control antibody pre-treated rabbits (n=6) resulted in a significant increase in eosinophil and neutrophil numbers ($P<0.05$). In contrast, anti-CD18 pretreatment resulted in a significant reduction in both eosinophil and neutrophil numbers. Airway responsiveness to histamine was enhanced after Ag in animals pre-treated with control antibody as assessed by R_l (before challenge 22.6±1.3 mg ml^{-1}, decreased to 6.65±1.31 mg ml^{-1} after challenge, $P<0.05$) and C_{dyn} (before challenge 9.80±1.24 mg ml^{-1}) decreased to 4.67±1.13 mg ml^{-1} after challenge. There were no significant changes in either R_l or C_{dyn} in anti-CD18 antibody pre-treated rabbits.

These data show that neutralising the CD18 molecules reduces the numbers of inflammatory cells in the airways, abolishes the LAR and inhibits the AHR induced by allergen. This illustrates the importance of the CD18 molecules in the recruitment of pro-inflammatory cells into the airways.

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192P THE ROLE OF PROTEIN PHOSPHATASES IN THE MODULATION OF NEURONAL ACTIVITY IN GUINEA-PIG AIRWAYS

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We previously demonstrated that the type 4 phosphodiesterase isoenzyme inhibitor, RO-20-1724 selectively attenuates excitatory non-adrenergic non-cholinergic (eNANC) contractile responses (Spina et. al., 1995). Recently, it has been shown that the protein phosphatase 1 and 2A inhibitor, okadaic acid augments the capsaicin-induced release of sensory neuropeptides from rat DRG neurones (Hingtgen & Vasko, 1994). In the present study we have investigated whether protein phosphatases are involved in regulating the eNANC response in guinea-pig isolated bronchus, using okadaic acid and its inactive analogue 1-Nor okadaone.

Guinea pigs were killed by cervical dislocation and the lungs removed. Isolated main bronchus was cut into 2 mm rings and suspended on two 'L' - shaped stainless steel holders and placed in 8 ml organ baths under a tension of 500 mg and stimulated electrically [electrical field stimulation (EFS) 3 Hz, 20 sec, 0.5 ms, 90 V]. The tissues were bathed in Krebs-Henseleit solution aerated with 95 % O_2 at 37 °C, in the presence of indomethacin (5 μM), propranolol (1 μM), thiorphan (10 μM) and atropine (1 μM). Results are expressed as mean ± s.e.mean. Data was analysed by student's paired and non-paired t-test. Values were considered significant if $P < 0.05$.

Neither okadaic acid (0.01 - 1 μM), 1-Nor okadaone (0.3 μM) or the vehicle [dimethylsulphoxide (DMSO) 0.008 - 8%] had any significant effect on baseline tone ($P > 0.05$). However, okadaic acid significantly attenuated eNANC contractile responses, whilst both the vehicle and inactive analogue had no significant affect (% inhibition: okadaic acid 0.3 μM 59.5 ± 8.7 %; 1-Nor okadaone 0.3 μM -1.25 ± 8.5 %; DMSO 0.25% 1.25 ± 13.29 %; $P < 0.05$ n = 4). We also tested the effect of okadaic acid on cholinergic responses in guinea-pig isolated bronchus. Okadaic acid (0.3 μM) in contrast failed to significantly alter the cholinergic component of the electrical field induced contraction (1 Hz control 22 ± 7.9 % MCh Emax vs. okadaic acid 18 ± 7.7%; 3 Hz control 26 ± 6.9 % MCh Emax vs. okadaic acid 27 ± 9.1 %; 10 Hz 36 ± 7.6 % MCh Emax vs. okadaic acid 33 ± 8.9 %; 30 Hz 50 ± 7.6 % MCh Emax vs. okadaic acid 42 ± 14 %; $P > 0.05$ n = 4). Furthermore, okadaic acid (0.3 μM) did not significantly alter the contractile potency (pD_2) to methacholine (okadaic acid 6.4 ± 0.2 vs. DMSO 6.4 ± 0.3, $P > 0.05$ n = 4).

The results would suggest that protein phosphatases 1 and 2A are involved in the prejunctional modulation of eNANC but not cholinergic responses in guinea pig airways.

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193P RELAXANT ACTIONS OF PROSTANOID AND NON-PROSTANOID IP-RECEPTOR AGONISTS ON THE HUMAN PULMONARY ARTERY

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Prostanoid IP-receptors in neural tissues may differ from the classical blood platelet IP-receptor (Wise *et al.*, 1995; Takechi *et al.*, 1996). We now report relaxant potencies of both prostanoid and non-prostanoid IP-receptor agonists on human intra-pulmonary artery, which we believe define the classical IP-receptor and may aid the definition of receptor subtypes. Ring preparations were bathed in Krebs-Henseleit solution - 95% O_2 / 5% CO_2, containing indomethacin (1 μM). Contractile tone (force transducer-MacLab recording) was induced by phenyl-ephrine (1 - 2 μM) in the presence of the TP-receptor antagonist GR 32191 (200 nM). The standard agonist was cicaprost (IC_{50} range = 0.3 - 1.2 nM) and relative potencies were expressed as equi-effective molar ratios (EMR, Table 1).

Table 1. Relaxant potencies of IP-receptor agonists on human isolated intrapulmonary artery (± s.e.mean, n = 4)

Prostanoid	EMR	Non-prostanoid	EMR
Cicaprost	1.0	BMY 45778	3.74 ± 0.76
TEI-9063	0.89 ± 0.23	BMY 42393	102 ± 19
Iloprost	2.37 ± 0.15	Octimibate	368 ± 31
Taprostene	22.7 ± 5.5	CU 23	1270 ± 130
Benzodioxane -prostacyclin	380 ± 141	CU 602	partial agonist (?)
TEI-3356	2370 ± 510		

Although TEI-9063 is the most potent relaxant, its highly potent EP_1 agonist actions make it much less selective than cicaprost. The related isocarbacyclin TEI-3356 is claimed to be "a highly selective EP_3-receptor agonist" (Negishi *et al.*, 1994); on 2 of 4 artery preparations, EP_3 contractile activity was indeed observed at 25 - 250 nM, but this evolved into relaxation at 1 - 5 μM.

BMY 45778 was the most potent of the non-prostanoids tested, agreeing with a reported Ki of 5.4 nM for displacement of [^{3}H]-iloprost binding to human platelet membranes (Meanwell *et al.*, 1993). Although much less potent, octimibate also behaved as a full agonist against tone induced by phenylephrine, and in addition by U-46619 (4 nM), and KCl (40 mM). This contrasts with its partial agonist profile on human coronary, mesenteric and skin/subcutaneous artery rings (Merritt *et al.*, 1991).

The novel agent CU 23 (8-[2,3-diphenyl-1-indolyl]octanoic acid) has one of the simplest non-prostanoid structures for an IP-agonist. Its Ki on the human platelet membrane radio-receptor assay is 2.5 ± 0.4 μM (our data). A second chemically simple compound CU 602 (9-[4,5-diphenyl-2-oxazolyl]-8-oxa-nonanoic acid) has a Ki on the platelet assay of 7.8 ± 0.6 μM. Its log concentration-response curves (0.1 - 5 μM) on the pulmonary artery showed considerable variation in slope; we are investigating whether this is due to partial agonism.

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194P SUSTAINED PLATELET ENTRAPMENT IN RABBIT PULMONARY VASCULATURE FOLLOWING ACTIVATION OF THE INTRINSIC COAGULATION PATHWAY WITH ELLAGIC ACID

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Thrombin is a serine protease generated as a result of activation of both the intrinsic and extrinsic coagulation pathways. We have previously described the ability of exogenous thrombin to induce ^{111}Indium (^{111}In) labelled platelet accumulation (PA) in the pulmonary (PV) and cerebral vasculature (CV) of rabbits (May et al., 1990) and have demonstrated the effectiveness of antithrombin agents in this model (Liu et al., 1994). In the present study, we have examined the effects of the intrinsic coagulation pathway activator, ellagic acid (EA), on rabbit PA in vivo.

Rabbit platelets labelled with ^{111}In-oxine were injected into male NZW rabbits (2.1-2.8 kg) anaesthetised with diazepam (4mg/kg i.p.) followed by Hypnorm (0.4ml/kg i.m.). ^{111}In levels in selected vascular beds were monitored continuously as described previously (May et al., 1990). Platelet responses in a given vascular bed have been expressed as mean ± s.e. mean of the % change in ^{111}In counts from stable baseline values or of the area under the curve (AUC) of the plot of % change against time. The significance of differences between means was assessed by analysis of variance followed by the Tukey Kramer test or by an unpaired t-test. There were 3 to 5 animals per group.

Intravenous infusion of vehicle (0.15M Tris HCl, pH 7.4) for 30min produced changes in platelet parameters which were <5% of baseline values. Infusion of EA (0.5-2mg/kg/min) caused dose-related increases in PA in the PV measured at the end of the infusion [EA 0.5mg/kg/min: 2.3 ± 0.4%; EA 1mg/kg/min: 17.7 ± 3.5% (P<0.05); EA 2mg/kg/min: 45.9 ± 4.9% (P<0.01)]. Accumulation in the PV was accompanied by a fall in ^{111}In counts in the CV [e.g. EA 2mg/kg/min: -40.8 ± 6.4% (P<0.01)] and in peripheral blood (PB) samples [e.g.EA 2mg/kg/min: -54.6 ± 2.1% (P<0.01)]. PA induced by EA (2mg/kg/min) in the PV was sustained as shown by the AUC over a 2h period from commencement of the EA infusion [vehicle: 1 ± 1; EA: 3529 ± 478, (P<0.01)] and by the % change from baseline at 2h [EA: 24.8 ± 3.5%, (P<0.05)]. At the end of 2h, the EA-induced decreases in counts in the CV and PB were -21.5 ± 7.1% (P=NS) and -42.9 ± 2.6% (P<0.01), respectively. The thrombin inhibitors Argatroban (1mg/kg bolus, 1min before EA), unfractionated heparin (200U/kg + 20U/kg/min for 45min, starting 15min before EA) and hirulog (0.2mg/kg/min for 45min, starting 15min before EA) had no significant effect on peak PA in the PV induced by EA 2mg/kg/min (data not shown) but produced significant (P<0.05) reductions in the AUC over 2h (Argatroban: 1657 ± 365, control: 3529 ± 478; heparin: 1973 ± 505, control: 4177 ± 562; hirulog: 2788 ± 345, control: 4670 ± 711).

These results show that ellagic acid induces a prolonged accumulation of platelets in the PV of rabbits and a concomitant thrombocytopaenia. Platelet accumulation appears to be at least partially a consequence of endogenous thrombin generation since it is susceptible to inhibition by thrombin inhibitors.

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195P EFFECT OF ISOPRENALINE ON ELECTROGENIC ION TRANSPORT ACROSS THE MUCOSA OF HUMAN SMALL AND LARGE INTESTINE

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It has been found that atypical β-adrenoceptors are located in the mucosa of human intestine (Summers *et al.*,1995). In the present study, we have used the agonist isoprenaline (ISO) to investigate a possible role for β-adrenoceptors on secretion in different regions of human intestine. The selective antagonists atenolol (β_1) and ICI 118,551 (β_2, Arch & Kaumann, 1993) were used to further elucidate the receptor responsible for the effects.

Sections of human terminal ileum, ascending colon and sigmoid colon were obtained fresh from specimens removed at operation for carcinoma. Mucosal preparations were stripped of muscle layers and set up as flat sheets in Ussing chambers, where changes in short-circuit current (I_{sc}; an indicator of electrogenic fluid secretion) were monitored. After 90 min equilibration, ISO (10 or 100μM) was applied to the serosal side of each preparation, either in the absence or presence of antagonist (applied to both sides of the preparation and incubated for 30 min prior to application of ISO), and the resulting changes in I_{sc} were recorded. An increase in I_{sc} indicates fluid secretion whereas a decrease indicates absorption. Data are expressed as mean ± s.e.mean; *n*=number of specimens. Statistical analysis was by Mann-Whitney U-test, with $P<0.05$ taken to indicate significance.

In the ileum, application of ISO caused a biphasic response consisting of an immediate transient increase in I_{sc} (maximal within 2 min), followed by a sustained decrease, measured 10 minutes after injection. ISO (10 and 100μM, both *n*=4) produced a significant increase in I_{sc} of 12.9 ± 7.4 and 14.0 ± 8.3 μAcm^{-2}, respectively. This effect of ISO was not significantly affected by the presence of ICI 118,551 (1μM, *n*=4), whereas it was significantly reduced by prior application of atenolol (10μM, $P < 0.05$, *n*=4). After 10 min exposure to ISO, the I_{sc} had significantly decreased below its pre-drug value (-1.7 ± 2.6 and -18.5 ± 6.2 μAcm^{-2} for 10 and 100μM ISO, respectively). Application of atenolol (10μM) or ICI 118,551 (1μM) had no significant effect on this pro-absorptive response.

In ascending colon, in contrast to the ileum, there was no initial increase in I_{sc} upon application of ISO (100μM). There was, however, a significant decrease in I_{sc}, which reached -13.8 ± 3.5 μAcm^{-2}, 10 min after applying ISO (*n*=4). Neither atenolol (10μM) nor ICI 118,551 (1μM) had any significant effect on this response. Preliminary data from sigmoid colon indicate a similar pro-absorptive response to ISO but the changes failed to reach statistical significance. All tissues responded to carbachol (100μM) with a significant secretory response, proving tissue viability.

In conclusion, ISO causes a secretory response in small intestine, which cannot be detected in colon. This effect appears to be mediated by β_1-adrenoceptors. In addition, ISO causes a delayed and sustained absorptive response in both small and large intestine which cannot be attributed to stimulation of β_1- or β_2-adrenoceptors.

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196P INVESTIGATION OF THE EFFECTS OF SB 204741 AT THE 5-HT_{2B} RECEPTOR IN THE LONGITUDINAL MUSCLE LAYER OF HUMAN SMALL INTESTINE

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It has previously been shown that application of 5-HT induces contraction of both circular and longitudinal muscle layers of human small intestine (Borman & Burleigh, 1994). On the basis of sensitivity to SB 200646 (a 5-$HT_{2B/2C}$ receptor antagonist; Baxter *et al.*, 1994) and yohimbine (a 5-HT_{2B} receptor and adrenoceptor antagonist), the response in longitudinal muscle has been ascribed to stimulation of a 5-HT_{2B} receptor (Borman & Burleigh, 1995). The present study has investigated the effects of a novel, selective 5-HT_{2B} receptor antagonist, SB 204741 (Baxter *et al.*, 1995), on 5-HT-induced contraction of the longitudinal muscle layer of human terminal ileum.

Strips of longitudinal muscle from human terminal ileum were prepared as described previously (Borman & Burleigh, 1995). Two cumulative concentration-response curves to 5-HT were obtained, the second curve being obtained 60 min after the first and in the presence of antagonist or control vehicle (incubated for 30 min). Contractile responses to 5-HT were expressed as a percentage of the maximum contractile response to carbachol (30 μM, applied at the end of each experiment). Data were analysed using the Mann Whitney U Test, with $p<0.05$ being taken to indicate a significant difference. EC_{50} values are given as geometric mean with 95% confidence limits (95% C.L.). Concentration ratios were calculated from these values.

Application of 5-HT produced a contraction of longitudinal muscle strips from human ileum, with an EC_{50} for 5-HT of 31.0 nM (95% C.L. 21.7-44.1, n=4). For a second control response curve, there was a significant increase in the EC_{50} for 5-HT, with a concentration ratio between curves of 2.2 (95% C.L. 1.8-2.6, n=4). Application of SB 204741 (0.1, 0.3, 1 and 3 μM, n=4 at each concentration) produced a significant, parallel and dextral displacement of the second response curve to 5-HT, but with no significant alteration in the maximum response to 5-HT. This compound had no significant effect on the basal tone of the tissue, indicating that it possesses negligible intrinsic activity at the human 5-HT_{2B} receptor. Schild analysis of this antagonism gave a Schild plot with a slope of 0.90 (not significantly different from unity), allowing a pK_B of 7.02±0.20 to be estimated (slope constrained to unity). This value is lower than has been reported for SB 204741 in rat jugular vein and stomach fundus (7.3 and 7.9 respectively), but is higher than has been reported for an interaction at other 5-HT receptor sub-types (pKi <6.0 at the 5-HT_{2C} receptor and <5.0 at other 5-HT receptors; Baxter *et al.*, 1995).

In conclusion, by the use of SB 204741 as a silent, competitive antagonist for the 5-HT_{2B} receptor, we have confirmed the classification of the 5-HT_{2B} receptor in human ileal longitudinal muscle.

We are grateful to Dr T.P. Blackburn of SmithKline Beecham for the generous donation of SB 204741. Supported by MRC ROPA award G9507991.

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197P RESPONSE OF THE ISOLATED INTESTINE OF *SUNCUS MURINUS* TO 5-HT AND CHARACTERISATION OF THE 5-HT RECEPTOR(S) MEDIATING CONTRACTION

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Suncus murinus has been used for the study of the mechanism of action of emetic and antiemetic drugs (Ueno *et al.*, 1987). Disturbance of gastrointestinal function is central to the induction of emesis although the mechanisms involved in gastrointestinal motility in *Suncus murinus* have received little attention (Muraki *et al.*, 1988). The aim of the present study is to elucidate the smooth muscle response of different regions of the intestine of *Suncus murinus* to 5-HT.

Two segments (about 1 cm length) taken from the intestine (2 cm distal to the pyloric sphincter and 2 cm proximal to the anal region) of adult Japanese House Musk shrew, *Suncus murinus* (38-88 g) of either sex were mounted in 10 ml organ baths containing Krebs' solution (37°C, 95% O_2, 5% CO_2). The tissues were allowed to equilibrate for 60 min and washed every 20 min. The resting tension was maintained at 0.5 g and recorded isometrically. Non-cumulative concentration-response curves to 5-HT were established with a 1 min contact time and 22 min intervals. The procedure was repeated in the constant presence of methysergide (1 μM), ritanserin (0.1 μM) or atropine (1 μM). Tissues were allowed to equilibrate for 1 h in the presence of antagonist before the application of 5-HT. Data were expressed as the mean±s.e.mean of n=5-8 and analysed using one-way ANOVA followed by Bonferroni/Dunnett's t-test.

5-HT (0.003-30 μM) produced a concentration-dependent contraction in both segments. The terminal segment of the intestine produced a greater response of 6.2 ± 0.9 g as compared to the proximal response of 1.4±0.3 g. In both segments, methysergide and ritanserin significantly ($p<0.05$) shifted the lower part (0.01-0.3 μM) of the concentration-response curves to the right, without affecting the maximum response, Fig.1. Atropine antagonised the 5-HT responses in the terminal region but not in the proximal segment with a progressive reduction of maximum response to 47.7% of control response (Fig. 1).

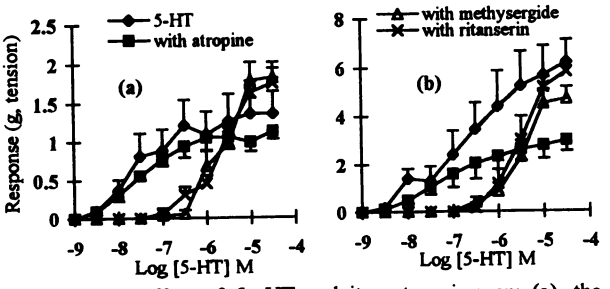

Figure 1- The effect of 5 - HT and its antagonism on: (a) the proximal and (b) the terminal region of *Suncus murinus* intestine.

These data suggest the involvement of 5-HT_2 receptors in the contractile responses to 5-HT in both regions of the intestine of *Suncus murinus*. A 5-HT-induced release of acetylcholine is also implicated in the contractile action of 5-HT in the terminal region with the possible involvement of other receptors such as the 5-HT_3 receptor.

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198P *IN VIVO* PROFILE OF DARIFENACIN, A SELECTIVE MUSCARINIC M3 RECEPTOR ANTAGONIST

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Darifenacin is a potent and selective M_3 muscarinic receptor antagonist, that *in vitro* displays 100-fold selectivity over M_2 and 30-fold selectivity over M_1 receptors (Wallis et al., 1995). Darifenacin is as potent as atropine at inhibiting carbachol-induced contractions of the guinea-pig ileum, yet it is 6-fold weaker at inhibiting carbachol-induced ^{86}Rb efflux in the guinea-pig salivary gland (Wallis et al., 1995). Studies were undertaken to determine the selectivity of darifenacin and atropine *in vivo*.

Male beagle dogs (11-14.5kg) (n=3-4) were fasted overnight and prepared as previously described (Quinn et al., 1991). Motility was stimulated by infusions of sulphated cholecystokinin octapeptide (CCK-8s) at 75ng.kg^{-1}.min^{-1} for 7 minutes. The left submandibular gland duct was cannulated to allow collection of saliva; secretion was stimulated by bipolar electrical stimulation for 1 minute (0.5ms, 20Hz and 20V). Results are expressed as the geometric mean with 95% confidence limits. The activity of compounds against oxotremorine-induced tremor and salivation was assessed in male mice (25-30g, Charles River CD1, n=5-6), using the method of Bevan et al (1988). Test compounds were injected intravenously via the tail vein 10 minutes prior to adminstration of oxotremorine (200μg.kg^{-1}) , 5 minutes later the salivation and tremor were scored visually using a scale of 0-3.

In the dog, darifenacin potently inhibited CCK-8s induced jejunal motility (ED_{50} 0.32 (0.19-0.53) μg.kg^{-1}.min^{-1}), whilst being significantly (paired t-test $p<0.05$) weaker at inhibiting salivation (ED_{50} 2.16 (0.52-8.95) μg.kg^{-1}.min^{-1}). No effect on heart rate was observed at doses up to 10μg.kg^{-1}.min^{-1}. In contrast atropine potently, but non-selectively, inhibited both motility (ED_{50} 0.26 (0.06-1.08) μg.kg^{-1}.min^{-1}) and salivation (ED_{50} 0.36 (0.12-1.01) μg.kg^{-1}.min^{-1}) and this was accompanied by a marked tachycardia (+90%). In the mouse, darifenacin inhibited oxtremorine-induced tremor (ED_{50} 4.1 (0.5-33) mg.kg^{-1}) and salivation (ED_{50} 0.3 (0.2-0.4) mg.kg^{-1}) but was significantly weaker than atropine (ED_{50} 0.09 (0.06-0.16) and 0.036 (0.009-0.14) mg.kg^{-1})

Thus, darifenacin was equipotent with atropine as an inhibitor of motility in the dog, but was markedly weaker as an inhibitor of salivation. Likewise in the mouse, darifenacin was weaker than atropine as an inhibitor of oxotremorine-induced salivation. These data are consistent with darifenacin's selectivity for the gut and reduced potency on the salivary gland *in vitro*. Oxotremorine-induced tremor is believed to be mediated by central M_1 receptors (Bevan et al, 1988), since darifenacin readily accesses the brain its weak activity is consistent with its weak M_1 activity *in vitro*. In conclusion, darifenacin is a novel M_3 antagonist which demonstrates potent inhibition of gut motility with selectivity over effects on heart rate and salivation.

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199P AGONIST INTERACTIONS AT PRESYNAPTIC HISTAMINE H_3-, $GABA_B$- AND SOMATOSTATIN RECEPTORS ON THE GUINEA-PIG ILEUM

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The coexistence of two or more populations of presynaptic receptors within a tissue provides the opportunity for interactions in terms of the control of transmitter release. Activation of histamine H_3- (Hew et al 1990), $GABA_B$ - (Bowery et al 1981) and somatostatin (Furness and Costa 1979) receptors have been shown to inhibit electrical field stimulated contractions of the guinea-pig ileum as a consequence of the reduction of neuronal release of acetylcholine. The present study examines possible interactions resulting from activation of these receptors.

Segments of distal ileum 2cm in length were obtained from Dunkin Hartley guinea-pigs (600-800g) of either sex and mounted under a tension of 1g in a 25ml organ bath containing Krebs solution at 37°C and gassed with 95% O_2/5% CO_2. Tissues were continuously stimulated coaxially by electrical field stimulation (5ms, 0.1Hz) with parallel platinum electrodes delivered from a Grass stimulator at 10V and isometric contractions were measured by a force transducer. Results are expressed as mean ± s.e. and statistical analysis was by Student's t- test.

The selective agonists R - (α) - methylhistamine (RAMH) (H_3, 4-124nM) and baclofen ($GABA_B$ 3.2-190μm), added cumulatively, and somatostatin (8-200nm), added non-cumulatively to the bath, caused concentration dependent inhibition of the twitch contraction with maximum inhibitions of 65.8 ± 6.7% (n = 8), 25.2 ± 4.5% (n = 8) and 50.2 ± 4.0% (n = 12 to 20) respectively. Increasing the bath Ca^{2+} concentration from 2.5mM to 10mM had no effect on the twitch height (2.8± 0.14g and 2.9 ± 0.13g (n = 22) respectively, but reduced the inhibitory effect of RAMH (28nM), baclofen (22μm) and somatostatin (32nM) from 39.3± 5.0% to 23.7 ± 3.2%, 16.2 ± 2.1% to 9.5 ± 0.71% (n = 5) and 29.7 ± 3.2% to 7.0 ± 1.2% (n = 11) respectively. When a maximum inhibition was achieved to one agonist, addition of a second agonist produced a further inhibitory effect e.g. in the presence of a concentration of RAMH producing a maximum inhibition of 59.4 ± 4.4%, addition of baclofen produced a further inhibition of 16.5 ± 2.7% (n = 14).

To study possible interactions between the agonists, the inhibitory effect of pairs of agonists were studied alone and in combination (Table 1). The effects of RAMH and baclofen were additive, those of RAMH and somatostatin synergistic and an inhibitory interaction between baclofen and somatostatin was observed.

Table 1 Interactions between RAMH, baclofen and somatostatin in inhibiting electrically evoked contractions of the guinea-pig ileum. * p< 0.001 compared to the sum of the individual drug effects.

% Inhibition				
RAMH 8nM	Somatostatin 8nM	Baclofen 8μm	Combined	n
12.2± 2.1	11.9 ± 1.9	-	36.2 ± 4.5*	13
18.2 ± 1.9	-	17.5 ± 1.6	40.7 ± 3.0	11
-	16.4 ± 2.4	15.3 ± 1.7	26.1 ± 2.7*	21

The data shows that none of the agonists can produce complete abolition of the twitch contraction which may reflect a limited receptor population. The activity of all three agonists is Ca^{2+} dependent but despite this they act independently and the interaction studies may indicate that separate transduction pathways are involved.

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200P CHARACTERISATION OF POTASSIUM CURRENTS IN RAT URETER SMOOTH MUSCLE CELLS

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In guinea-pig ureter smooth muscle, calcium (Ca)-sensitive and A-type ($I_{K(A)}$) potassium currents have been described (Imaizumi *et al.*, 1989; Lang, 1989). However, no delayed-rectifier current ($I_{K(V)}$) was identified in these studies. In the present investigation the K-currents in rat ureter have been characterised by their voltage-sensitivity and pharmacology.

Smooth muscle cells were isolated by enzymatic treatment and whole-cell K-currents were measured under Ca-free conditions. Stepping for 500ms from a holding potential of -100mV to test potentials positive to -50mV induced a rapidly–activating and –inactivating current characteristic of $I_{K(A)}$. On holding at –50mV, $I_{K(A)}$ became inactivated to reveal a current with activation and inactivation characteristics similar to those of $I_{K(V)}$ in rat bladder cells (Green *et al.*, 1995). Holding at -10mV inactivated both $I_{K(A)}$ and $I_{K(V)}$ leaving a small, non-inactivating current. On stepping to more negative test potentials (up to -140mV) no evidence of an inwardly-rectifying current was obtained.

At a holding potential of -100mV, exposure to 20 mM TEA had no effect on the instantaneous peak current ($I_{K(A)}$) but the current present at the end of the test pulse ($I_{K(V)}$) was reduced (by 73 ± 11% at +50mV, n=5). In contrast, 4AP (5mM) inhibited both $I_{K(A)}$ and $I_{K(V)}$ by 33 ± 6% and 34 ± 9% at +50mV, respectively (n=4). When cells were held at -10mV, exposure to 10μM levcromakalim induced $I_{K(ATP)}$. This agent increased the holding current by 83 ± 25pA (n=6) and hyperpolarised the zero current potential by 24 ± 6mV (n=6), effects which were inhibited by glibenclamide (10μM). As previously observed in rat bladder (Green *et al.*, 1995), $I_{K(V)}$ was simultaneously reduced in the presence of levcromakalim.

Exposure to 33μM NS1619 (Edwards *et al.*, 1994) induced a noisy, iberiotoxin (250nM)-sensitive outward current at -10mV. On stepping to a test potential of +50mV the induced current was 908 ± 292pA (n=4) indicating an increase in current flowing through large conductance, Ca-sensitive K-channels ($I_{BK(Ca)}$). The induction of $I_{BK(Ca)}$ by NS1619 was accompanied by a marked reduction in $I_{K(V)}$.

These data show that $I_{K(A)}$, $I_{K(V)}$, $I_{K(ATP)}$ and $I_{BK(Ca)}$ are all present in rat ureter. Since K_{ATP} may comprise an inwardly-rectifying K-channel coupled to a sulphonylurea binding site (Inagaki *et al.*, 1995), it is surprising that no inwardly-rectifying current could be detected in this tissue.

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201P THE EFFECTS OF THE $GABA_B$ AGONISTS BACLOFEN AND 3-APPi ON MICTURITION PARAMETERS IN A CONSCIOUS RAT CYSTOMETRY MODEL

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The $GABA_B$ agonist baclofen inhibits micturition parameters in the conscious rat (Igawa *et al.*, 1993). In a small scale clinical trial baclofen was effective in relieving the symptoms of urinary urge incontinence (UUI), (Taylor and Bates, 1979), suggesting that the $GABA_B$ receptor may have a role in the control of micturition. The aim of this study was to examine the role of central and peripheral $GABA_B$ receptors in the modulation of micturition in the conscious rat using baclofen and the non-CNS penetrant $GABA_B$ agonist 3-aminopropylphosphinic acid (3-APPi) (Bolser *et al.*, 1994).

Rats (Male, CD, Charles River, 250-350 g) were anaesthetised (fentanyl/medetomidine *ip*, reversed with atipamezole/nalbuphine *ip*) and the bladder exposed *via* a mid-line incision and cannulated. A jugular vein was also cannulated. The cannulae were exteriorised *via* a sub-cutaneous tunnel to a retroscapular sealable injection port. Rats were left to recover for at least 72 h prior to cystometrical investigation. For each investigation the rat was placed in a Bollman's cage and the bladder infused with 0.9% saline (200 µl/minute, room temperature) *via* the bladder cannula. Pressure recordings were made *via* a transducer in series with the infusion line. At micturition, the time to micturition (micturition interval, MI), micturition volume (MV) and peak micturition pressure (MP) were recorded. When a series of three consistent micturitions had been recorded, the study compound or vehicle was injected *via* the jugular vein cannula. MV, MI and MP were measured for 1 h post dose. Initial studies indicated that a maximum response was reached within 1 h. Effects were expressed as a percentage of the mean pre-dose value. Statistical significance was tested using a non-paired Student's t-test.Re-use of animals was limited to two cystometrical investigations with at least 24 h rest intervals.

Baclofen and 3-APPi doses were based on their reported potencies, in the rat *in vivo* (Igawa *et al.*, 1993; Chapman *et al.*, 1993). Baclofen (0.3, 1 and 3 mg/kg *iv*, n=4-6) was without significant effect on MP (maximum 22.5 ± 11.4% at 3 mg/kg, n=4) but caused a significant ($p<0.01$) dose related decrease in MV and MI ($ED_{50}s$ = 2.3 ± 0.9 and 1.1 ± 0.3 mg/kg respectively). 3-APPi (0.3, 1 and 3 mg/kg *iv* n=4) caused no dose related changes in any parameter, however a significant ($p<0.05$) decrease was recorded in MV (34.0 ± 2.7%) and MI (37 ± 5.3%) at 1 mg/kg (all results expressed as mean ± sem).

The potency of the peripherally selective $GABA_B$ agonist 3-APPi on micturition parameters in the conscious rat is much less than would be predicted from its reported 9-fold greater *in vivo* $GABA_B$ potency relative to baclofen (Chapman *et al.*, 1993). Thus, it may be concluded that the effects of baclofen on micturition parameters observed in the current study are predominately centrally mediated. Therefore $GABA_B$ receptors appear to be involved in the control of micturition at the level of the CNS.

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202P BRADYKININ IN MILK FROM COWS WITH CLINICAL AND SUBCLINICAL MASTITIS

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Bradykinin (BK) and kininogen are present in normal bovine milk (Peeters et al., 1979) and kallikrein has been detected in healthy bovine mammary tissue (William et al. 1989). In this study levels of BK, a potent inflammatory mediator, have been evaluated in milk from animals with different stages of mastitis.

Samples of milk with low somatic cell count (SCC< $0.1x10^6ml^{-1}$) were obtained from a group of cows with clinically healthy udders in which there was no record of previous mastitis (Control). A second group termed "clinically mastitic" showed symptoms of mastitis (udder hardness, tenderness, oedema, and milk clotting) and an SCC>$1x10^6ml^{-1}$. A third group termed " sub-clinical " showed no mastitic symptoms, but had an SCC > $0.25 x10^6ml^{-1}$. In each animal the milk from each udder quarter was sampled separately. The number of bovine somatic cells was recorded by a Fosomatic cell counter. The milk was centrifuged at 3000 g, 4°C, 15 min. The supernatant was acidified with acetic acid to pH 4.6 and centrifuged at 75000 g, 4°C, 15 min. Bradykinin was extracted using Sep-Pak Vac cartridges (C18) and the eluates were dried under nitrogen. Reconstituted aliquots were radioimmunoassayed against synthetic BK (Moshi et al, 1992). The mean recovery of radiolabelled Bk was 92.3±1.3%. The Mann-Whitney test was used for statistical comparisons. Values are given as mean ± sd. unless otherwise stated. The assay antibody cross reacted identically with BK and Lys-BK and the milk kinin, but did not bind with Des-Arg-BK or Des-Phe-Arg-BK.

The mean immunoreactive BK (IR-BK) level in milk from 40 sub clinically inflamed quarters in 19 cows (279.2 ± 52 pg ml^{-1}) was 131 % higher ($p=0.018$) than that from 28 quarters in 13 healthy control animals (120.7 ± 21). That in 17 clinically inflamed quarters from 17 cows (790 ± 112 pg ml^{-1}) was 544 % higher ($p<0.001$) than that in the control group.

IR-BK was measured in quarter milk from 20 udders with at least one subclinically inflamed quarter and compared with milk from 13 clinically healthy animals (Fig. 1). The kinin level showed no simple relationship to the presence of inflammatory cells.

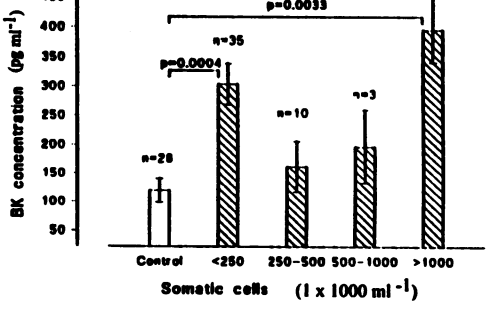

Fig. 1. IR-BK levels in milk from quarters of udders with at least one subclinically inflamed quarter (Hatched). Controls (Open) have only low cell count, clinically healthy quarters. (Mean, sd., n=No. of quarters).

In high cell count milk from subclinically mastitic udders, without the presence of any clinical symptoms of udder inflammation, the BK levels increased more than 100% compared to controls. When the number of inflammatory cells in the milk was raised and inflammatory symptoms were also present in affected quarters, the BK increase was much greater (more than 500%) compare to controls. The kinin level was not found to be dependent on the presence in the milk of inflammatory cells.

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203P INHIBITION OF NEUROGENIC OEDEMA FORMATION BY A TACHYKININ NK_1 RECEPTOR ANTAGONIST, SR140333, BUT NOT BY A BRADYKININ ANTAGONIST, HOE140, IN RAT SKIN

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Plasma extravasation induced by electrical stimulation of the rat saphenous nerve has been shown to be attenuated by an NK_1 receptor antagonist (Lembeck *et al.*, 1992). Using two different stimulation parameters (Escott, & Brain, 1993; Morton & Chahl, 1980) we have now compared the effect of SR140333 (Emonds-Alt *et al.*, 1993) with the bradykinin B_2 receptor antagonist HOE140 (Hock *et al.*, 1990). Morton and Chahl, (1980), have provided evidence that kinins are involved in the response.

Male Wistar rats (200-250g), anaesthetised with sodium pentobarbitone (50mgkg^{-1}, i.p.), were prepared for intradermal (i.d.) test agents and/or electrical stimulation of the saphenous nerve of one hind leg, with the other hind leg serving as sham control. The nerve was stimulated either at 10V, 1ms, 2Hz for 0-5 mins, and accumulation measured 0-30 mins, or, at 25V, 2ms, 10Hz for 0-15 mins, and accumulation measured 0-15 mins. Plasma extravasation was assessed in skin by the extravascular accumulation of i.v. ^{125}I-albumin. Statistics by ANOVA and Bonferroni's modified t-test.

The results (table 1) show that the NK_1 receptor antagonist abolished neurogenic oedema formation induced by both sets of stimulation parameters, suggesting that both induce a predominantly NK_1-mediated plasma extravasation. HOE140 was without effect. Further, bradykinin (i.d.) induced extravasation was not affected by the NK_1 antagonist. These results indicate that, in normal rat skin, neurogenic oedema formation does not involve a bradykinin receptor mediated component. It can also be concluded that bradykinin induced oedema does not involve an NK_1 receptor -mediated component.

P.K.Towler is the recipient of an MRC/Pfizer studentship.

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Table 1. Plasma extravasation induced by electrical stimulation of the saphenous nerve and i.d test agents. Results as µl plasma 100mg tissue^{-1} and µl plasma site^{-1} respectively. Mean ± s.e.mean., n = animals . **p<0.01, ***p<0.001 treated vs control.

Test agent	Saphenous nerve stimulation Type 1 (10V, 1ms, 2Hz)			Saphenous nerve stimulation Type 2 (25V, 2ms, 10Hz)			i.d. test agent (0.1ml site^{-1})		
	Stimulated	Sham	n	Stimulated	Sham	n	Tyrode	BK (1nmol)	n
Control (saline) i.v.	22.9 ± 1.8	3.6 ± 0.5	8	14.4 ± 2.3	2.7 ± 0.6	7	5.1 ± 0.9	20.4 ± 4.0	4
SR140333 100µgkg^{-1} i.v.	2.5 ± 0.4***	1.6 ± 0.2	4	4.3 ± 0.8**	2.4 ± 0.4	4	5.3 ± 0.9	31.2 ± 9.9	3
HOE140 80nmolkg^{-1} i.v.	26.0 ± 2.9	3.7 ± 1.1	6	15.8 ± 1.0	3.3 ± 0.4	4	7.2 ± 1.4	6.1 ± 0.6 **	4

204P SPINAL ANALGESIA BY ADENOSINE AGONISTS AND INHIBITORS OF ADENOSINE METABOLISM, WITH SYNERGY BETWEEN AN ADENOSINE KINASE AND AN ADENOSINE DEAMINASE INHIBITOR

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Directly and indirectly acting adenosine agonists produce antinociception in thermal threshold tests as well as in the formalin test. Release of adenosine from the rat spinal cord is greatly enhanced by the combination of an adenosine kinase inhibitor (AKI) and an adenosine deaminase inhibitor (ADI) (Golembiowska *et al.*, 1995). The current study further examined the spinal analgesic properties of directly and indirectly acting adenosine receptor agonists, as well as a possible synergistic interaction between an AKI and an ADI, using a carrageenan (CARRA) thermal hyperalgesia model.

Male Sprague-Dawley rats were used. Drugs were administered intrathecally (i.t.) either by acute percutaneous lumbar puncture or, in antagonist experiments, through a surgically implanted catheter. For acute i.t. injections under halothane anaesthesia (100-125 g rats), 10 µl of drug solution was injected via a 30G needle inserted between L5 and L6. In catheterized rats (300-350 g), an i.t. catheter was surgically implanted under halothane anaesthesia, and the rat was tested 5 to 7 days after surgery. Thermal hyperalgesia testing was performed as described by Hargreaves *et al.* (1987). Unilateral hindpaw inflammation was induced by a subcutaneous injection of 2µg CARRA in 100 µl saline into the plantar surface following determination of baseline paw withdrawal latencies (PWL). Drugs were administered three hours post-CARRA, a time by which thermal hyperalgesia was evident. PWLs were measured repeatedly at 13, 30, 60, 120, 180, 240 min post-drug. To evaluate drug effect, the "area under curve" values (AUC) from 0 to 60 min was calculated according to the formula AUC = (PWL_{13}-PWL_0) + (PWL_{30}-PWL_0) + (PWL_{60}-PWL_0). Statistical significance against control group (P<0.05, denoted *) was established by analysis of variance followed by the Sheffe post-hoc test.

Table 1 shows AUC values for N^6-cyclohexyladenosine (CHA, A_1 selective), 2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS-21680, A_2 selective), 5'-amino-5'-deoxyadenosine (NH_2dAdo), iodotubercidin (ITU) (both AKIs), and deoxycoformycin (dCF, an ADI). Antinociception produced by 300 nmol NH_2dAdo (inflamed: 10.2±2.1) was antagonized by 242 nmol of the A_1-selective antagonist 8-cyclopentyl-1,3-dimethylxanthine (2.5±1.6; P<0.05). 100 nmol dCF, inactive by itself, greatly enhanced the analgesic effects of 10 and 30 nmol NH_2dAdo in both paws when co-administered (Table 1).

We conclude that (a) both directly and indirectly acting adenosine agonists, except for dCF, produce spinal antinociception in the current model, with rank orders of potency: CHA>CGS21680 (direct); ITU>NH_2dAdo (indirect); and (b) dCF and NH_2dAdo act synergistically to produce spinal analgesia, probably through enhanced release of endogenous adenosine (Golembiowska *et al.*, 1996).

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Table 1

		Dose (nmol) 0.03	0.1	1	10	100	300
CHA	I	11.9±2.2*	15.3±1.4*	42.4±3.6*	-	-	-
	C	3.5±2.36	10.9±0.9*	22.1±5.4*	-	-	-
CGS21680	I	-	-0.7±1.2	0.1±0.4	18.4±4.2*	-	-
	C	-	1.4±1.2	1.2±1.4	4.9±1.7	-	-
NH_2dAdo	I	-	-	-	3.8±1.0	11.4±2.2*	17.8±1.3*
	C	-	-	-	1.9±0.9	6.9±1.7	14.5±1.3*
ITU	I	-	1.8±1.5	6.7±2.9	9.5±2.9*	33.0±2.4*	-
	C	-	0.5±1.5	3.6±1.7	6.5±1.5	8.1±1.6	-
dCF	I	-	-	-	-	2.4±0.8	1.5±1.0
	C	-	-	-	-	0.9±0.7	2.6±1.5

100 nmol dCF+10 nmol NH_2dAdo I : 22.3±2.5* C : 10.4±1.2*
100 nmol dCF+30 nmol NH_2dAdo I : 37.2±3.9* C : 18.7±4.5*
I=inflamed paw C=contralateral paw n=3-13 per group

205P THE EFFECTS OF TACRINE AND THE IMIDAZOLINE 2 SITE LIGAND BU224 IN THE RAT DELAYED T MAZE

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The α_2 adrenoceptor antagonist / imidazoline 2 (I_2) site ligand idazoxan facilitates performance in animal models of learning and memory (Sara, 1991). Idazoxan and the selective I_2 site ligand 2BFI (2-(2-benzofuranyl)-2-imidazoline) improve passive avoidance in rats (Dickinson *et al.*, 1989; Mirza *et al.*, 1996) suggesting that I_2 sites may be involved in cognition. BU224 (2-(4,5-dihydroimidaz-2-yl)-quinoline) exhibits a high affinity and selectivity for I_2 sites over α_2 adrenoceptors in rat brain (Lione *et al.*, 1996) thus providing an opportunity to further explore possible cognitive functions for these sites. This present study assesses the effects of the anticholinesterase, tacrine (THA) and BU224 in the delayed rat T-maze.

The delayed T maze reinforced alternation procedure was used to measure short term spatial memory in Male Hooded-Lister rats (350-400g, Charles River, U.K.). Animals were trained to perform this task to criterion (>85% correct) on a no-delay schedule. A time-course study was then carried out to demonstrate a delay-dependent effect on performance. Trained rats (n=14) received either BU224 (1 or 3mgkg^{-1}), tacrine (THA; 1mgkg^{-1}) or saline (1mlkg^{-1}) i.p. BU224 was administered 30min prior to task and tacrine was administered 20min prior to task in a balanced cross over design, with 24 hours between consecutive treatments. Performance of the animals was assessed in terms of percentage correct responses and number of trials to criterion (10 correct trials). Data were analysed by analysis of variance.

The time course study revealed a 40sec delay between trials induced a maximal deficit in performance. Analysis of variance showed that there was a significant effect of 40sec delay on performance in this drug study (Figs. 1 & 2). THA was able to attenuate the delay induced deficit in performance on the trials to criterion measurement, although the attenuation of % correct trials did not reach significance at the 5% level (Figs.1 & 2). By contrast 1 and 3 mgkg^{-1} BU224 did not significantly reverse the delay induced deficit on either measure (Figs. 1 & 2).

These results indicate that THA can facilitate cognition in the rat T maze and suggest that trials to criteria may provide a more sensitive measure than % correct trials in agreement with Davey *et al.* (1995). The lack of effect of BU224 suggests that I_2 sites play no role in this paradigm, although whether BU224 is an I_2 site agonist or antagonist is still unknown.

Fig. 1

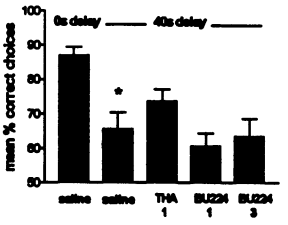
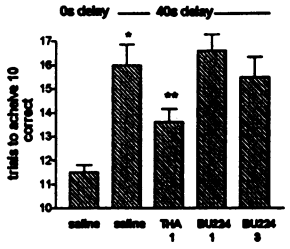


Fig. 2



* one way ANOVA [$F_{1,26}$=16.6, p<0.001] ** one way ANOVA [$F_{1,25}$=5.18, p<0.05]

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206P INTRA-ACCUMBENS DOPAMINE INFUSION INDUCES BEHAVIOURAL SENSITIVITY TO 7-OH-DPAT IN THE RAT

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Chronic intraaccumbens (IACB) dopamine infusion has been shown to increase behavioural sensitivity to subsequent acute challenge with dopamine agonists in the rat (Costall *et al.*, 1984). The dopamine D_3 receptor has been localised in limbic areas of the rat brain (Landwehrmeyer *et al.*, 1993) and implicated in the behavioural effects of the putative D_3/D_2 receptor agonist 7-OH-DPAT (Damsa *et al.*, 1993). The present studies investigated the effects of IACB dopamine infusion on sensitivity to acute challenge with IACB 7-OH-DPAT.

Sprague-Dawley rats (female 250±10g, n=7 per group) were subject to standard stereotaxic surgery under pentobarbitone anaesthesia (60mg/kg i.p.) for implantation of chronically indwelling guide cannulae into the nucleus accumbens. Following a 14 day recovery period rats received bilateral IACB infusions (infusion rate 12µl/24h per hemisphere) of vehicle (0.1% sodium metabisulphite) or dopamine (50µg/24h) for 12 days. Locomotor activity was assessed daily for 60 min (08.00-09.00 h) in photocell cages; data are shown as mean±s.e.m activity counts/ day over the 12 day infusion period. Infusions were withdrawn 14 days before commencement of the acute studies. Acute bilateral IACB injections (1µl/hemisphere) of vehicle or 7-OH-DPAT 0.5-10 µg to rats withdrawn from infusion or to non-infused rats were followed by locomotor assessment for 80 min; data are shown as mean±s.e.m total activity counts/80 min. Treatment with vehicle or (-) sulpiride (10 mg/kg) IP was given 45 min prior to IACB 7-OH-DPAT/vehicle. Data were analysed by ANOVA with Dunnett's t-test.

Dopamine infusion IACB induced a significant hyperactivity response in the rat (275±27 counts/day; p<0.001 compared to vehicle infusion IACB 82±5 counts/day). Subsequent acute IACB injection of 7-OH-DPAT 0.5-2.5 µg significantly reduced locomotor activity in dopamine-infused rats (168±19-276±14 counts/80 min; p<0.001-0.05 compared to vehicle IACB 399±46 counts/80 min); the response to 7-OH-DPAT 0.5 µg was not antagonised by (-)sulpiride (196±36 counts/80 min; p<0.001). 7-OH-DPAT 10 µg IACB caused a significant hyperactivity response (837±72 counts/80 min; p<0.001) which was antagonised by (-)sulpiride (414±76 counts/80 min; p<0.001). In vehicle-infused rats 7-OH-DPAT 1.0-2.5 µg IACB significantly reduced locomotor activity (144±13-150±32 counts/80 min; p<0.001 compared to vehicle IACB 311±43 counts/80 min); this response was not antagonised by (-)sulpiride. 7-OH-DPAT 0.5 µg and 10 µg IACB (343±55 and 307±24 counts/80 min) had no effect on locomotor activity (compared to vehicle IACB 311±43 counts/80 min). No significant difference in response to 7-OH-DPAT IACB was found for vehicle-infused compared to non-infused rats. At the dose used (-)sulpiride alone had no effect on locomotor activity.

The results indicate that IACB dopamine infusion increases behavioural sensitivity to subsequent acute IACB challenge with the D_3/D_2 receptor agonist 7-OH-DPAT. Antagonism of the hyperactivity response to 7-OH-DPAT by sulpiride may indicate an enhanced agonist action of 7-OH-DPAT at D_2 receptors. The failure of sulpiride to antagonise the increased sensitivity to the locomotor inhibitory effects of 7-OH-DPAT suggests a potentiated agonist action at D_3 receptors. Supersensitivity of mesolimbic D_3 receptors secondary to dopaminergic excess may result in behavioural inhibition; this may be relevant to the study of the negative symptomology of schizophrenia.

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207P 8-OH-DPAT ENHANCES THE EXPRESSION OF COCAINE-INDUCED PLACE PREFERENCE IN THE MOUSE

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The reinforcing properties of cocaine have been demonstrated using a number of paradigms and are believed to be mediated via the dopaminergic system (Roberts & Ronaldi, 1995). However, 5-hydroxytryptamine (5HT) may also have a role in cocaine reward since 8-OH-DPAT has recently been shown to decrease the response rates for cocaine self-administration in the rat (Peltier & Schenk, 1993). The aim of the present investigation was to examine the effects of 8-OH-DPAT on the expression of a conditioned place preference (CPP) response to cocaine as this may be reflective of the self-administration procedure since the drug of interest is not administered until after conditioning has been achieved (Cunningham *et al*, 1992).

Male adult BKW mice (32-50g) were used and cocaine CPP was assessed in a 3 chambered apparatus. Baseline preferences were determined by allowing each mouse access to the apparatus on 3 separate occasions. The time spent in the outer two chambers was recorded for each 15 min session, and the mean of the 3 sessions was taken as the pre-conditioning time (s±sem). During the conditioning phase mice were treated with cocaine (5.0 mg/kg, s.c.) or vehicle (0.9% saline) and confined to one of the two outer chambers for 30 min. On alternate days the mice received the other treatment and were confined to the opposing chamber such that each mouse received 4 cocaine and 4 saline conditionings. 24 h later each group (n=8) received one of three doses of 8-OH-DPAT (0.125, 0.25 or 0.5 mg/kg, s.c.) or vehicle (0.9% saline) 15 min prior to being tested for changes in preference behaviour. Data were analysed by 1 and 2 way ANOVA followed by post-hoc t-test analysis.

Cocaine conditioning produced a significant ($F(2,14)=8.1$, $p < 0.05$) increase in the time spent in the cocaine paired chamber from 265.4 ± 16.8 s to 357.2 ± 26.6 s. Saline pretreatment had no effect on the response. 8-OH-DPAT (0.125 and 0.5 mg/kg) significantly ($F(3,21)=4.0$, $p < 0.05$ and $p < 0.01$, respectively) enhanced the time spent in the cocaine paired side from 287.4 ± 9.9 s to 569.1 ± 69.1 s and 253.9 ± 14.3 s to 604.0 ± 63.0 s, respectively when compared with the vehicle control (Figure 1).

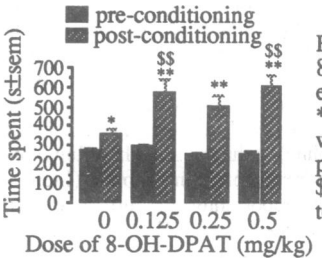

Figure 1. The effect of 8-OH-DPAT on the expression of cocaine CPP. *p < 0.05, **p < 0.01 when compared to the pre-conditioning preference. $$p < 0.01, when compared to vehicle control.

The demonstration of an enhancement in cocaine induced preference behaviour by 8-OH-DPAT is contradictory to the findings of Peltier & Schenk (1993) suggesting that the present procedure may not reflect the self-administration model as previously suggested. However, these data do support the findings of Montgomery *et al* (1991) who demonstrated that low doses of 8-OH-DPAT enhanced responding for brain self-stimulation in the rat and suggested that this may be due to facilitation of dopaminergic neurotransmission by 5HT to promote motivational arousal.

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208P SITE OF ANTI-CATALEPTIC ACTION OF DIZOCILPINE IN THE RAT

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Side effects of neuroleptics include parkinson-like symptoms of akinesia and rigidity which are relieved by the NMDA receptor antagonist dizocilpine (Moore et al., 1993). The present study investigates the site of dizocilpine's anticataleptic activity in the brain, and addresses the hypothesis that this involves blockade of glutamatergic transmission in the corpus striatum (CS) and/or striatal output pathways.

Catalepsy was induced in male Wistar rats (160-300g; n≥6 per group) with systemic (1 mg/kg i. p.) or intrastriatal (7 µg/0.5 µl) haloperidol, or muscimol injected into the globus pallidus (GP; 25 ng/0.5 µl) or ventromedial thalamus (VMT; 50 ng/0.5 µl). Intracerebral injections were made acutely under halothan anaesthesia. Catalepsy was assessed by the horizontal bar test (8 cm elevation), by measuring descent latencies (DL; maximum 360 sec). The t values given represent the time after injection of the catalepsy-inducing agent. Analysis was by ANOVA and Tukey's post hoc test.

Catalepsy induced by systemic haloperidol was inhibited by dizocilpine (0.2 mg/kg i.p.) injected 10 min before (DL 360±0 vs 31.0±22.3 at t=30 min, p<0.001) but not 45 min after the neuroleptic (DL 360±0 vs 341.5±18.5). Thus dizocilpine *prevents* but does not *reverse* haloperidol-induced rigidity by the systemic route. Systemic dizocilpine also reversed catalepsy elicited by haloperidol deposited in the CS (DL 360±0 vs 61.3±29.1 at t=120 min, p<0.001), but not that evoked with muscimol from the GP (DL 346±14 vs 321±39 at t=75 min) or VMT (DL 359.2±0.8 vs 360±0 at t=75 min). These results suggest dizocilpine disrupts the catalepsy upstream of the GP and VMT, probably at the level of the striatum, in line with earlier reports (Yoshida *et al.*, 1994).

To further establish the contribution of glutamate transmission in the forebrain and subthalamic efferents to haloperidol-induced muscular rigidity, a further set of experiments was performed, in which rats were first anaesthetised with halothane and injected with dizocilpine (in 0.5 µl) in the CS, nucleus accumbens (NAc), entopeduncular nucleus (EPN) or substantia nigra pars reticulata (SNr). Haloperidol (1 mg/kg i.p.) was then administered after 45 min. Catalepsy was attenuated by the NMDA antagonist placed in the NAc (DL control 360±0 vs 13.7±3.7 at t=15 min, p<0.001), EPN (DL 360±0 vs 33.7±31.3, p<0.001), SNr (DL 360±0 vs 13.2±6.4, p<0.001) and the CS (DL 360±0 vs 10.0±2.4, p<0.001). Misplaced injections of dizocilpine and control injections of saline were ineffective. Minimum effective doses of dizocilpine were lower in the SNr and EPN (1 µg and 5µg respectively), and had a shorter duration of action (15-30 min) compared to the NAc and CS (10 µg, duration 45-90 min). These data support the notion that blockade of dopamine D_2 receptors by haloperidol in the CS leads to muscular rigidity through overactivity of glutamatergic synapses both in the CS itself, and in the striato-pallidal-subthalamo EPN/SNr output circuit, which can be reversed by focal blockade of NMDA receptors in these structures (Gerfen, 1992).

H. Özer is supported by the British Council.

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209P COMPARISON OF CONTINUOUS *VS* INTERMITTENT NICOTINE ADMINISTRATION ON THE RESPONSIVENESS OF THE TERMINAL REGIONS OF ASCENDING DOPAMINE PATHWAYS TO A SUBSEQUENT LOCAL NICOTINE CHALLENGE *IN VIVO*

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Daily nicotine injections (0.4mg/kg/day s.c) can ameliorate the release of dopamine from mesolimbic neurones produced by a subsequent nicotine injection (Benwell and Balfour, 1992). This study did not distinguish between an effect of nicotine on cell body or terminal region nicotinic acetylcholine receptors (AChRs). Given that nicotinic AChRs in the terminal regions of neurones are thought to play an important role in the central actions of nicotine (McGehee *et al.*, 1995), we have compared the effect of two chronic nicotine regimes on the responsiveness of nicotinic AChRs in the terminal regions of the rat mesolimbic, mesocortical and nigrostriatal pathways to a local application of nicotine using *in vivo* microdialysis.

On day one, Alzet osmotic pumps containing nicotine (0.4mg/kg/day) or saline control, were implanted subcutaneously into male Sprague-Dawley rats (250-350g). On day seven, the pumps were removed and dialysis probes implanted into the striatum, accumbens or cortex. The following day the effect of a local application of nicotine (delivered to the terminals via the dialysis probe) on dopamine levels was determined as previously described (Marshall *et al*, 1996). A challenge dose of $3x10^{-3}$M nicotine was used for the striatum and accumbens, and 10^{-2}M nicotine for the cortex, administered over 15 min. Another group of rats received daily subcutaneous injections of nicotine (0.4mg/kg/day) or saline for seven days and were then subjected to in vivo microdialysis as described above. Differences between nicotine and saline control responses were compared using two way ANOVA for repeated measures. *=$p<0.05$.

Table 1: Effect of Nicotine Administration on Responsiveness of Dopaminergic Terminals to Local Nicotine Challange

	Injections		Pumps	
	Saline	Nicotine	Saline	Nicotine
Striatum	372±122%	1370±317%*	397±173%	264±73%
Accumbens	368±128%	663±199%	349±137%	351±151%
Cortex	173±32%	283±102%	211±47%	199±72%

Data shown are peak dopamine levels (expressed as % of basal, mean ± s.e.m.) after local nicotine challange, n=3-8.

Thus, daily nicotine injections produce a 4 fold increase in the overflow of dopamine in the striatum in response to a subsequent nicotine challange administered locally. This is similar to the results obtained by Benwell and Balfour (1992) in the mesolimbic system following a systemic nicotine challange. This suggests that intermittent exposure to peak nicotine sensitises nicotinic AChRs in the striatum whereas steady state, trough levels, produced by chronic infusion, do not.

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210P LACK OF EFFECT OF THE DOPAMINE D_4 RECEPTOR ANTAGONIST, L-745,870 ON AMPHETAMINE-INDUCED BEHAVIOURS IN RODENTS

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It is now well established that drug (e.g. amphetamine)-induced activation of mesolimbic dopamine systems increases locomotor activity in rodents whereas activation of nigrostriatal dopamine systems induces stereotyped behaviours. Furthermore, whilst classical neuroleptics (e.g. haloperidol) block both responses, the atypical neuroleptic clozapine selectively blocks amphetamine induced hyperactivity in the rat. Recent studies have now shown that clozapine has high affinity for the human dopamine D_4 receptor suggesting that this receptor subtype may contribute towards the 'atypical' neuroleptic-like profile seen in behavioural tests (Van Tol *et al.*, 1991). To examine this possibility, we have determined whether the selective dopamine D_4 receptor antagonist L-745,870 (3-{[4-(4-chlorophenyl) piperazin-1-yl]methyl}-1*H*-pyrrolo[2,3b]pyridine; Patel *et al.*, this meeting) blocks amphetamine-induced behaviours in rodents.

Male BKTO mice (20-30 g; n = 8-11/group) were habituated to individual photocell activity cages for 2h and then orally dosed with L-745,870 (870; 0.01 - 1 mg/kg), haloperidol (0.0625 - 1 mg/kg), clozapine (0.03 - 10 mg/kg) or vehicle (10 ml/kg). Animals received either saline (10 ml/kg, s.c.) or d-amphetamine (amph; 5 mg/kg, s.c.) 10 min (L-745,870) or 30 min later and the number of cage crosses recorded in 10 min intervals for 2h. Stereotyped behaviour was examined in male Sprague Dawley rats (180 - 310 g; n = 8/group) previously habituated to individual observation cages for 30 min prior to oral administration of L-745,870 (0.01 - 1 mg/kg), haloperidol (0.05 - 1 mg/kg), clozapine (30 - 100 mg/kg) or vehicle (3 ml/kg). All animals received apomorphine (1 mg/kg, s.c.) 20 min (L-745,870) or 60 min later and the time spent sniffing and licking/biting recorded 20-25 min after apomorphine injection. All data were analysed by analysis of variance followed by Dunnett's t test, *$P<0.05$.

D-amphetamine (5 mg/kg, s.c.) induced a marked increase in the number of cage crosses recorded in the mouse (mean no. cage crosses (2h) ± s.e. mean from 3 separate experiments: vehicle = 32 ± 22; amph = 898 ± 143*). Pretreatment with haloperidol or clozapine dose-dependently antagonised this response (ED_{50} = 0.14 ± 0.013 and 3.13 ± 0.63 mg/kg, p.o. respectively) whilst oral administration of L-745,870 failed to block amphetamine-induced hyperactivity (mean no. cage crosses (2h) ± s.e. mean: vehicle/vehicle = 76 ± 15*; vehicle/amph = 1136 ± 196; 0.01 mg/kg 870/amph = 1114 ± 144; 0.03 mg/kg 870/amph = 1208 ± 155; 0.1 mg/kg 870/amph = 1209 ± 142; 0.3 mg/kg 870/amph = 1076 ± 165; 1 mg/kg 870/amph = 928 ± 166; *$P<0.05$ compared to vehicle/amph). Pretreatment with haloperidol also dose-dependently and significantly attenuated apomorphine-induced stereotypy in the rat (ED_{50} = 0.37 ± 0.08 mg/kg, p.o). In contrast, only a modest, 37% reduction was observed following high doses of clozapine (mean duration (sniffing + licking/biting) ± s.e. mean: vehicle = 530 ± 34 sec; 100 mg/kg = 334 ± 76 sec*) whilst L-745,870 failed to alter this response (vehicle = 543 ± 16 sec; 0.01 mg/kg = 527 ± 10 sec; 0.1 mg/kg = 527 ± 20 sec; 1 mg/kg = 557 ± 37 sec).

In conclusion, the potent, selective dopamine D_4 receptor antagonist, L-745,870, failed to attenuate amphetamine-induced behaviours in rodents. Thus it seems unlikely that the blockade of amphetamine-induced hyperactivity seen with clozapine is mediated through dopamine D_4 receptor antagonism.

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211P THE EFFECT OF ALPRAZOLAM ON 20 kHz ULTRASOUND-INDUCED DEFENCE BEHAVIOUR

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Ultrasonic vocal calls between 20-32kHz are emitted as part of the rats defence response to aversive situations (Blanchard *et al.* 1991). Recent studies have demonstrated that artificially generated 20kHz ultrasound, can induce defence-like behaviour in rats. This behaviour is associated with activation of nuclei that comprise the brain aversive system (Aspley *et al.* 1995). Furthermore, the response can be enhanced by anxiogenic and attenuated by anxiolytic drugs (Beckett *et al.* 1995a, 1996). The present study examines the effect of the anti-panic benzodiazepine, alprazolam, on ultrasound induced behaviour.

Male Lister hooded rats (250-300g) pretreated (30min i.p.) with vehicle (50:50 mixture of PEG and saline) or alprazolam (0.5 and 2 mg/kg), were placed in an open field arena (75cm diameter) with a wall mounted piezoelectric speaker. After a 2 min basal period they were exposed to a 1 min 20kHz square wave ultrasound tone (65, 72 and 75 dB intensity, randomised) followed by a further 2 min without sound. This procedure was repeated for each intensity with a 1 min inter-procedure interval. Locomotor behaviours were recorded and analysed via a computer tracking system (Beckett *et al.* 1995b) as distance travelled and speed.

Exposure to the ultrasound tone produced intensity related locomotor behaviour similar to that previously reported (Beckett *et al.* 1995a, 1996). Treatment with 0.5 and 2.0 mg/kg alprazolam produced a significant reduction in maximum speed values (Fig 1). This was most marked at the 75 dB stimulus intensity. Pre-stimulus values revealed no effects on basal locomotor activity. Value obtained for average speed and distance travelled showed similar changes. Data not shown.

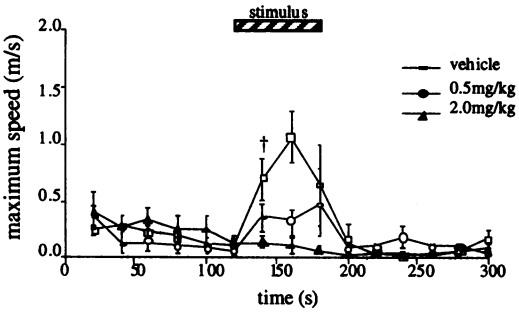

Figure 1. Effect of 30 min pretreatment with alprazolam (0.5 and 2.0 mg/kg i. p.) on maximum locomotor speed per 20 second time bin in response to a 20kHz ultrasound stimulus (75dB) (mean ± s.e.m.) $p<0.05$ vs vehicle (*0.5mg/kg, †2.0mg/kg, n=7-9). One way ANOVA, post-hoc Duncans NMR.

These results demonstrate that ultrasound induced defence-like behaviour can be attenuated by the benzodiazepine alprazolam at doses which do not effect basal locomotor activity. This is consistent with the known anti-panic properties of alprazolam in humans.

This work was supported by the Medical Research Council

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212P ELECTROPHYSIOLOGICAL ACTIVITY IN THE HIPPOCAMPUS IS REGULATED BY MINERALOCORTICOID RECEPTORS IN URETHANE-ANAESTHETIZED RATS

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Hippocampal theta activity is a rhythmic, sinusoidal waveform with a frequency range of 3-10 Hz that occurs in alert, immobile rats presented with threatening stimuli (Bland, 1986). This type of theta is abolished by cholinergic receptor antagonists and is reliably modelled using urethane-anaesthetized rats (Smythe *et al.*, 1992). Recently, we reported that cholinergic blockade increased corticosterone (CORT) secretion elicited by acute stress (Bhatnagar *et al.*, 1994). In the present study we sought to ascertain whether or not CORT modulates cholinergic theta activity.

Adult male, Lister hooded rats weighing between 350-500 g, served as subjects (n=10). Rats were anaesthetized with isofluorane and implanted with jugular catheters. They were then switched to urethane (0.8 g/ml) anaesthesia and placed in a stereotaxic frame. A theta recording electrode was placed in the stratum moleculare of the hippocampus (A-P -3.3, M-L 2.5, D-V 2.7 mm). An indifferent electrode was placed in the anterior cortex, and a bipolar stimulating electrode was positioned in the dorso-medial posterior hypothalamus (DMPH) to activate theta. Baseline recordings of spontaneous and DMPH-stimulated activity were obtained. Rats were then administered the mineralocorticoid receptor (MR) antagonist spironolactone (50 mg/kg IP) and basal and DMPH-stimulated activities were monitored for 30 min. Changes in theta frequency (Hz) were analyzed using ANOVA followed by a Bonferroni corrected t-test, and by simple linear regression.

ANOVA revealed a main effect of DMPH stimulation intensity on theta frequency with $F(3,40)=55.9$, $P<.001$. Figure 1 shows that theta frequencies increased in response to tail pinch and with increasing stimulation intensities prior to drug administration. ANOVA also demonstrated a significant effect of time post spironolactone with $F(3,40)=3.32$, $P<.03$. Spontaneous theta was absent by 5 min following spironolactone. Overall, maximal theta frequencies elicited by DMPH stimulation declined with the passage of time post injection. A regression analysis on the 0.1 mA level looking at the time x frequency relationship revealed a significant negative correlation $F(1,12)=8.3$, $P<.02$ ($r= -0.64$).

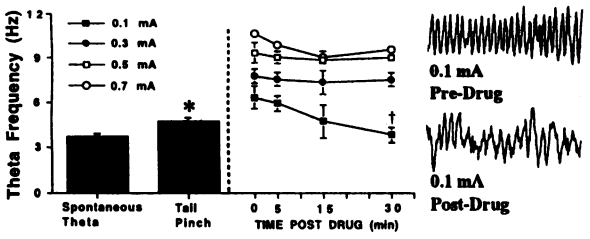

Figure 1. Hippocampal theta frequencies under basal and stimulated conditions (Means ±SEM). Left panel shows that tail pinch elevated frequencies compared to basal values (*$P<.05$). The middle panel shows stimulation levels and frequency at various times following spironolactone injection (†$P<.05$ compared to 0 time point). Right panel shows examples of analogue data.

Hippocampal cholinergic theta activity is modulated by CORT acting through MR. It is possible that CORT alters anaesthetic levels to affect basal but not stimulated theta responses. In conclusion, the present findings support the contention that hippocampal cholinergic systems are targeted by CORT.

Bhatnagar, S. *et al.* (1994) *Neurosci. Abs.* 20, 935.
Bland, B.H. (1986) *Prog. Neurobiol.* 54, 1-54.
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213P EFFECTS OF INTRAHIPPOCAMPAL SPIRONOLACTONE AND RU38486 ON ANXIETY-LIKE BEHAVIOUR (ALB) IN RATS: TEST SPECIFICITY

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Corticosterone (CORT) modulates anxiety-like behaviour (ALB) in rats (Smythe *et al.*, in press), but the brain region and mechanism remains unknown. The hippocampus has the highest density of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) where CORT is known to act (Reul and De Kloet, 1985). In the present study we investigated the effects of intrahippocampal infusions of MR and GR antagonists on ALB as measured by the black-white box and social interaction tests.

Adult male, Lister hooded rats (350-500 g) were implanted bilaterally with hippocampal cannulae (A-P -3.3; M-L ±2.5; D-V -2.3 mm) under pentobarbital anaesthesia (60 mg/kg IP), 3 weeks prior to testing. On the test day, rats were infused with 3µl vehicle (VEH; artificial CSF with 5% ethanol), 150 ng of spironolactone (MR antagonist), or 150 ng RU38486 (GR) antagonist), either 10 min or 3 hr prior to being placed into the white chamber of the Black-White box (n=9-11/group). Rats were scored for time spent in the white chamber and intercompartmental crosses. Because of the results following spironolactone given at 10 min prior to testing a separate group of rats was injected with VEH or spironolactone (n=10-12) and tested in a social interaction test using these parameters. Pairs of rats, both injected with VEH or spironolactone, were placed into an open field and scored for time spent engaged in interactive behaviours (sniffing, following, physical contact). Data were assessed by ANOVA and *post hoc* testing was performed using a Bonferroni corrected t-test.

ANOVA on the black-white box data revealed a significant drug effect on time spent in the white chamber at 10 min F(2,28)=3.5, P<.04, but not at 3 hr. As shown in Fig. 1, spironolactone significantly increased time in the white area (P<.05). ANOVA on crossing behaviour similarly showed a drug effect F(2,28)=5.4, P<.01, at 10 min where spironolactone increased crossing behaviour (P<.01), and an effect at 3 hr F(2,25)=3.7, P<.04, where spironolactone decreased crossing behaviour (P<.01). ANOVA revealed no effect of spironolactone on social interaction (Fig. 2).

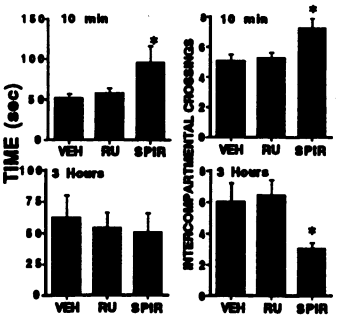

Fig. 1. (at right) Left panel shows time spent in white, while right panel shows crossing behaviour. Means ±SEM are shown. *significantly different from control group (P's<.01-.05).

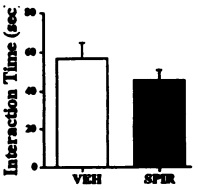

Fig. 2.(at left) Social interaction following spironolactone. Means ± SEM are shown.

These data show that CORT affects ALB via hippocampal MR, but not GR, on some tests. In conclusion, CORT can modify ALB by acting directly on hippocampal steroid receptors.

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Smythe, J.W. et al. (in press) *Br. J. Pharmacol.*

214P DOPAMINE D2, BUT NOT D1, RECEPTORS MODULATE ANXIETY-LIKE BEHAVIOUR (ALB) IN RATS TESTED IN THE BLACK-WHITE BOX

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Previous research has demonstrated that the cholinergic antagonist scopolamine (SCOP) increases anxiety-like behaviour (ALB) in rats (Smythe *et al*, in press) and that intrahippocampal SCOP administration increases corticosterone secretion elicited by stress (Bhatnagar et al., 1994). Dopamine (DA) systems are known to modulate hippocampal cholinergic function (Day and Fibiger, 1994) and we hypothesized that DA receptor antagonists might similarly affect ALB as assessed by the Black-White box.

Adult male, Lister hooded rats were injected with either the D1 antagonist SCH23390 (0, 0.1 or 0.25 mg/kg IP) or the D2 antagonist raclopride (0, 0.05, or 0.10 mg/kg IP) 20 min prior to being placed into the white chamber of the Black-White box (n=10/group). Rats were videotaped and the tapes were scored for latency to exit the white chamber, latency to re-enter the white chamber, and time spent in the white chamber. Data were assessed by ANOVA and *post hoc* testing was performed using a Bonferroni corrected t-test.

ANOVA revealed no effect of the D1 antagonist SCH23390 on any behavioural measure. However, the D2 antagonist raclopride significantly affected time to exit the white area F(2,27)=6.1, P<.007, and pairwise comparisons showed that drug-treated rats left the white area sooner than control rats (P's< .01). These data are shown in Fig. 1A. ANOVA also revealed an effect of raclopride on times to re-enter the white area F(2,27)=4.6, P<.02. Raclopride-treated rats exhibited delayed re-entry times compared to control rats (P's< .01). These data are illustrated in Fig. 1B. Fig. 1C shows the data for total time spent in the white chamber. ANOVA again demonstrated a significant effect of drug treatment F(2, 27)=5.5, P<.01. *Post hoc* comparisons showed that raclopride significantly decreased time spent in the white chamber (P's<.05). Motor behaviour (line crossings) was unaffected by these agents at these doses in the black F(2,27)=1.3, ns, and white F(2,27)=0.9, ns, chambers.

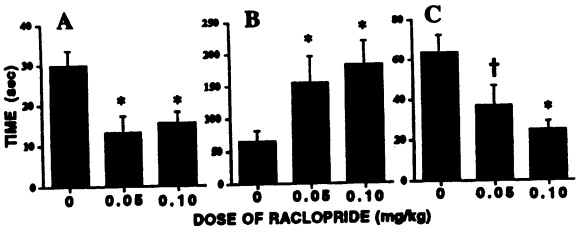

Fig. 1. Means and SEMs for the data obtained. Panel A shows time to exit the white arena, panel B shows time to re-enter the white chamber and panel C shows total time spent in the white compartment. *significantly different from control group (P<.01) and †(P<.05).

Day and Fibiger (1994) showed that D2 antagonists decreased hippocampal acetylcholine release *in vivo* and together with our previous findings that SCOP increases ALB (Smythe et al., in press), suggests that D2 antagonists increase ALB by inhibiting hippocampal cholinergic function. In conclusion, we have shown that DA can modulate ALB in rats via D2 but not D1 receptors, perhaps via actions on cholinergic systems, although we have no empirical support for this contention.

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Day, J.C.and Fibiger,H.C. (1994) *J. Neurochem.* 63, 2086-2092.
Smythe, J. W. *et al.* (in press) *Pharmacol. Biochem. Behav.*

215P LACK OF EFFECT OF THE CCK B ANTAGONIST CI-988 ON BENZODIAZEPINE WITHDRAWAL-INDUCED WEIGHT LOSS

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CCK B antagonists alleviate some benzodiazepine (BZ) withdrawal signs in rodents (Hughes et al., 1990; Singh et al., 1992). However, we found no effect of L-365-260 on BZ withdrawal-induced hypophagia (Goudie and Leathley, 1995). We have assessed the actions of CI-988 on BZ withdrawal-induced weight loss. The experiment (Table 1) involved 6 groups (ns=10) treated chronically (i.p.) with saline (S) or chlordiazepoxide (C). S or C (10 mg/kg escalated to 30 mg/kg at 2 mg/kg/day) was administered b.i.d. for 21 days to female Wistar rats (208-320g) maintained at 21 degrees. During a withdrawal phase of the study up to day 28, groups were treated b.i.d. with saline (S) or CI-988 (CI) at 4 doses (0.01, 0.1, 1, or 10 mg/kg).

During chronic treatment the overall mean body weight of the 5 groups treated with C rose from 303 - 330 g, while that of the group treated with S rose from 306 - 328 g. ANOVA [$F<1$] revealed that C treatment did not increase weight. As groups differed at the end of chronic treatment, to assess withdrawal weights were calculated as percentages of those recorded on day 22 (a.m. - i.e. prior to withdrawal). Planned pairwise ANOVAs (2 groups x 6 days) revealed that controls (S-S) differed from group C-S [$F=16.94$), $p<0.001$]. Thus BZ withdrawal induced weight loss. Comparisons of group C-S with the C-CI groups allowed evaluation of the effect of CI-988 on withdrawal. None of these groups differed from C-S [largest $F=1.47$ for group C-CI 1, $p>0.24$]. Thus CI-988 had no effect on withdrawal. The failure of CI-988 to alleviate weight loss accords with our earlier negative results with L-365,260. Given positive effects reported for other BZ withdrawal signs with CCK B antagonists it would appear that BZ withdrawal is mediated by a number of independent mechanisms. (Goudie and Leathley 1995).

Acknowledgements:- Supported by Parke-Davis Neuroscience Research Centre.

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Hughes, J., Boden., P., Costall, B. *et al.* (1990) *Proc. Nat. Acad .Sci. U.S.*A. 87, 6728-6732.

Singh, L., Field, M.J., Vass, V.A. *et al.* (1992) *Br. J. Pharmacol.* 105, 8-10.

Table 1. Details of experimental design and group mean (s.e.) weights (pre-withdrawal percentages)

Gp Day	23	24	25	26	27	28
S-S	100.4 (0.3)	100.4 (0.4)	100.8 (0.2)	100.9 (0.2)	101.5 (0.2)	101.7 (0.3)
C-S	94.4 (0.5)	95.5 (0.9)	95.4 (1.3)	95.7 (1.3)	95.8 (1.7)	95.7 (2.1)
C-CI 0.01	95.5 (0.4)	95.8 (0.7)	94.7 (0.7)	93.7 (0.5)	95.2 (0.9)	95.8 (0.8)
C-CI 0.1	95.4 (0.5)	94.5 (0.4)	94.1 (0.6)	94.6 (0.5)	95.2 (0.7)	95.6 (1.0)
C-CI 1	96.7 (0.5)	94.9 (0.8)	93.8 (1.3)	92.8 (1.7)	92.6 (1.5)	92.0 (1.8)
C-CI 10	96.3 (0.6)	95.2 (0.7)	94.9 (0.8)	94.8 (0.9)	94.7 (0.8)	94.2 (0.8)

216P EFFECT OF *RHAZYA STRICTA* EXTRACT ON FORCED SWIMMING TEST IN RATS

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Immobility induced by forced swimming is well known as an animal model of depression (Willner, 1990). Using this paradigm, we have, in the present work, tested the possibility that the medicinal plant *Rhazya Stricta*, which has previously been found to have a sedative (Ali *et al*, 1995) and variable effects on the activity of the endogenous monoamine oxidase inhibitor tribulin (Ali *et al*, in preparation) may have an antidepressant-like action. Rats were pretreated with various doses (0.1-6.4 $g.kg^{-1}$) of the lyophilized extract of plant leaves (which was subjected to phytochemical finger printing to ensure uniformity of used material), or with desipramine (10, 20 and 40 $mg.kg^{-1}$) or buspirone (0.06 and 0.12 $mg.kg^{-1}$) and were then subjected to forced swimming test.

The results (Table 1) indicate that the plant extract produced a biphasic (bell-shaped) effect on the immobility time. The lower doses (0.1, 0.2 and 0.4 $g.k^{-1}$) elicited a highly significant and inversely dose-dependent decrease in immobility time, and the higher doses (0.8, 1.6 and 6.4 $g.kg^{-1}$) showed a dose-dependent decrease in immobility time. Statistical analysis was carried out using one-way analysis of variance followed by Scheff's and Dunnett's test.

Desipramine (20 and 40 $mg.kg^{-1}$) produced dose-dependent significant decreases in immobility time. Buspirone treatment produced statistically insignificant decreases in immobility time amounting to 6-16%. The mechanism of the bimodal action of *R. Stricta* on immobility in the forced swimming test is thus far uncertain and warrants further investigation.

Table 1. Effect of *Rhazya stricta* on immobility time during forced swimming test in rats

Treatment	Immobility time (sec)
Control	135.00 ± 10.33
0.1 $g.kg^{-1}$	0.45 ± 0.03
0.2 $g.kg^{-1}$	21.82 ± 1.75
0.4 $g.kg^{-1}$	93.16 ± 12.19
0.8 $g.kg^{-1}$	9.88 ± 3.50
1.6 $g.kg^{-1}$	0.14 ± 0.11
6.4 $g.kg^{-1}$	0.09 ± 0.05

Values are mean ± s.e.m. (n=7). All values from treated rats were significantly different from the controls.

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Willner, P. (1990). Animal models of depression. *Pharmac. Therap.* 45, 425-455.

217P THE EFFECT OF SEROTONERGIC AGONISTS ON THE DISCRIMINIATIVE STIMULUS CUE PRODUCED BY ETHANOL IN THE RAT

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Previous studies have shown that 5-hydroxytryptamine (5-HT) is involved in mediation of ethanol ingestion (Sellers *et al.*, 1992) and in the discriminative stimulus cue induced by ethanol (Grant, K. A. and Barrett, J. E. 1991). The aim of the present study was to examine the 5-HT receptor subtypes involved in mediation of the ethanol discriminative stimulus cue, using an operant drug discrimination paradigm. The ability of the 5-HT re-uptake inhibitor fluoxetine, the 5-HT_{1A} receptor agonist 8-OH-DPAT, the 5-HT_2 receptor agonist DOI and the 5-HT_{1B} receptor agonist TFMPP to generalise to the ethanol cue were examined. 15 Female Sprague Dawley rats (275-325g) were food deprived to 85% free-feeding body weight. They were subsequently trained to press one of two available levers for delivery of a food pellet, on an FR10 schedule of reinforcement while under the influence of either 0.75g/kg ethanol or saline, according to a previously described protocol (Sanger D. J. 1993). Ethanol or saline was administered (i.p.) 30 min prior to training. Animals were considered to have reached criterion if they correctly selected the appropriate lever for 8 out of 10 consecutive training days. Correct responding was defined as no more than 15 responses being made before delivery of the first pellet, and 90% correct responding over the duration of the session. Following acquisition of criterion, fluoxetine (0.5-10 mg/kg, i.p.), 8-OH-DPAT (0.05-1 mg/kg, s.c.), DOI (0.25-5 mg/kg, s.c.) or TFMPP (0.05-0.5 mg/kg,i.p.) were administered 30 min prior to testing and their effects on lever selection and responding were examined over the 15 min test session. Data were analysed by a one-way ANOVA with Dunnett's t-test.

8-OH-DPAT (0.1-1 mg/kg), DOI (1-5 mg/kg) and TFMPP (0.3-0.5 mg/kg) all showed partial generalisation to the ethanol stimulus cue, while fluoxetine (0.5-10 mg/kg) had no effect. Fluoxetine (5-10 mg/kg), 8-OH-DPAT (0.5-1mg/kg) and DOI (5 mg/kg) all had a significant effect to reduced response rates during the session. (Table 1). In summary, the present results indicate that 5-HT is involved in the discriminative stimulus induced by ethanol. However, a general increase in 5-HT neurotransmission produced with fluoxetine was insufficient to result in ethanol-like responding, whereas activation of the 5-HT system with selective receptor agonists led to partial generalisation to the ethanol cue. The present results suggest that 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors are involved, at least partly, in mediation of the interoceptive effects of ethanol.

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Sanger, D. J. (1993) *Behav. Pharmacol.* 4, 523-528.
Sellers, E. M., Higgins, G. A. and Sobell, M. B. (1992) *TiPS* 13, 69-75

Table 1 The effect of 5-HT agonists on the ethanol discriminative stimulus cue

Fluoxetine (mg/kg)	a	% control rate	8-OH-DPAT (mg/kg)	a	% control rate	TFMPP (mg/kg)	a	% control rate	DOI (mg/kg)	a	% control rate
0	0/12	100	0	0/12	100	0	0/12	100	0	0/12	100
0.5	2/12	98.6±4.3	0.05	4/12	110.4±3.0	0.05	0/12	89.4±8.4	0.25	3/12	111.2±4.0
1.0	3/12	104.3±2.9	0.1	7/12	91.2±10.6	0.1	2/12	97.9±7.6	0.5	2/12	91.8±7.1
5.0	2/12	78.7±3.9 **	0.5	10/12	66.9±5.3 **	0.3	7/12	100.4±7.6	1.0	5/12	83.6±7.8
10.0	2/12	72.0±5.6 **	1.0	9/12	39.2±5.7 **	0.5	8/12	87.0±7.5	5.0	7/12	74.3±5.8 *

Data are mean±s.e.m. (n = 12). Significant effect of drug on response rates compared with saline * $p<0.01$, ** $p<0.001$ (Dunnett's t-test) a = n ethanol responding / n responding

218P MUSCARINIC RECEPTOR-MEDIATED RESPONSES OF GUINEA-PIG DORSAL RAPHE NEURONES *IN VITRO*

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Muscarinic cholinergic receptors are present in the dorsal raphe nucleus (DRN) which has been implicated in a number of functions including sleep regulation and pain modulation (Baghdoyan *et al.*, 1994; Wang & Nakai, 1994); however, the direct effects of cholinergic receptor activation have not been examined. We have used electrophysiological recording techniques to determine the effects of the muscarinic receptor agonists carbachol and muscarine on neurones of the guinea pig dorsal raphe nucleus *in vitro*.

Transverse midbrain slices (350 μm) were prepared from anaesthetized male Dunkin Hartley guinea pigs (200 - 400 g, 100 - 250 mg kg^{-1} pentobarbitone), submerged in a recording chamber and perfused with an artificial cerebrospinal fluid (aCSF) at 30 °C (see Craven *et al.* 1994). Intracellular recordings were made using microelectrodes filled with 2 M KCl (40 - 120 MΩ). 'Classical' presumed 5-hydroxytryptamine (5-HT) containing DRN neurones were identified according to the shape and duration of action potentials (1 - 2 ms at 50% peak) and their hyperpolarization by 5-HT (30 - 100 μM) (see VanderMaelen & Aghajanian, 1983). 'Nonclassical' DRN neurones gave diverse responses to 5-HT (30 - 100 μM); these cells tended to have shorter duration action potentials (0.4 - 1.8 ms) and were generally faster firing (maximum >30 spikes s^{-1} with depolarizing current delivered via the recording electrode) than classical neurones. Carbachol or muscarine (both 10 μM, 1 - 2 min) and tetrodotoxin (TTX) or atropine (both 1 μM, 10 - 100 min) were dissolved in aCSF and applied via the perfusion system. Results are expressed as the median value (range, n).

In 25 of 54 classical 5-HT neurones carbachol induced a depolarizing, often excitatory response which was mimicked by muscarine (n=4). The carbachol induced depolarization persisted in the presence of TTX [13 mV (6 to 18, n=7)] but was reduced or abolished by treatment with the muscarinic receptor antagonist atropine [100% (75 to 100, n=6)]. The remaining 29 classical neurones gave little or no detectable response to carbachol. Carbachol induced effects were recorded in 23 of 33 nonclassical neurones; 14 gave a predominantly hyperpolarizing response which persisted in the presence of TTX [-14 mV (-6 to -25 mV, n=5)], whereas 9 gave a depolarizing, excitatory response. The carbachol induced responses of nonclassical neurones were abolished by atropine (n=3 in both cases).

These results provide direct electrophysiological evidence of a diversity of muscarinic receptor mediated responses of DRN neurones. Depolarizing responses were observed in classical 5-HT containing neurones, and hyperpolarizing or depolarizing responses were recorded in nonclassical cells. A similar variety of muscarinic receptor mediated responses has been observed in the nucleus raphe magnus *in vitro* (Pan & Williams, 1994).

R.M.C. is a Wellcome Prize Student.

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219P THE EFFECTS OF CARBAMAZEPINE AND LAMOTRIGINE ON EXCITATORY AMINO ACID RESPONSES IN THE RAT CORTICAL WEDGE

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Lamotrigine and carbamazepine are antiepileptic drugs whose primary action involves use-dependent blockade of voltage-sensitive sodium channels (Leach et al., 1986; McLean and MacDonald, 1986). They have also been reported to have different effects on glutamate receptors. Carbamazepine inhibits NMDA-activated currents in cultured spinal cord neurones (Lampe & Bigalke, 1990) whereas lamotrigine is claimed to have no effects (Baxter et al., 1990; Leach et al., 1991). Here we compare the effects of lamotrigine and carbamazepine on NMDA and non-NMDA agonist responses, using the in vitro cortical wedge preparation (Harrison and Simmonds, 1985).

Cortical wedges, cut from 500 μm coronal sections of hemibrains removed from halothane-anaesthetised male Sprague-Dawley rats (80-120g; Charles River), were placed in a two-compartment chamber such that the cortical side was isolated from the callosal side by a grease-seal. The experimental protocol used was that previously described (Phillips et al., 1996). Following two-way analysis of variance, comparisons between control and drug groups were made using Williams' test.

Although carbamazepine weakly blocked NMDA and AMPA/quisqualate responses at NMDA and non-NMDA receptors respectively (IC_{50}s >100μM, n=4), it had no effect on spontaneous epileptiform discharges (SEDs) at concentrations ≤ 100 μM. The former findings are in agreement with the data of Lampe & Bigalke (1990). However, these authors did not determine the effects of carbamazepine on non-NMDA receptor-mediated responses. Lamotrigine reduced both the frequency and amplitude of SEDs; IC_{50}s (95% confidence limits): 254μM (152-425, n=5) & 141 μM (81-246, n=5) respectively), but in agreement with Baxter et al. (1990) and Leach et al. (1991), it had no effect on NMDA-evoked responses and only weakly attenuated responses to the non-NMDA agonists AMPA and quisqualate (IC_{50}s >100 μM, n=4). This weak effect at non-NMDA receptors is unlikely to explain the effect of lamotrigine on SEDs, because, the potent and selective non-NMDA antagonist, NBQX, has no effect on the frequency or amplitude of SEDs (Phillips et al., 1996).

These data show firstly that the ability, or lack of ability, to attenuate SEDs in the rat cortical wedge is not a reliable predictor of anticonvulsant efficacy in the clinic. Secondly, they show that the anticonvulsant effects of both drugs are unlikely to involve antagonism of ionotropic glutamate receptors.

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220P LEECH RETZIUS CELLS AS A MODEL FOR EPILEPTIFORM ACTIVITY

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The aim of this study was to examine the possible role of the Retzius (R) cells of the leech *Hirudo medicinalis* as a model for studying the underlying mechanism of epileptiform activity (EA), using selected convulsants and anticonvulsants. In R cells, which normally fire at 0.5-3 Hz, EA is characterised by paroxysmal depolarising shifts (PDSs) of membrane potential and rapid firing. Intracellular recordings were used (for method see Leake & Koubanakis 1995). Convulsants (EA agents) were applied in at least 4 effective concentrations, each being tested on 5 preparations from each of 3 animals. EA was quantified as the amplitude (mV), duration (sec) and frequency (Hz) of 3 PDSs, every minute. Once stable PDSs were achieved, anticonvulsants (10^{-5}-10^{-3}M) were added. Bemegride, cobalt chloride and α-cypermethrin were successful in inducing EA but picrotoxin, bicuculline and benzyl penicillin were not. Chemical isolation of R cells, using high (20mM) magnesium Ringer abolished the effects of bemegride, but did not affect those of other EA agents. In terms of threshold dose the order of potency of effective EA agents was: α–cypermethrin (10^{-6}M) > cobalt chloride (10^{-5}M) > bemegride ($2x10^{-3}$M). Anticonvulsants used were carbamazepine and sodium valproate. Carbamazepine partially (in a dose dependent manner) or totally reversed EA and normal activity resumed (see example in table 1). Sodium valproate was a very effective antagonist against bemegride but very poor against cobalt chloride and α-cypermethrin.

Table 1: Example of effects of 10^{-4} M carbamazepine on amplitude of PDSs caused by 3 EA agents (5 preparations from each of 3 animals; SE: standard error).

EA agent	Mean Amp. (mV)	S.E	Mean Amp. (mV)	S.E
Bemegride $2x10^{-3}$M	9.8	0.25	0.2	0.05
Cobalt chloride 10^{-5}M	11.9	0.33	0.2	0.15
α-cypermethrin 10^{-5}M	3.3	0.15	2.0	0.25
	A		B	

A: after development of stable PDS and prior to anticonvulsant application B: 5 minutes after application of carbamazepine. Carbamazepine (10^{-4}M) significantly reduced the amplitude of PDSs caused by bemegride ($p<0.001$), cobalt chloride ($p<0.001$) and α–cypermethrin ($p<0.05$; analysis by balanced hierachical ANOVA).

It was concluded that the three effective convulsants operate through at least two different modes of action; a direct membrane action and via synaptic activation. This makes R cells a possible model for the study of epileptiform activity.

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221P ROLE OF GLUTAMATE IN MALONATE-INDUCED DEGENERATION OF BASAL FOREBRAIN CHOLINERGIC NEURONS

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Deficits in energy metabolism have been implicated in the pathophysiology of neurodegenerative disorders (Beal, 1993). Previously, we have reported that intranigral infusions of malonate, an inhibitor of mitochondrial function, destroys the dopaminergic neurons of the nigrostriatal pathway (Connop *et al.*, 1996). Blockade of this neurotoxicity by dizocilpine (MK-801), N^{ω}-nitro-L-arginine methyl ester and 7-nitro indazole suggests an involvement of N-methyl-D-aspartate (NMDA) receptors and nitric oxide (NO) in the toxic action of malonate. In the present study, we examined whether malonate is toxic to basal forebrain cholinergic neurons *in vivo*. The potential role of glutamate release, NMDA receptor activation and NO formation in malonate action was examined by evaluating the effect of lamotrigine (an inhibitor of glutamate release), MK-801 and N^{ω}-nitro-L-arginine (L-NA) on this action.

Unilateral focal infusions of malonate (1.33 - 4.4 µmol/µl) were delivered into the nucleus basalis magnocellularis (nBM) of male Sprague-Dawley rats (250 - 300g) under halothane anesthesia (2%). Seven days following malonate infusions, choline acetyltransferase (ChAT) activity in the ipsilateral and contralateral cortex and amygdala was measured (Boegman *et al.*, 1992). Basal ChAT activity in the cortex and amygdala was found to be 25.8 ± 1.0 and 89.9 ± 3.9 nmol/pg/h respectively. Malonate infusion into the nBM produced a dose-related depletion of ipsilateral ChAT activity in both the cortex and amygdala (% decrease compared to the uninjected side). A maximal decrease in ipsilateral cortical and amygdaloid ChAT activity was observed following a dose of 4.4 µmol malonate (51.0 ± 8.0 and 64.5 ± 10.0%; n=4, respectively). Infusion of a 3 µmol dose of malonate into the nBM of vehicle treated animals resulted in a 41.3 ± 3.4 and 54.3 ± 4.0% (n=10) decrease in cortical and amygdaloid ChAT activity, respectively. Following systemic pretreatment with lamotrigine (16 mg/kg; i.p.), this dose of malonate produced a 15.0 ± 4.0 and 20.6 ± 6.3% (n = 5) decrease in cortical and amygdaloid ChAT activity, respectively. In MK-801 (5 mg/kg; i.p.) pretreated animals the corresponding decreases in cortical and amygdaloid ChAT activity were 12.6 ± 3.5 and 12.4 ± 4.2% (n = 6), respectively. Thus, both lamotrigine and MK-801 significantly attenuated the neurotoxic action of malonate on the cholinergic neurons of the nBM.

Following pretreatment with L-NA (2 and 10 mg/kg; i.p.), the toxic action of malonate on cortical and amygdaloid ChAT activity was not altered (n = 6). L-NA at doses of 2 and 10 mg/kg reduced nitric oxide synthase (NOS) activity in the nBM by 46.0 ± 3.2 and 73.2 ± 6.0% (n = 4), respectively, as measured by a sensitive spectrophotometric assay (Salter *et al.*, 1995). The level of NOS inhibition in the nBM following systemic treatment with L-NA (10 mg/kg) persisted for at least 13 h after a single injection (n = 4).

These results suggest an involvement of glutamate release and NMDA receptor activation but not NO formation in malonate-induced degeneration of the cholinergic neurons of the nBM.

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222P EFFECTS OF TEMPERATURE ON INOSITOL 1,4,5 TRISPHOSPHATE-STIMULATED Ca^{2+} MOBILIZATION

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Inositol 1,4,5-trisphosphate (IP_3) stimulates release of Ca^{2+} from intracellular stores. The kinetics of IP_3-stimulated Ca^{2+} release are unusual in that at physiological temperatures, even prolonged exposure to submaximal concentrations of IP_3 releases only a fraction of the IP_3-sensitive stores. The mechanisms responsible for such quantal Ca^{2+} mobilization have been reported to be inhibited at low temperature (Kindman & Meyer, 1993), but there is also conflicting evidence (Parys *et al.*, 1993).

Rat hepatocytes were permeabilized with saponin and the intracellular stores were loaded to steady state with $^{45}Ca^{2+}$ at 37°C in cytosol-like medium (CLM, free $[Ca^{2+}]$ = 200 nM). The cells were then diluted into a Ca^{2+}-free CLM containing thapsigargin (1 µM) at appropriate temperatures. The method allowed simultaneous inhibition of further $^{45}Ca^{2+}$ uptake, reduction of cytosolic $[Ca^{2+}]$ (to < 5 nM) and rapid changes of incubation temperature. After 30 s, IP_3 was added and at intervals thereafter the $^{45}Ca^{2+}$ contents of the stores were assessed by rapid filtration.

The half-maximal effect (EC_{50}) of IP_3 on the extent of Ca^{2+} mobilization occurred at progressively lower concentrations of IP_3 as the temperature was reduced (Table 1).

Table 1. Decreased temperature increases the sensitivity of the intracellular Ca^{2+} stores to IP_3 (means ± s.e.m.).

Temperature	2°C	10°C	20°C	30°C	37°C
EC_{50}, nM	37±1	71±7	130±11	198±13	265±36
(n)	(6)	(6)	(6)	(7)	(9)

At 37°C, the EC_{50} for the effect of IP_3 on unidirectional $^{45}Ca^{2+}$ efflux was similar after 30 s (265 ± 36 nM; n = 9) and 120 s (256 ± 44 nM; n = 9), confirming that responses to IP_3 are quantal at 37°C. At 2°C, the EC_{50} for IP_3-stimulated $^{45}Ca^{2+}$ efflux decreased from 76 ± 3 nM (n = 8) after a 30 s incubation with IP_3 to 46 ± 2 nM (n = 6) after 120 s. However, after more prolonged incubation with IP_3, the EC_{50} stabilised, such that after a 4 min exposure to IP_3 it was 41 ± 1 nM (n = 4) and after 7 min it was 37 ± 1 nM (n = 3). At 2°C, 37.5 nM IP_3 released 49 ± 2 % of the IP_3-sensitive Ca^{2+} stores with a half-time ($t_{1/2}$) of 85 ± 5 s (n = 6), whereas at 37°C the response to 250 nM IP_3, which released 48 ± 7 % (n = 9) of the stores, was complete within 30 s. At 10°C, the response to 75 nM IP_3, which released 49 ± 3 % (n = 4) of the Ca^{2+} stores, occurred with a $t_{1/2}$ of 37 ± 10 s. These results establish that responses to IP_3 are quantal at all temperatures between 2°C and 37°C, but the quantal behaviour takes much longer to become established at lower temperatures.

We conclude that the intracellular Ca^{2+} stores are more sensitive to IP_3 at reduced temperatures and that quantal responses are maintained albeit with slower kinetics.

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223P STUDIES OF INOSITOL 1,4,5-TRISPHOSPHATE EXPORT *IN VIVO* AND *IN VITRO*

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Recent *in vivo* microdialysis studies have indicated the presence of extracellular levels of inositol- 1,4,5 trisphosphate (IP3) which are increased after stimulation of muscarinic receptors (Minisclou *et al.*, 1994), presumably through an increase in its export out of cells. The aim of this present study was to set up an *in vitro* system for measuring IP3 efflux with a view to investigating the mechanism(s) involved.

In vivo microdialysis studies demonstrated an increase in extracellular IP3 after 100μM carbachol stimulation which reached a maximum 60 min after perfusion of the agonist, thus confirming the results of Minisclou *et al.* (1994).

Levels of extracellular and intracellular IP3 in the SH-SY5Y neuronal cell line 15 s, 1 min, and 5 min after stimulation with 1mM carbachol were measured using a radioreceptor binding assay (Challiss *et al.*, 1990). Intracellular IP3 levels were stimulated from 30.6 ± 11.0 pmoles mg protein^{-1} to 237.2 ± 46.8 pmoles mg protein^{-1} (means ± s. e. mean; n=3) after only 15 s. However, there was no apparent increase in extracellular IP3 accumulation up to 5 min after addition of the agonist.

IP3 release was also studied in rat brain cortical slices stimulated by 1mM carbachol. As with the SH-SY5Y cells, there was a rapid increase in internal IP3 above basal levels on stimulation with the muscarinic agonist (233.1 ± 45.9% at 1 min; P=0.01; n=5), but there was no increase in external IP3 above basal levels (106.1 ± 8.8% accumulated after 5 min). Basal levels of IP3 as measured using the radioreceptor binding assay were surprisingly high (16.0 ± 4.7 pmoles per assay in the extracellular fractions compared to 19.96 ± 1.1 pmoles per assay in the intracellular fractions after 1 min). Therefore, in a further set of experiments, cortical slices were prelabelled with [^{3}H]-myo-inositol for 45 min before stimulation with agonist. Inositol phosphates in the internal and external fractions were then separated out on formate columns. Internal levels of IP3 were once again raised above basal levels after carbachol stimulation (214.4 ± 31.7% after 5 min; P<0.05; n=4), although there was no change in external IP3 levels. Basal levels of external IP3 were apparently not dissimilar to internal basal levels as was found using the radioreceptor binding assay.

Recently we have discovered saturable binding of [^{3}H]-IP3 to rat brain cortical slices (25% displacement of 1nM [^{3}H]-IP3 by 10nM unlabelled IP3; P<0.05; n=3) which may reduce the levels of free external IP3 which could be the reason why we have been unable to demonstrate an increase in external IP3 in this system.

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224P CYCLOPROCTOLIN ANTAGONISES PROCTOLIN-STIMULATED INOSITOL TRISPHOSPHATE PRODUCTION IN THE LOCUST FOREGUT

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Proctolin (H-Arg-Tyr-Leu-Pro-Thr-OH), first identified by Brown (1967), has been shown to cause dose-dependent contraction of the isolated foregut of the locust *Schistocerca gregaria* (Banner *et al.*, 1987). Subsequent work (Gray *et al.*, 1994) showed that cycloproctolin (synthesised by forming a covalent bond between the *N*-terminal of arginine and the *C*-terminal of threonine) is an antagonist of proctolin-induced tissue contraction. Recently, Hinton & Osborne (1995) showed that in locust foregut homogenates proctolin-induced inositol trisphosphate (IP_3) production is potentiated by lithium. Here we describe the effects of cycloproctolin on proctolin-stimulated IP_3 synthesis.

Foregut homogenates from locusts were prepared and incubated with [^{3}H]-*myo*-inositol as described by Hinton & Osborne (1995) and challenged, for 2 min, with proctolin in the absence and presence of cycloproctolin. [^{3}H]-IP_3 was eluted from anion exchange columns (DOWEX-1, X8, formate form, mesh 100-200, Bio-Rad) as described by Berridge *et al.* (1983). Anion exchange chromatography yielded a peak corresponding to the known elution profile of IP_3. Radioactivity was quantified by liquid scintillation counting and the results, which have been normalised by subtracting the Tris buffer treated controls, are expressed as d.p.m. mg^{-1} protein.

Proctolin caused dose-dependent IP_3 production with an EC_{50} value of 4.0 ± 0.5 nM (n = 6) at concentrations ranging from 0.1 nM to 1 μM. Cycloproctolin (0.1 nM - 1 μM) caused a marked reduction in proctolin stimulated IP_3 production with an IC_{50} value of 2.5 ± 0.2 nM. The maximal antagonistic effect of cycloproctolin occurred at 0.5 μM and was not enhanced by higher doses.

These data show that cycloproctolin, which is a potent antagonist of proctolin-induced foregut contraction, is also capable of markedly reducing proctolin-stimulated IP_3 synthesis. Furthermore it also suggests that the locust foregut contains a proctolin receptor which, when activated, causes production of IP_3 thereby leading to tissue contraction. This possibility is being investigated further by evaluating the effects of protein kinase C inhibitors and sarcoplasmic reticulum channel blockers on proctolin-induced tissue contraction.

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225P Ca^{2+} RELEASE AND BINDING STUDIES OF COMPOUNDS STRUCTURALLY RELATED TO INOSITOL 1,4,5-TRISPHOSPHATE AND ADENOPHOSTIN

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Adenophostins A and B, metabolic products of the fungi *Penicillium brevicompactum*, are potent inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3]-receptor agonists. The adenophostins incorporate features that enable them to mimic Ins(1,4,5)P_3 but also have additional components, which enhance their affinity for Ins(1,4,5)P_3 receptors (Takahashi *et al.*, 1994).

A structural basis for the exceptional potency of the adenophostins is not clear therefore three compounds, whose structures represent different aspects of the construction of adenophostin A, were examined for their biological activity. These compounds were 2'-adenosine monophosphate (2'-AMP) which is the nucleoside half of the adenophostins, and the synthetic analogues (2-hydroxyethyl)-α-D-glucopyranoside-2',3,4-trisphosphate [Gluc(2',3,4)P_3] and α,α'-trehalose-3,4,3',4'-tetrakisphosphate [Trehal(3,4,3',4')P_4]. Gluc(2',3,4)P_3 is based on the phosphorylated glucopyranose component of adenophostin A, while the sterically larger and conformationally more rigid Trehal(3,4,3',4')P_4 consists of two copies of this component in a C_2 symmetrical molecule. Ca^{2+} mobilisation was examined by evaluating release of $^{45}Ca^{2+}$ from permeabilised rabbit platelets (Mills *et al.*, 1993). Binding to the Ins(1,4,5)P_3-receptor was determined by displacement of [^{3}H]Ins(1,4,5)P_3 from its binding site on rat cerebellar membranes (Challis *et al.* 1994).

All the compounds studied, except 2'-AMP, were able to release Ca^{2+} from permeabilised platelets and to displace [^{3}H]Ins(1,4,5)P_3 from its binding site on rat cerebellar membranes (Table 1). Ca^{2+} release by these compounds was inhibited by >85% with the Ins(1,4,5)P_3-receptor antagonist heparin (25 μg/ml; 3 min). Adenophostin A was some 50 fold more potent than Ins(1,4,5)P_3 in both $^{45}Ca^{2+}$ release and binding studies. Gluc(2',3,4)P_3 released $^{45}Ca^{2+}$ and inhibited [^{3}H]Ins(1,4,5)P_3 binding with a 6 fold lower potency than Ins(1,4,5)P_3, this compares

Table 1 Comparison of Ins(1,4,5)P_3, adenophostin A and analogues on $^{45}Ca^{2+}$ release and displacement of [^{3}H]Ins(1,4,5)P_3 binding. Values are mean ± S.E.M. (n = 3-10)

Compound	$^{45}Ca^{2+}$ release (EC_{50}) [μM]	Displacement of [^{3}H]Ins(1,4,5)P_3 IC_{50} [μM]
D-Ins(1,4,5)P_3	0.4 ± 0.08	0.038 ± 0.005
Adenophostin A	0.0073 ± 0.0036	0.00074 ± 0.00042
2'-AMP	no release (1 mM)	-
Gluc(2',3,4)P_3	2.05 ± 0.35	0.21 ± 0.07
Trehal(3,4,3',4')P_4	100 ± 65	0.37 ± 0.17

with the 10-12 fold lower potency previously demonstrated for $^{45}Ca^{2+}$ release in SH-SY5Y and MDCK cells and the 5 fold lower affinity reported for binding to pig cerebellar membrane Ins(1,4,5)P_3-receptors (Wilcox *et al.*, 1995). Trehal(3,4,3',4')P_4 was some 10 fold weaker at binding, whilst some 250 fold weaker at releasing $^{45}Ca^{2+}$ than Ins(1,4,5)P_3. This study demonstrates that alone, the nucleoside half of the adenophostins is inactive, but partial removal of this structure from the adenophostin molecule, as in Gluc(2',3,4)P_3 and Trehal (3,4,3',4')P_4, produces compounds considerably weaker than adenophostin.

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226P ACTIONS OF cADP-RIBOSE ON CONTRACTIONS IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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cADP-ribose (cADPR) is thought to regulate Ca^{2+} release from endoplasmic reticulum stores in a wide variety of cells (Lee et al, 1994). The aim of the present study was to test whether cADPR (applied via a patch pipette or photoreleased from 'caged' cADPR) might influence contraction in cardiac ventricular cells.

Myocytes were isolated from guinea-pig ventricular muscle and superfused with a balanced salt solution containing 2.5 mM Ca^{2+} (36°C). Patch electrodes contained, in mM: KCl, 150; $MgCl_2$, 5; K_2ATP, 1; HEPES, 3 (pH 7.2). In some experiments cADPR was added to the patch pipette; in others a 'caged' derivative (1-(1-(2-nitrophenyl)ethyl)ester) was added at 300 μM and cADPR released by exposure to uv light (for a period of 5 to 20 s; wavelength <400 nm, selected by a dichroic mirror) delivered via a quartz fibre optic. Current-clamp conditions were used for whole-cell recording and action potentials were stimulated to fire at 1 Hz. The accompanying contractions were measured from the video image of the myocytes viewed microscopically using an edge-detection system (Annetts et al 1990).

Contraction was consistently enhanced by cADPR applied to the cytosol of the myocytes via the patch pipette. The mean increase of contraction (measured 5 min after rupture of the patch membrane) was 22±11% at 5 μM cADPR (n=6 cells, $P<0.05$, paired t test) and 23±9% at 10 μM cADPR (n=6 cells, $P<0.05$). Application of cADPR often caused spontaneous oscillating contractions (5 of 15 cells at 5 μM and 4 of 14 cells at 10 μM). In cells dialysed with caged cADPR, photorelease of cADPR increased contraction by 12±3% (n=7 cells, $P<0.05$).

The observations are consistent with enhancement by cADPR of the Ca^{2+} sensitivity of Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (as has been shown for Ca^{2+} release from the endoplasmic reticulum in other cell types, including sea urchin eggs and mammalian neurones, Lee et al, 1994). This effect of cADPR, whether applied in the patch pipette or photoreleased, might underlie the observed increases in contraction.

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227P ACTIONS OF 8-Br-cADP RIBOSE ON CONTRACTIONS IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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cADP-ribose (cADPR) has been shown to play an important role in the regulation of calcium release from endoplasmic reticulum stores in a wide variety of cells (Galione, 1993). 8-NH_2-cADPR has been shown to act as a competitive antagonist of cADPR in sea urchin egg preparations (Walseth & Lee, 1993) and to reduce calcium transients and contractions in cardiac ventricular cells (Rakovic et al 1995). The aim of the present study was to test whether 8-Br-cADPR, another antagonist of cADPR (Walseth & Lee, 1993) might influence contraction in cardiac ventricular cells.

Myocytes were isolated from guinea-pig ventricular muscle and superfused with a balanced salt solution containing 2.5 mM Ca (36°C). Patch electrodes (containing, in mM: KCl, 150; $MgCl_2$, 5; K_2ATP, 1; HEPES, 3; pH 7.2) were used for whole-cell recording under current-clamp conditions and action potentials were stimulated at 1 Hz. The accompanying contractions were measured from the video image of the myocytes viewed microscopically using an edge-detection system (Annetts et al 1990).

Figure 1 shows that contraction was reduced by 5 μM 8-Br-cADPR applied to the cytosol of the myocytes via the patch pipette. The mean reduction of contraction (measured 7 min after rupture of the patch membrane) at this concentration was 23±4% (n=7 cells, $P<0.05$, paired t test). In 4 cells exposed to 50 μM 8-Br-cADPR in the pipette, contraction was reduced by 37±8% (7 min, $P<0.05$). When 8-Br-cADPR was omitted from the patch pipette solution, contraction was well maintained over the same time period (contraction was 98±3% of control, $P>0.05$, n=4).

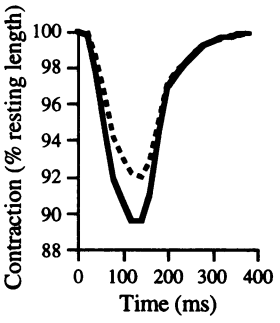

Figure 1 Contractions before (solid line) and after (dotted line) cytosolic application of 5 μM 8-Br-cADPR.

The observations are in agreement with previous work with 8-NH_2-cADPR (Rakovic et al, 1995) and are consistent with an inhibition by 8-Br-cADP-ribose of endogenous cADPR which might regulate the Ca^{2+} sensitivity of Ca^{2+}-induced Ca^{2+} release.

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228P IDENTIFICATION OF mRNA ENCODING AN N1/*cif* NUCLEOSIDE TRANSPORTER IN RAT BRAIN

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Background: Na^+-dependent, concentrative nucleoside transport has been identified in a number of cell types and tissues. These transport processes are thought to be involved in the salvage of endogenous nucleosides, providing cells with nucleosides required for metabolism. Alternatively, they may serve to regulate adenosine receptor activation by controlling extracellular adenosine levels. This could be of considerable importance during cerebral ischemia given the neuroprotective effects of endogenous adenosine. Previously, we have shown that mRNA encoding a pyrimidine-selective Na^+-dependent nucleoside transporter (cNT1) is present in rat brain (Anderson *et al.*, 1995). SPNT is a purine-selective member of the same gene family. In the present study, we endeavoured to verify the existence of mRNA encoding the SPNT transporter in rat brain.

Objective: The objective of the present study was to detect mRNA for SPNT in rat brain, and determine the relative abundance of the mRNA in different brain regions using reverse transcriptase PCR (RT-PCR)

Methods: Brains were removed from male Sprague-Dawley rats (300 g) and dissected. Total RNA was isolated from cortex, cerebellum, hippocampus, striatum, superior colliculus, brain stem, choroid plexus and posterior hypothalamus. RNA samples were reverse transcribed in the presence or absence of reverse transcriptase and PCR was performed with primers designed to amplify the SPNT nucleotide sequence from base 1228 to 1462 (235-bp). Products were agarose gel purified, subcloned into the pCR-Script™ phagemid, and sequenced at The University of Alberta Nucleotide Sequencing Facility.

Results: Agarose gel electrophoresis of RT-PCR reaction products indicated the presence of the expected 235-bp product in every brain region tested, with similar relative abundance in each region. Digestion of the product with Alw44 I produced a shift in fragment size consistent with the formation of the predicted 162-bp and 73-bp pieces, and nucleotide sequencing revealed that the sequence was identical to that predicted by the published SPNT sequence. A second product (~300-bp) was also seen. Nucleotide sequencing showed that the sequence was identical to that of the 235-bp product except for an 81-bp insert after base 115. While RNase treatment of RNA samples prior to RT-PCR abolished the 235-bp product, the 316-bp product was unaffected, indicating that it is from a genomic DNA source and suggesting that the 81-bp sequence is an intron. The prevalence of this product in non-reverse transcriptase treated samples supports this conclusion.

Conclusion: The presence of the 235-bp RT-PCR product in all brain regions tested indicates that mRNA encoding SPNT is present throughout rat brain, and suggests that such functional transporters may play an important role in transmembrane shuttling of nucleosides in rat brain.

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229P ADENOSINE-MEDIATED ENHANCEMENT OF THE FUNCTIONAL ACTIVITY AND STABILITY OF SOLUBILIZED/RECONSTITUTED NUCLEOSIDE TRANSPORTERS

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The duration and magnitude of the extracellular receptor-mediated effects of adenosine are determined partly by its rate of uptake into the surrounding cells. A number of subtypes of plasma membrane-located adenosine transporters have been defined by their dependence on sodium and their sensitivity to inhibition by nitrobenzylthioinosine (NBMPR) (Cass, 1995). However, attempts to purify and characterize these transporters have been hampered by the functional instability of the detergent-solubilized proteins. The aim of the present study was to determine whether the presence of transporter ligands, such as adenosine, in the solubilization media could enhance the functional stability of the solubilized transport proteins.

Plasma membranes from Ehrlich ascites tumour cells (containing both the NBMPR-sensitive and -resistant forms of equilibrative nucleoside transporter; Hammond, 1991) were solubilized with 1% (w/v) octylglucoside (±stabilizing agents) and reconstituted into liposomes, as described by Hammond (1994), immediately after solubilization ("fresh") and after storage for 48 h/6°C (data shown as mean±s.e.m., n=5). Storage of the solubilized membranes prior to reconstitution resulted in a parallel loss (≈60%) of [^{3}H]NBMPR binding (B_{max}=9.0±0.6, fresh; 3.5±0.6 pmol/mg, stored) and [^{3}H]uridine (20 μM) uptake activities (initial rate, V_i = 36±7 versus 12±2 pmol/mg/s). In addition, the relative amount of NBMPR-resistant [^{3}H]uridine influx decreased from 20±2% to 8±2% of the total transporter-mediated activity. The inclusion of 20 mM adenosine in the solubilization media both enhanced the rate of [^{3}H]uridine uptake in liposomes prepared from the freshly solubilized preparations, and reversed the storage-induced loss of [^{3}H]NBMPR binding (B_{max}=9.9±0.4, fresh; 8.1± 0.6 pmol/mg, stored) and [^{3}H]uridine influx activity (V_i=53±8, fresh; 46±6 pmol/mg/s, stored). Adenosine (20 mM) also led to an increase in the relative amount of NBMPR-resistant [^{3}H]uridine uptake in liposomes prepared using freshly solubilized proteins (34±2% with adenosine, versus 20±2% in its absence), and completely prevented the storage-induced loss of NBMPR-resistant transport activity (32±1% of total after 48 h/6°C). These actions of adenosine were concentration dependent with a partial protective effect observed using 5 mM adenosine. A partial stabilization of the solubilized [^{3}H]NBMPR binding activity was also seen with uridine, and with the adenosine analogues 2'-deoxyadenosine and 2-chloroadenosine, but not with cytidine, inosine, dipyridamole, dilazep, or diazepam.

These data suggest that both the sensitivity of the transporter to NBMPR and the stability of the solubilized proteins are dependent on protein conformation. The NBMPR-resistant form of the transporter also appears more susceptible than the NBMPR-sensitive system to the deleterious effects of detergent solubilization. This may reflect a property integral to the transport protein, or may be due to differential phosphorylation or other associated regulatory components. The protective effects of nucleosides are likely due to their binding to the substrate translocation site thereby effectively "locking" the transporter in a stable conformation.

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230P FACILITATION OF β-ADRENOCEPTOR- AND FORSKOLIN-STIMULATED cAMP ACCUMULATION BY METHACHOLINE IN CHINESE HAMSTER OVARY CELLS CO-EXPRESSING M_3-MUSCARINIC AND β_2-ADRENOCEPTORS

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We have previously investigated the potential crosstalk between M_3-muscarinic cholinoceptor (M_3-mAChR) and β_2-adrenoceptor signalling pathways in Chinese hamster ovary (CHO) cells co-expressing these receptor populations (CHO-m3β2 [clone #27] expressing M_3 and β_2 receptors at levels of 1456 ± 240 (n=4) and 224 ± 33 fmol/mg protein (n=6) respectively). In these cells, M_3-receptor activation causes a substantial enhancement of β_2-adrenoceptor-stimulated cyclic AMP accumulation (Ellis *et al.*, 1995). Here, we have studied the mechanism by which M_3-mAChR activation may cause this modulatory effect.

Incubations were carried out using confluent cell monolayers of CHO-m3β2 cells maintained at 37°C in Krebs-Henseleit buffer (equilibrated with 95% O_2/5% CO_2). Drug additions were made for 10 min unless otherwise stated, and at the concentrations, indicated. Reactions were terminated by the addition of ice-cold trichloroacetic acid (0.5 M) and samples were extracted on ice before being neutralized. Measurements of cyclic AMP were carried out using a standard radioreceptor binding assay.

In agreement with previous data, addition of isoprenaline (ISO; 1 μM) or methacholine (MCh; 1 mM) substantially elevated cyclic AMP levels (basal, 36 ± 11; +ISO, 1592 ± 130; +MCh, 147 ± 23 pmol mg^{-1} protein; n=3). Co-addition of increasing concentrations of MCh (0.1-1000 μM) caused a concentration-dependent potentiation of the ISO (1 μM) response (ISO+MCh (1 mM), 4091 ± 334 pmol mg^{-1} protein; EC_{50} 2.4 μM (-log EC_{50} (M) 5.62 ± 0.31; n=3). The effects of MCh *per se*, and MCh facilitation of ISO-stimulated cyclic AMP accumulations were completely blocked by atropine (1 μM). Omission of extracellular Ca^{2+} did not affect the ISO- (1 μM), MCh- (1 mM) or ISO+MCh-stimulated cyclic AMP accumulations. Although phorbol myristate acetate (PMA; 1 μM) caused a significant enhancement of the cyclic AMP response to ISO (136 ± 10%, n=4; P<0.05, Student's t-test), this amounted to <20% of the potentiation evoked by MCh, and the PMA effect was not enhanced further by co-addition of ionomycin (1 μM).

A facilitatory effect of M_3-mAChR stimulation was also seen with respect to forskolin (FK)-stimulated cyclic AMP accumulation in CHO-m3β2 cells (basal, 15 ± 4; +FK (10 μM), 1011 ± 77; +MCh (1 mM), 115 ± 33; FK+MCh, 3837 ± 125 pmol mg^{-1} protein; n=3). Surprisingly, the cyclic AMP accumulations evoked by FK and FK+MCh were partially suppressed by the β-adrenoceptor antagonist ICI 118551 (1 μM). Careful comparison of FK-stimulated cyclic AMP responses in CHO-m3β2 cells and the parent cell-line, CHO-m3 (see Tobin *et al.*, 1992) demonstrated that the CHO-m3β2 cells exhibited a greater response to sub-maximally effective concentrations of FK, whilst in the presence of ICI 118551 the concentration-response curves for FK-stimulated cyclic AMP in CHO-m3β2 and CHO-m3 cells were essentially identical.

The present study provides evidence that the facilitation of cyclic AMP accumulation in CHO-m3β2 cells by M_3-mAChR activation occurs by a protein kinase C- and Ca^{2+}-independent mechanism through a direct effect on adenylyl cyclase. Preliminary evidence also suggests that the β-adrenoceptor expressed in this cell-line may affect cyclic AMP generation independently of agonist-occupation.

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231P USE OF [^{35}S]-GTPγS BINDING TO ASSESS TYPE 1α METABOTROPIC GLUTAMATE RECEPTOR ACTIVITY IN BABY HAMSTER KIDNEY CELL MEMBRANES

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The metabotropic glutamate receptor (mGluR) family share little sequence homology with other G-protein coupled receptors, and it has been proposed that the glutamate binding site is contained within the extracellular domain(s) and G protein coupling may be substantially determined by the second intracellular loop of mGluRs (Gomeza *et al.*, 1996). Here we present evidence that type 1α mGluR activity can be assessed using [^{35}S]-GTPγS binding which has been widely used to determine agonist-mediated guanine nucleotide exchange at Gα proteins (see Lazareno *et al.*, 1993).

Baby Hamster kidney (BHK) cells expressing recombinant mGlu1α receptors were maintained in DMEM supplemented with 5% dialysed calf serum, 2 mM glutamine, 50 μg ml^{-1} gentamicin, 500 μg ml^{-1} G418 and 1 μM methotrexate (Thomsen *et al.*, 1993). Membranes were prepared and binding experiments were performed according to the methods of Lazareno *et al.* (1993). Optimum assay conditions were found to be 60 min incubations at 30°C with 70 pM [^{35}S]-GTPγS, 1 μM GDP, 10 mM $MgCl_2$, 100 mM NaCl and 100 μg ml^{-1} membrane protein.

A clear elevation over basal of [^{35}S]-GTPγS binding upon stimulation with 300 μM glutamate was observed (in the presence of 10 μM GDP) after 5 min, with a maximum stimulation above basal of 41.4 ± 6.2% at 60 min (n=4). Total inhibition of 100 μM glutamate-stimulated [^{35}S]-GTPγS binding was achieved, under optimal conditions (in the presence of 1 μM GDP), upon co-incubation with 300 μM (S)-4-carboxy-3-hydroxyphenylglycine. Upon stimulation with a maximally effective concentration of glutamate (300 μM), in the presence of 0.1 μM to 10 μM GDP, large increases in [^{35}S]-GTPγS binding above basal were seen at all GDP concentrations, with the maximum increase (62.4 ± 1.6% above basal, n=3) being observed in the presence of 1 μM GDP. Lowering the concentration of NaCl (to 10mM) in the assay reduced the response at 1 μM GDP to 15.4 ± 0.4% (n=3). Glutamate increased [^{35}S]-GTPγS binding in a concentration-dependent manner under the optimal assay conditions with an apparent EC_{50} value of 3.7 ± 0.5 μM (n=4). This response was partially sensitive to pertussis toxin (PTx). The basal level of [^{35}S]-GTPγS binding was 173.6 ± 4.2 fmol mg^{-1} protein (n=3) which increased to 286.3 ± 6.4 fmol mg^{-1} protein upon stimulation with 300 μM glutamate (an increase of 112.7 ± 2.8 fmol mg^{-1} protein). Pre-treatment with PTx (100 ng ml^{-1}, 22-24 h) reduced the basal binding to 69.6 ± 0.4 fmol mg^{-1} protein (n=3), and also reduced glutamate-stimulated binding to 97.4 ± 2.5 fmol mg^{-1} protein (n=3) (an increase of 27.8 ± 2.2 fmol mg^{-1} protein). Thus, PTx causes a decrease in basal [^{35}S]-GTPγS binding and more marked decrease in agonist-stimulated binding.

We conclude that [^{35}S]-GTPγS binding can be used as a measure of receptor activation in this BHK-mGluR1α recombinant membrane system, and that this process is mediated by both PTx-sensitive and insensitive G proteins.

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232P EFFECT OF THE MEK INHIBITOR, PD 098059, ON AII-STIMULATED THYMIDINE INCORPORATION IN VASCULAR SMOOTH MUSCLE CELLS

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We have previously reported that vascular smooth muscle cells derived from spontaneously hypertensive rats (SHR) produce an increase in :- (i) tyrosine phosphorylation, (ii) MAPK activity and (iii) DNA synthesis, when stimulated with angiotensin II (AII). The same is not true for their normotensive derived control cells, WKY (Wilkie *et al*, 1996). In this report we show that all of the above form part of the mitogenic signalling cascade within these SHR cells.

SHR cells were cultured from 12 week old rats and used between passages 8 - 14. Cells were stimulated with 100nM AII (for 4 min for the assays and Western blots and for 1 hour for the [^{3}H] thymidine studies) with or without a 30 min preincubation with varying concentrations of the specific MEK inhibitor, PD 098059 (PD) (Alessi *et al*, 1995). MAPK activity was quantified using a kinase assay which measured the ^{32}P incorporation into a partially selective nonapeptide substrate (Wilkie *et al*, 1995). Phosphorylation of MAPK was estimated using Western blot analyses using an antibody directed against the phosphorylated form of tyrosine residue 204 of MAPK followed by densitometric scanning of the autoradiographs. Measurement of [^{3}H] thymidine incorporation was used as an index of DNA synthesis. All results show mean ± s.e.mean from at least 4 experiments, significance was measured using a students t - test.

AII stimulation of MAPK tyrosine phosphorylation was shown to be inhibited by the MEK inhibitor, PD : unstimulated = 0.02 ± 0.01, AII stimulated = 0.47 ± 0.03, AII + 100μM PD = 0.11 ± 0.02. Effect of PD, $p < 0.05$ (data expressed as $p42^{mapk}$ optical density units / μg protein). This shows that AII stimulated MAPK phosphorylation via an increase in MEK activity and that this stimulation was inhibited by PD. MAPK activity was also inhibited by PD : unstimulated = 8425 ± 181, AII = 19404 ± 1710, AII + 100μM PD = 10065 ± 815, $p < 0.05$ (data expressed as c.p.m / μg protein). Over the series of these experiments, PD was found to inhibit AII stimulated MAPK activity with a mean IC_{50} of 15.9 ± 2.3μM. AII stimulated [^{3}H] thymidine incorporation could also be inhibited using PD : unstimulated = 5604 ± 309, AII = 15487 ± 742, AII + 100μM PD = 4693 ± 198. $p < 0.01$ (data expressed d.p.m / μg protein). PD inhibited the DNA synthesis with a mean IC_{50} of 17.8 ± 3.1μM.

Taken together these results show that AII is stimulating MAPK activity via increasing the activity of the upstream regulator of MAPK, MEK, and that when MEK is inhibited by PD 098059, the AII stimulation of MAPK and [^{3}H] thymidine incorporation are also blocked. This therefore provides evidence that AII acting via G - protein linked receptors, can stimulate the MEK / MAPK pathway, which in turn can stimulate an increase in DNA synthesis in vascular smooth muscle cells derived from spontaneously hypertensive rats.

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233P ACTIONS OF 8Br-cGMP ON THE DELAYED RECTIFIER POTASSIUM CURRENT IN GUINEA-PIG ISOLATED VENTRICULAR MYOCYTES

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Several biochemical pathways have been reported to be involved in the regulation of L-type calcium currents by cGMP, by influencing the activity of a number of enzymes including: i) cGMP-activated phosphodiesterase, ii) cGMP-inhibited phosphodiesterase iii) cGMP-activated protein kinase. In contrast, regulation of the delayed rectifier potassium current (I_K) by cGMP is not well documented. We have therefore investigated the influence of 8-Br cGMP on basal and isoprenaline stimulated delayed rectifier potassium currents in guinea-pig isolated ventricular myocytes.

Myocytes were enzymatically isolated from guinea-pig ventricle and were superfused with a balanced salt solution at 36° C. Delayed rectifier potassium currents were activated by step depolarisations from -40mV to +40mV for 10 to 800 ms and were measured as outward tails on repolarisation to -40mV (switched voltage-clamp, in presence of 50mM BAPTA in electrode solution to suppress calcium transients).

Superfusion of cells with 10μM LY83583 (a guanylate cyclase inhibitor, Schmidt et al. 1985) did not significantly alter I_K, so that at 10 minutes currents were 105±4% of control. In addition, exposure of cells to 10μM 8-Br cGMP appeared not to significantly alter basal I_K over a period of 10 minutes (at 10 mins, currents at 400ms were 99±4% of control, figure 1A). In contrast,when I_K was enhanced by exposure to 5nM isoprenaline, I_K was further increased following exposure to 10μM 8-Br cGMP so that at 10 minutes currents at 400 ms were 131±11% of isoprenaline control (P<0.001, paired 't' test), figure 1B.

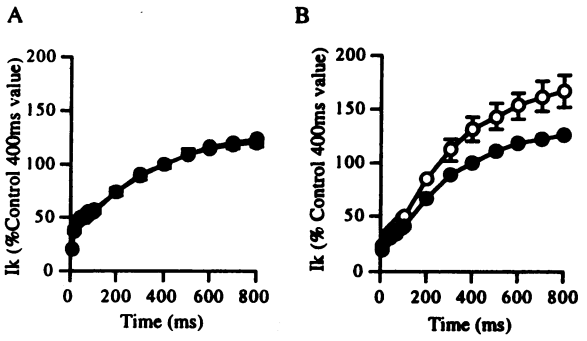

Fig.1. Activation of delayed rectifier potassium currents (expressed as % control value at 400 ms) at the start of experiment (filled circles) and after 10 minutes exposure to 10μM 8-Br cGMP (open circles) under basal conditions (A) or isoprenaline stimulated conditions (B). Mean control current amplitudes: 270±19pA (basal) and 710±82pA (isoprenaline stimulated).

It has previously been reported that cGMP does not influence basal L-type calcium currents although it enhances isoprenaline stimulated L-type calcium currents in guinea pig ventricular myocytes through inhibition of a cGMP-inhibited phosphodiesterase (Ono & Trautwein, 1991). Hence, it seems possible that a similar mechanism of inhibition of a cGMP-inhibited phosphodiesterase may underlie the observed effects of 8-Br cGMP on isoprenaline stimulated I_K.

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234P DIFFERENTIAL ACTIVITY OF CLONED $P2Y_1$, $P2Y_2$ AND $P2Y_4$ PURINOCEPTORS TO SURAMIN

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We have previously reported that cloned $P2Y_1$- and $P2Y_2$-purinoceptors, transfected into 1321N1 cells, show different sensitivity to the P_2- antagonist suramin (Charlton et al, 1996). Suramin acts as an antagonist at both receptors, but is more potent at $P2Y_1$ than $P2Y_2$. Here we report a recent study comparing these two receptors with a third cloned receptor, also transfected into 1321N1 cells, the human pyrimidinergic receptor or $P2Y_4$.

We used cloned turkey $P2Y_1$-, human $P2Y_2$- and human $P2Y_4$-purinoceptors, transfected into a 1321N1 subclone which showed no native response to the nucleotide agonists (Parr et el, 1994; Filtz et al, 1994; Nguyen et al, 1995). Phospholipase C activity was indicated by the accumulation of total [^{3}H]inositol (poly) phosphates as previously described (Charlton et al, 1996). Suramin, when included, was present for a 1 hour preincubation as well as the 15 min incubation period in the presence of agonist. Data were compared using Student's t-test (n=3).

Concentration response curves to several nucleotide agonists on the three receptors revealed agonist potencies consistent with those previously reported. At the $P2Y_1$, 2-methylthio-ATP (2MeSATP) and ATP had log EC_{50} values of -7.62 and -5.97 respectively, whilst UTP gave no response. At the $P2Y_2$, 2MeSATP was ineffective, with UTP and ATP having log EC_{50} values of -5.83 and -5.11 respectively. UTP was an effective agonist at cloned $P2Y_4$ receptors (log EC_{50} -5.94). Suramin (100 μM) reduced the response to maximally effective agonist concentrations to 18.5 ± 2.1% of the maximal response of the $P2Y_1$ (significant at P<0.025) and 64.1 ± 5.1% of the maximal response of the $P2Y_2$ (significant at P<0.05), but had no effect on the $P2Y_4$-purinoceptor (Figure 1). Thus, the receptors have a clear rank order of sensitivity to suramin ($P2Y_1$ > $P2Y_2$ >> $P2Y_4$).

In summary, we have shown that suramin is capable of distinguishing between the $P2Y_1$-, $P2Y_2$- and $P2Y_4$- purinoceptors. Previous reports of suramin sensitive and suramin insensitive $P2Y_2$ receptors, characterised using UTP, have lead to the proposal of the existance of two or more $P2Y_2$ subtypes. However, the data presented here suggests that, in some cases, suramin insensitive P_2 purinoceptor responses to UTP may be due to a $P2Y_4$ receptor.

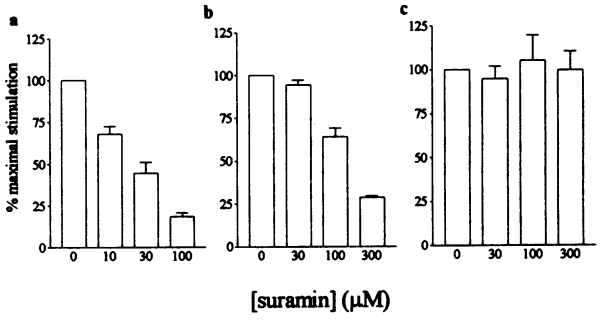

Figure 1. The effect of increasing concentrations of suramin on responses to **a)** 0.3 μM 2MeSATP on turkey $P2Y_1$, **b)** 10 μM UTP on human $P2Y_2$ and **c)** 10 μM UTP on human $P2Y_4$, all transfected into 1321N1 cells.

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235P PPADS REVEALS P2Y PURINERGIC RESPONSES IN TRANSFECTED 1321N1 CELLS

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Extracellular ATP interacts with P2Y purinoceptors. Recently a number of cDNAs encoding a variety of P2Y purinoceptors have been isolated and stably expressed in human astrocytoma 1321N1 cells having no native purinergic response. The ability of these cells to release nucleotides such as ATP in response to shear stress has recently been reported (Lazarowski et al 1995). In this study we report that the transfection of 1321N1 cells with P2Y purinoceptors is itself sufficient to enhance activity of phospholipase C (PLC), and provide evidence that this is caused by release of endogenous nucleotides.

1321N1 cells were transfected with either the turkey $P2Y_1$ (P_{2Y}) or the human $P2Y_2$ (P_{2U}) purinoceptor. Bovine aortic endothelial (BAE) cells co-express native $P2Y_1$ and $P2Y_2$ purinoceptors. Cells were in 24 well multiwells; total [^{3}H]inositol phosphate levels were determined as described (Charlton et al 1996). Pyridoxalphosphate-6-azophenyl-2,4-disulphonic acid (PPADS), an antagonist at $P2Y_1$ purinoceptors (Brown et al 1995) was included for 10 min prior to, as well as during the agonist stimulation period. Data were compared using Students t-test (n=3)

Using 1321N1 cells transfected with either $P2Y_1$ or $P2Y_2$ purinoceptors we showed that basal accumulation of inositol phosphates was elevated over those in non transfected cells (10.4 ± 1.8 and 3.1 ± 0.4 fold over non transfected for $P2Y_1$ and $P2Y_2$ 1321N1 cells respectively). In 1321N1 $P2Y_2$ cells the mean stimulation of inositol phosphates by UTP (100μM) (expressed as fold over basal) was 3.8 ± 0.9. However in 1321N1 $P2Y_1$ cells, a maximally effective dose of the selective agonist 2 methylthio-ATP (2MeSATP) (3μM) resulted in a smaller stimulation (1.51 ± 0.02 fold over basal). To further investigate whether the basal accumulation of inositol phosphates was due to release of endogenous nucleotides we used PPADS. At 30μM, PPADS significantly reduced the basal accumulation of inositol phosphates in $P2Y_1$ cells (5829 ± 664 vs 2340 ± 55 dpm for control vs PPADS treated respectively $p<0.05$). Furthermore, under conditions in which no stimulation of $P2Y_1$ purinoceptors could be seen, PPADS revealed a significant stimulation mediated by 100μM ADP (In the absence of PPADS, 6239 ± 430 vs 5951 ± 707, in the presence of PPADS (30μM), 2428 ± 178 vs 6013 ± 733 $p< 0.01$ for basal vs ADP stimulated respectively). In BAE cells, the basal accumulation of inositol phosphates was dose dependently inhibited by PPADS (0.3-10μM) (significantly different $p<0.001$ on analysis of variance of inhibition by PPADS). To reduce the high basal accumulation of inositol phosphates observed in 1321N1 $P2Y_1$ cells, the stimulation procedure was modified to one where the cells were not washed, and LiCl and agonists were added directly to the labelling medium. This modification resulted in a substantial reduction in basal accumulation of inositol phosphates and revealed a significantly larger stimulation mediated by 2MeSATP (1.51 ± 0.02 vs 4.18 ± 0.17 $p<0.01$ expressed as fold over basal).

These observations reveal that both 1321N1 and BAE cells release endogenous nucleotides into the extracellular medium in response to shear stress, and that this can stimulate PLC coupled to both native and P2Y transfected purinoceptors.

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236P EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR ON NITRIC OXIDE SYNTHASE AND CYCLOOXYGENASE EXPRESSION IN ENDOTHELIAL CELLS

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Angiogenesis is an essential part of a number of pathophysiological processes including wound healing, tumourogenesis and rheumatoid arthritis (Folkman and Shing 1992). Vascular endothelial growth factor (VEGF) is a cytokine that stimulates angiogenesis by unknown cellular mechanisms (Leung et al., 1989). Here we have investigated the effect of VEGF on the expression of nitric oxide synthase (NOS) and cyclooxygenase (COX) in primary bovine and human endothelial cells.

Bovine aortic endothelial cells (BAEC) were isolated and cultured in Dulbecco's modified minimal essential medium containing 10% fetal calf serum (FCS). Human endothelial cells (HUVEC) were isolated from umbilical cords and grown in endothelial cell growth medium (ECGM; Promocell, Germany). BAEC and HUVEC (both passage 1-4) were treated with VEGF (0.001-10 ng/ml) for 24 h. Equal quantites of protein from the cell extracts were subjected to Western blot analysis using primary antibodies for endothelial NOS (eNOS), inducible NOS (iNOS; 1:1000, 1:2000 respectively, Bryant et al., 1995), COX1 (1:7500, Merck, Sharpe and Dohme) and COX2 (1:10000, Cayman Chemical Co.). Protein bands were visualised using diaminobenzidine or enhanced chemiluminescensce (ECL, Amersham, UK) and were quantified using densitometric analysis. Cellular proliferation was measured using a fluorometric assay for DNA content. To measure the effect of VEGF on HUVEC proliferation, cells were placed in basic ECGM containing 2% FCS for 12 h to render them quiescent. HUVEC were then cultured for a further 48 h in the presence of VEGF (10 ng/ml).

Western blot analysis of untreated BAEC demonstrated constitutive expression of eNOS and COX1, but not of iNOS or COX2. VEGF did not change the expression of eNOS or induce the expression of either iNOS or COX2 (n=4). Conversely VEGF increased COX1 expression. For instance 10 ng/ml VEGF produced an increase of 68±20% in COX1 expression (n=4). Similarly VEGF upregulated COX1 expression in HUVEC by 84±23% at a concentration of 10 ng/ml (n=4). A preliminary time course study suggested that COX1 protein is upregulated after 2 h in HUVEC (n=2). Treatment of HUVEC with VEGF at a dose of 10 ng/ml produced a 32±9% increase in cellular DNA confirming that VEGF can cause cellular proliferation (n=4). There was no basal or VEGF stimulated expression of either iNOS or COX2 in HUVEC (n=3).

This study shows that VEGF causes cellular proliferation in HUVEC and upregulates COX1 protein expression in both HUVEC and BAEC. VEGF did not affect the expression of eNOS, iNOS or COX2. This data suggests that COX1 may play an important role in the response of endothelial cells to VEGF. It also suggests that VEGF-mediated angiogenesis may be more dependent on the activity of COX1 than COX2.

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237P MICROASSAY FOR THE ASSESSMENT OF NOVEL SUPEROXIDE DISMUTASE-MIMETICS *IN VITRO*

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The detection of superoxide anion by the reduction of ferricytochrome c is a popular biochemical method. Originally described by McCord & Fridovich (1969) in their study of the action of the enzymic superoxide anion scavenger, superoxide dismutase (SOD), the method has lent itself to the assessment of SOD-mimetics such as the catechol species, 1,2-dihydroxybenzene-3,5-disulfonate (tiron) and the stable nitroxide, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Miller & Rapp, 1973; Samuni *et al.*, 1990). Our aim was to develop a rapid, reliable and economical, platereader-based, microassay that would expedite the assessment of putative SOD-mimetics *in vitro*.

The assay mixture consisted of (final concentration): 50 µl ferricytochrome c (100 µM); 10 µl xanthine oxidase (5-20 mU/ml); 20 µl hypoxanthine (100 µM); and 20 µl, isotonic phosphate (10 mM)-buffered saline (pH 7.4) to make a total volume of 100 µl in a 96-well plate. The reaction was conducted at room temperature and initiated by the addition of hypoxanthine. The increase in optical density at 550 nm was measured over a 3 min period at 30 s intervals using a kinetic platereader and the initial reaction rate determined. Data are mean±s.e. mean.

There was a linear relationship between the initial reaction rate and the concentration of xanthine oxidase (5-20 mU/ml) ($r=0.9982$, $P<0.002$, $n=4$). Blank controls lacking either enzyme or substrate showed no activity. Xanthine oxidase at 20 mU/ml, which provided an initial reaction rate of 28.3±1.7 mOD/min (n=4), was adopted for subsequent inhibition studies (see McCord & Fridovich, 1969). The initial reaction rate was depressed by SOD (200 U/ml) by 95.3±1.1% ($n=4$, $P<0.01$) and abolished by tiron (10 mM, n=3) and TEMPO (0.3 mM, n=7). The pIC_{50} values for tiron and TEMPO were determined to be 3.47±0.04 (n=3) and 4.29±0.05 (n=7), respectively. In contrast, catalase (CAT, 200 U/ml:inhibition=-2.2±2.4%, n=3, $P>0.05$) and dimethylthiourea (DMTU, 10 mM:inhibition=-0.1±3.8%, n=3, $P>0.05$) had no significant effect.

The linear dependence of the initial reaction rate on the concentration of xanthine oxidase, indicates that both substrate and detecting species were in adequate excess. Furthermore, the obligatory participation of superoxide anions was confirmed using established scavengers of this reactive oxygen species while there was no evidence of a role for either the hydroxyl radical or hydrogen peroxide. The poor potency exhibited by tiron and TEMPO is consistent with the high scavenging concentrations employed in recent studies (Münzel *et al.*, 1995; Gelvan et al., 1991; Samuni et al., 1990) and reflects the stoichiometric nature of superoxide anion inhibition. We anticipate this microassay will allow the evaluation of novel SOD-mimetics for possible use in the treatment of conditions associated with oxidant stress.

This work was supported by Lipha s.a., Lyon, France.

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238P A NITRIC OXIDE ADDUCT MAY BE PRODUCED BY THE METABOLIC ACTIVATION OF GLYCERYL TRINITRATE IN VASCULAR SMOOTH MUSCLE

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Glyceryl trinitrate (GTN) is a vasodilator that has enjoyed extensive clinical use in the treatment of angina pectoris. Associated with its chronic use is the development of tolerance, which appears to stem from impaired vascular biotransformation of GTN to nitric oxide (NO), the putative active moiety that activates guanylyl cyclase to effect a cascade of events resulting in vascular dilation. This study was designed to test the hypothesis that NO is the relaxant metabolite produced by metabolic activation of GTN in vascular tissue. A tissue bioassay of rabbit taenia coli strip (RTCS) can be relaxed by a diffusible factor produced by adjacent rabbit aortic strip (RAS) treated with GTN (Hussain *et al*, 1994). The hypothesis predicts that superoxide ($O_2^{\cdot-}$) should scavenge the relaxing factor (presumably NO) and attenuate RTCS relaxation. When superoxide, generated by xanthine (10 µM) / xanthine oxidase (20 mU/ml), was included in an oxygenated 37°C tissue bath containing RTCS and RAS incubated in Krebs buffer, no significant change in RTCS sensitivity to GTN (0.1 nM - 10 µM) was observed compared to the same preparation in the absence of $O_2^{\cdot-}$ (EC_{50} values of relaxation were 78.1±5.2 nM and 84.1±8.7 nM, respectively, n=5). In contrast, the EC_{50} for 3-morpholinosydnonimine (SIN-1, which forms NO spontaneously) relaxation of RTCS was significantly increased by $O_2^{\cdot-}$ (0.2±0.1µM vs 7.5±2.0 nM, n=5, $P<0.01$). NO gas (200 µL, 5%) caused a 78.3±5.3% relaxation of K^+-precontracted RTCS, whereas tissues in the presence of $O_2^{\cdot-}$ relaxed only 14.2±8.4% and this attenuation of RTCS relaxation to NO gas was reversed by superoxide dismutase (100 U/ml). These observations were interpreted to indicate that some factor resistant to inactivation by superoxide is formed during GTN biotransformation. It is possible that such a factor is a nitric oxide adduct rather than NO *per se*. As nitrosothiols and other NO conjugates are susceptible to homolytic cleavage by UV light, such photosensitivity could be exploited to form NO from a putative adduct, which in turn could be detected by chemiluminescence (Brien *et al*, 1991). Bovine pulmonary artery (BPA) incubated with 100 µM GTN produced a time-dependent increase in NO formation which was increased in the presence of long wave UV light. After 0.5 and 2 min of incubation, no NO was detected from GTN incubated BPA; however, in the presence of UV light, signals of 159±45 and 171±37 pmol NO/g tissue were observed, respectively (n=4). Longer incubation times of 5 and 10 min UV light exposure resulted in greater levels of NO compared to untreated controls (276±39 and 729±170 pmol NO/g vs 101±20 and 118±19, $P<0.01$, n=4). Overall, these data are consistent with an adduct of NO being produced by vascular biotransformation of GTN.

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239P CYCLIC GMP-INDEPENDENT RESPONSES TO NITRIC OXIDE IN HUMAN PLATELETS

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Nitric oxide (NO) donors inhibit agonist-induced calcium mobilization in human platelets (MacIntyre *et al.*, 1985). NO-induced decreases in basal intracellular calcium concentrations ($[Ca^{2+}]_i$) in fibroblasts are reported to be cyclic GMP (cGMP)-independent (Garg & Hassid, 1991). We have, therefore, investigated whether the NO donor, 3-morpholinosydnonimine (SIN-1) could produce cGMP-independent inhibition of agonist-induced Ca^{2+} mobilisation in human platelets.

Human platelet rich plasma was incubated with fura-2/AM (2 μM; 45 min; 37°C). A washed platelet preparation was prepared (2 x 10^8 platelets/ml in HEPES (5mM) Tyrodes). Fluorescence was measured using a Perkin-Elmer spectrophotometer and $[Ca^{2+}]_i$ calculated (Grynkiewicz *et al.*, 1985). Platelet cGMP levels were measured using a scintillation proximity assay. A fibrinogen receptor antagonist, GR144053 (1 μM; Eldred *et al.*, 1994), was added to all experiments to prevent platelet aggregation. Data are expressed as mean ± s.e.mean.

The thromboxane A_2 mimetic, U46619, evoked biphasic elevations of $[Ca^{2+}]_i$. The plateau phase of the increase in $[Ca^{2+}]_i$ was 63.8 ± 3.7 nM (n=15) above resting $[Ca^{2+}]_i$ (46.2 ± 9.7 nM; n=17). The effects of SIN-1 on the plateau phase of the U46619 response were investigated. SIN-1 (10 μM) inhibited U46619 (1 μM)-induced rises in $[Ca^{2+}]_i$ by 71.1 ± 2.1% (n=15). This was unaffected by either the small or large conductance Ca^{2+}-dependent potassium (K^+) channel blockers apamin (100 nM; 30 mins) and iberiotoxin (30nM; 30min), respectively. However, the soluble guanylyl cyclase inhibitor, 1*H*-[1,2,4]Oxadiazolo [4,3-*a*]quinoxalin-1-one (ODQ; 10 μM; 15 mins), partially reduced the SIN-1-induced decrease in $[Ca^{2+}]_i$. In the presence of ODQ and apamin the SIN-1 response was abolished, whilst iberiotoxin did not potentiate the inhibitory effects of ODQ. Forskolin (10 μM)-induced changes in $[Ca^{2+}]_i$ were unaffected by ODQ, apamin or iberiotoxin.

Table 1 Effects of various agents on the inhibition of U46619-induced $[Ca^{2+}]_i$ (plateau) by SIN-1 and Forskolin. Responses are expressed as % inhibition of the plateau phase induced by U46619 (n=5-10, *indicates p <0.01, data was analysed using Student's paired t-test).

Pre-treatment	U46619 (1μM) + SIN-1 (10 μM)	U46619 (1μM) + Forskolin (10 μM)
Control	71.1 ± 2.1	64.0 ± 5.9
ODQ (10 μM)	43.7 ± 6.3 *	62.3 ± 10.9
Apamin (100 nM)	69.5 ± 3.2	73.4 ± 7.9
Apamin and ODQ	5.8 ± 3.7 *	75.5 ± 2.7
Iberiotoxin (30 nM)	75.2 ± 1.7	57.3 ± 8.0
Iberiotoxin and ODQ	48.4 ± 9.7 *	78.0 ± 7.4

In the presence of 3-isobutyl-1-methyl-xanthine (0.3 mM), and U46619 (1 μM), SIN-1 (10 μM) increased cGMP levels from 834 ± 56 to 1882 ± 186 pmol/10^8 platelets (n=5; p <0.01). ODQ (± apamin) abolished the SIN-1-induced elevation of cGMP (n=5; p <0.01), while apamin alone had no effect (1617 ± 36 pmol/10^8 platelets, n=5, p >0.01).

These data suggest that NO-evokes cGMP-dependent and -independent decreases in $[Ca^{2+}]_i$ in human platelets. The cGMP-independent pathway may involve the activation of apamin-sensitive K^+ channels.

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240P PROTECTION OF LOW DENSITY LIPOPROTEIN AGAINST COPPER-MEDIATED OXIDATIVE MODIFICATION: THE IMPORTANCE OF LIPOXYGENASE INHIBITION AND ANTIOXIDANT ACTION

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Antioxidants are effective in reducing low density lipoprotein (LDL) oxidation (Esterbauer *et al.*, 1991). Nordihydro-guaiaretic acid (NDGA) is a potent inhibitor of lipoxygenase and also an effective antioxidant (Shappell *et al.*, 1990). The present study was designed to examine the importance of lipoxygenase inhibition and antioxidant action in the protec-tion of LDL against copper-mediated oxidative modification.

Human LDL were prepared by ultracentrifugation and oxida-tion of LDL was produced in the presence of 5 μM Cu^{2+} as described previously (Liu *et al.*, 1994) The effect of NDGA were compared with other antioxidants (vitamin(V) E, vitamin C and butylated hydroxytoluene, BHT). Lipoxygenase inhibitors with (phenidone) and without (diethylcarbamazine, DEC, and L-739,010) antioxidant activity were also tested. Oxidative modification of LDL was assessed by monitoring the formation of thiobarbituric acid-reactive substances (TBARS), lysophosphatidylcholine (LPC) production and relative changes in electrophoretic mobility (REM). Data were analyzed by ANOVA and presented as mean ± SD (n=4 to 6).

Pre-incubation with 50 μM NDGA was effective in preventing TBARS formation and significantly reduced REM and LPC formation for 24 h (figure 1). 50 μM of VE, VC and BHT also reduced the production of TBARS, LPC and REM. However, their effects were less than that produced by NDGA (figure 1).

Pre-incubation of the LDL with non-antioxidant lipoxygenase inhibitors, L-739,010 and DEC, showed little or no inhibition of the oxidative modification (figure 2). Addition of L-739,010 did not increase the protective effect of VC against oxidative modification of LDL.

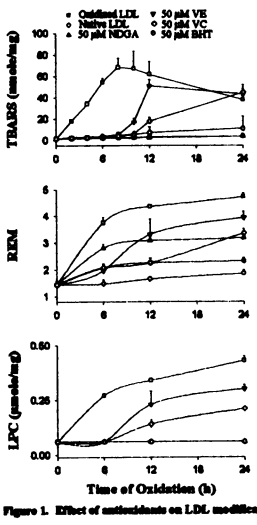

Figure 1. Effect of antioxidants on LDL modification

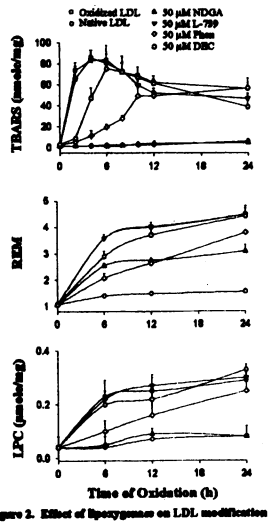

Figure 2. Effect of lipoxygenase on LDL modification

This study demonstrates that the protective effect of NDGA on oxidation of LDL, as assessed by several measures of the modification process, is a function of its antioxidant properties, rather than its inhibition of lipoxygenase.

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241P POTENCIES OF NONSTEROID ANTIINFLAMMATORY DRUGS AS INHIBITORS OF PGE_2 PRODUCTION BY COX-2 CONTAINING A549 CELLS VARY WITH CELLULAR STIMULI

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It has recently been reported that the potency of nonsteroid drugs (NSAIDs) as inhibitors of cyclooxygenase-2 (COX-2) in intact human airway epithelial (A549) cells varies with assay conditions (Saunders et al., 1996). In particular, that NSAIDs more potently inhibit the accumulated production of prostanoids over hours than the acute production stimulated by exogenous arachidonic acid. We have examined this further using as an additional, acute cell stimulus, calcium ionophore (A23187).

Confluent A549 cells (grown in Dulbecco's modified Eagle's medium) were washed and incubated for 24 h with fresh medium in the presence of IL-1β (10 ng ml^{-1}), to induce COX-2 (Mitchell et al., 1994). In some experiments drugs were added together with the IL-1β, to assess their effects on the accumulation of PGE_2 (an indicator of COX-2 activity; measured by radioimmunoassay) and the expression of COX-2 protein (determined by Western blot analysis). In other experiments the medium was removed from the A549 cells after 24 h and replaced with fresh medium containing test compounds or vehicles. After a further 30 min arachidonic acid (AA, 30 μM) or A23187 (10 μM) was added and the cells incubated for a further 15 min to permit the stimulated, acute production of PGE_2.

Ibuprofen, indomethacin or diclofenac all more potently inhibited the accumulation of PGE_2 over 24 h than the acute formation of PGE_2 induced by AA or A23187 (Table 1). None of the agents affected the expression of COX-2 as assessed by Western blot analysis. Dexamethasone incubated together with IL-1β for 24 h potently inhibited the accumulation of PGE_2 (IC_{50} <1 pg ml^{-1}, n=12) but unlike the NSAIDs this was associated with inhibition of the expression of COX-2 protein.

Thus, in A549 cells NSAIDs inhibit more potently the accumulated formation of PGE_2 than the acute stimulated formation following exposure to AA or A23187. This may well be because the availability of AA to COX-2 is much lower during the period of the PGE_2 accumulation assay than following the acute exposure to exogenous AA or A23187. Thus, our results support the suggestion (Saunders et al., 1996) that the potencies of NSAIDs may vary with the availability of AA to COX-2.

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Table 1. IC_{50} (μg ml^{-1}) values of NSAIDs as inhibitors of PGE_2 formation (n=18-24).

NSAID	accumulation	acute AA	acute A23187
ibuprofen	0.38	0.49	6.8
indomethacin	0.005	0.2	0.07
diclofenac	0.00009	0.006	0.006

242P PREVENTION OF THE EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE BY AMONOGUANIDINE OR AMINOETHYL-ISOTHIOUREA

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An enhanced formation of nitric oxide (NO) following the induction of the inducible isoform of NOS (iNOS) has been implicated in the pathogenesis of circulatory shock and inflammation (Miller et al., 1993; Thiemermann, 1994). This study elucidates the effects of the NOS inhibitors aminoethyl-isothiourea (AE-ITU), aminoguanidine (AG), N^G-methyl-L-arginine (L-NMMA) or N^ω-Nitro-L-arginine methyl ester (L-NAME) on the expression of iNOS protein in macrophages and the rat by endotoxin.

Murine macrophages (J774.2) were cultured in DMEM containing L-glutamine (3.5 mM) and 10% foetal calf serum. To induce iNOS, fresh culture medium containing *E.coli* 0127:B8 lipopolysaccharide (LPS; 1 μg ml^{-1}, serotyp: 0127:B8) was added. Fifteen min prior to LPS, cells were treated with vehicle (saline), AE-ITU (100 μM, n=6), AG (1 mM, n=6), L-NMMA (1 mM, n=6), or L-NAME (1 mM, n=6). After 24 h, nitrite accumulation in the cell culture medium was measured by the Griess reaction and expression of iNOS by Western blot analysis following SDS-PAGE electrophoresis (7.5% polyacrylamide).

Male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and the femoral vein for the administration of compounds. At time 0, rats received saline (0.9% NaCl; 1 ml kg^{-1} i.v., n=6) or LPS (10 mg kg^{-1} i.v., n=6). At 2 h, a continuous infusions of vehicle (0.6 ml kg^{-1} h^{-1} saline, i.v., n=6), AE-ITU (1 mg kg^{-1} h^{-1} i.v., n=6), AG (5 mg kg^{-1} h^{-1} i.v., n=6), L-NMMA (3 mg kg^{-1} h^{-1} i.v., n=6), or L-NAME (0.3 mg kg^{-1} h^{-1} i.v., n=6) was started until the end of the experiment 6 h after the administration of LPS. At 6 h after LPS, rats were killed and lungs were removed for the measurement of the expression of iNOS protein by Western blot analysis as described above. Blots were densitrometrically analysed using a GS 700 Imaging Densitrometer (Bio-Rad) and the computer programm Molecular Analyst® (Macintosh).

AE-ITU, AG, L-NMMA and to a lesser extent L-NAME inhibited the increase in nitrite production elicited by LPS (Table1). LPS resulted in the expression of iNOS protein in macrophages, which was significantly inhibited by pretreatment of cells with AE-ITU or AG, but not by L-NMMA or L-NAME (Table1). In addition, LPS also caused an increase in the expression of iNOS protein in lungs obtained from rats at 6 h after endotoxin, which was significantly reduced by treatment of LPS-rats with AE-ITU or AG, but not with L-NMMA or L-NAME. (Table1).

Table 1

Treatment	Blot Density (OD*mm^2*10^{-2})		Nitrite (J774.2)
	J774.2	lung	(μM)
vehicle (saline)	0.4 ± 0.4	8 ± 3	2 ± 0.3
LPS	45 ± 8	76 ± 15	54 ± 5
+ AE-ITU	3 ± 1*	28 ± 5*	4 ± 1*
+ AG	10 ± 3*	38 ± 7*	6 ± 1*
+ L-NMMA	39 ± 5	61 ± 9	8 ± 2*
+ L-NAME	45 ± 9	64 ± 7	21 ± 5*

mean ± s.e.mean. *p<0.05 vs LPS, unpaired Student's *t* test.

Thus, AE-ITU or AG inhibit not only iNOS activity, but also the induction of iNOS *in vitro* (macrophages) and *in vivo* (rat) caused by endotoxin.

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243P LIPOTEICHOIC ACID FROM VARIOUS GRAM-POSITIVE BACTERIA EITHER ALONE OR IN COMBINATION WITH A FRAGMENT OF PEPTIDOGLYCAN INDUCE NITRIC OXIDE SYNTHASE IN MURINE MACROPHAGES

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The cell wall components of *Staphylococcus aureus*, lipoteichoic acid (LTA) and peptidoglycan (PepG), synergise to induce nitric oxide synthase (iNOS) activity in murine macrophages (De Kimpe *et al.*, 1995). A moiety of PepG, N-acetyl-D-glucosaminyl-β-[1→4]-N-acetylmuramyl-L-ala-D-isoglutamine (NAG-AP) accounts for the ability of PepG to synergise with LTA to induce iNOS activity in macrophages (Kengatharan *et al.*, 1996). However, structural differences in the LTAs from *S.aureus* (a pathogenic bacterium) and *B.subtilis* (a non-pathogenic bacterium) may be responsible for the lack of synergy between *B.subtilis* LTA and PepG (or NAG-AP) in inducing iNOS activity in macrophages (this meeting, Kengatharan *et al.*, 1996). Here, we investigate whether structurally different LTAs from various Gram-positive bacteria induce iNOS activity either alone or in combination with NAG-AP.

Murine macrophage cells (J774.2) were cultured in 96-well plates with culture medium (DMEM) containing foetal calf serum (10%) and glutamine (4mM) until cells reached confluence. LTAs from *Staphylococcus aureus*, *Streptococcus sanguis*, *Streptococcus mutans* or *Streptococcus faecalis* or *Streptococcus pyogenes* were added to the cells either alone or in combination with NAG-AP (1-10μg.ml^{-1}) and/or N^{G}-methyl-L-arginine (L-NMMA, 10-300μM). During the preparation of LTA solution, care was taken to prevent contamination with endotoxin. In one experiment, cells were incubated with dexamethasone (10μM) for 90 min before the addition of LTAs. Nitrite accumulation, an indicator of NO formation, was measured 24h later in the supernatant of J774.2 cells by the Griess method.

LTAs from *S.aureus, S.sanguis, S.mutans, S.faecalis* and *S.pyogenes* induced a concentration-dependent (0.01-30μg.ml^{-1}) increase in the nitrite formation in the supernatant of J774.2 macrophages from baseline (2±1μM, n=12) (Table 1). However, the *S.pyogenes* LTA was less potent in eliciting nitrite formation compared to the other LTAs tested. The nitrite formation induced by the LTAs were attenuated by dexamethasone (an inhibitor of iNOS expression) as well as L-NMMA (an inhibitor of iNOS activity). Furthermore, LTAs from *S.aureus, S.sanguis, S.mutans* and *S.faecalis* synergised with NAG-AP to induce nitrite formation (NAG-AP alone; at 1μg.ml^{-1}; 2.3±0.6μM, n=19) (Table 1). The synergy between *S.pyogenes* LTA and NAG-AP was observed at a higher concentration of the LTA (LTA at 10μg.ml^{-1}; 31±5μM vs. LTA+NAG-AP; 50±5μM, $P<0.05$, n=12-19). The increases in nitrite formation by LTAs+NAG-AP were also attenuated by L-NMMA.

Table 1. The enhanced formation of nitrite caused by various LTAs is potentiated by NAG-AP and attenuated by L-NMMA in J774.2 cells.

Bacteria	Synergy with NAG-AP		inhibition by L-NMMA	
	LTA (1μg.ml^{-1})	+NAG-AP at (1μg.ml^{-1})	LTA (10μg.ml^{-1})	+L-NMMA at (100μM)
S.aureus	37±7	74±2#	73±2	29±1*
S.sanguis	33±2	63±2#	54±2	18±1*
S.mutans	20±2	29±3#	50±1	20±2*
S.faecalis	3±1	21±2#	42±7	9±2*

Nitrite values (in μM) are in mean ± s.e.mean (n=9-19); #$P<0.05$ *vs* LTA (1μg.ml^{-1}) & *$P<0.05$ *vs* LTA (10μg.ml^{-1}) by unpaired *Student's* t-test.

These results show that structurally different LTAs can induce iNOS activity in macrophages either alone or in synergy with NAG-AP. The fact that NAG-AP is a moiety that is conserved in the structure of PepG from a wide range Gram-positive bacteria, synergy between LTA and NAG-AP, hence PepG, may contribute importantly to the mechanism by which Gram-positive bacteria cause the induction of iNOS in macrophages.

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244P L-ARGININE OR TIRON ATTENUATE THE INDUCTION OF HEMEOXYGENASE-1 IN LUNGS AND THE *IN VIVO* OXIDATION OF DIHYDRORHODAMINE 123 IN A MODEL OF GRAM-POSITIVE SHOCK

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Free radicals induce the expression of stress proteins such as hemeoxygenase-1 (HO-1; Keyse *et al.*, 1991). Nitric oxide (NO) synthases (NOSs) in the absence of their substrate, L-arginine, produce reactive oxygen species such as superoxide (see Griffith & Stuehr, 1996). In addition, NO and superoxide anions react to form a potent oxidant, peroxynitrite. We have previously shown that lipoteichoic acid (LTA) and peptidoglycan (PepG) from *Staphylococcus aureus* synergise in expressing iNOS protein and activity *in vivo* (De Kimpe *et al.*, 1995). Here, we study the effect of the superoxide scavenger, tiron, or modulators of NO synthesis on (i) the expression of HO-1 (in lungs), (ii) the expression and activity of iNOS (in lungs), and (iii) the *in vivo* oxidation of dihydrorhodamine 123 (DHR123) elicited by co-administration of LTA and PepG in rats.

Male Wistar rats (250-350g) were anaesthetised with thiopento-barbitone sodium (120mg.kg^{-1}, i.p.). The trachea was cannulated to facilitate spontaneous respiration. The jugular vein was cannulated for the administration of compounds. At 2 or 4h after the injection of LTA (3 mg.kg^{-1}) and PepG (5 mg.kg^{-1}) the animals were killed and (i) the lungs were removed to determine the expression of HO-1 and iNOS protein by Western blot analysis and for determining iNOS activity via the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline, and (ii) plasma was obtained for the determination of nitrite & nitrate by the Griess method. In addition, at 4h, animals received DHR123 (2μmol.kg^{-1} in 10% dimethylsulphoxide) and plasma was obtained after 20 min and the levels of rhodamine, an indicator of the formation of peroxynitrite *in vivo*, was determined (Szabo *et al.*, 1995). Some animals received tiron (300 mg.kg^{-1}), L-arginine (15mg.kg^{-1} + 30mg.kg^{-1}.h^{-1}), D-arginine (15mg.kg^{-1} + 30mg kg^{-1}.h^{-1}) or aminoguanidine (AMG, 5mg.kg^{-1} + 10mg.kg^{-1}.h^{-1}) at 2 h after LTA+PepG administration.

Injection of LTA and PepG resulted in (i) a delayed expression of HO-1 and iNOS proteins in the lungs, (ii) enhanced activity of iNOS in the lungs, (iii) increases in plasma levels of nitrite & nitrate, and (iv) increased *in vivo* oxidation of DHR123 to rhodamine (rhodamine; 0.05±0.3nM for sham *vs* 9.34±2.90nM for LTA+PepG, $p<0.05$, n=4-8) (Table 1). Treatment with tiron attenuated the expression of HO-1 protein and the rises in the plasma levels of rhodamine without affecting the expression of iNOS protein or activity. However, AMG reduced the iNOS activity and the rises in plasma levels of rhodamine without affecting the expression of HO-1. Interestingly, L-arginine reduced the expression of HO-1 as well as the rises in rhodamine levels without affecting iNOS expression or activity. In addition, L-arginine significantly increased the plasma levels of nitrite & nitrate. In contrast, D-arginine did not affect any of the parameters studied.

Table 1. Effect of tiron or modulators of NO synthesis on HO-1 and iNOS expression and iNOS activity and the oxidation of DHR 123.

Group	Protein density (% 4h control)		Plasma nitrite & nitrate (μM)	Plasma rhodamine (% control)
	HO-1	iNOS		
sham (4h)	29±17	0±0	35±2	1±5
LTA+PepG (2h)	20±7	24±8	70±5	2±5
LTA+PepG (4h)	100	100	202±14	100
+tiron (4h)	47±11#	101±38	194±6	37±5#
+L-arginine (4h)	67±8#	90±16	289±15#	65±13#
+AMG (4h)	85±18	164±22#	119±7#	42±16#

Values as mean ± s.e.mean (n=4-8); #$P<0.05$ *vs* LTA+PepG (4h) by One sample *t*-test or by ANOVA (Bonferroni's test) as appropriate.

These results show that LTA+PepG cause (i) the formation of free radicals in lungs as indicated by the expression of HO-1, and (ii) the generation of superoxide anions and NO which contributes to the formation of peroxynitrite *in vivo*. The fact that L-arginine, but not D-arginine, reduced expression of HO-1 and the rises in rhodamine levels suggests that formation of free radicals, possibly from NOS, can be attenuated by increasing the concentration of the substrate for NOS *in vivo*.

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245P EFFECTS OF L-ARGININE ON TUMOUR CELL GROWTH: A ROLE FOR POLYAMINES OR NO?

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L-Arginine is the precursor of the potent vasodilator, nitric oxide (NO) but it can also be metabolised through the urea cycle to urea and L-ornithine. L-Ornithine is the precursor of the polyamine growth factors, putrescine, spermidine and spermine, and hence arginine can have marked effects on cell growth as well as blood pressure. The effects of arginine on growth are variable with small tumours regressing and large tumours being stimulated in the the presence of arginine (Levy et al., 1954). One suggestion for this dual effect of arginine is that tumour growth may be promoted via stimulation of polyamine biosynthesis and growth inhibition may be mediated via production of NO. The aim of this study was to investigate this hypothesis by determining the effects of L-arginine on the growth of human tumour cells and the role of the polyamines and NO in producing these effects.

Human colonic cancer cells (HT115) were grown in culture in DMEM supplemented with 10% (v/v) horse serum. Cells were allowed to attach to the culture plates for 16 h and then grown for a further 48 h before exposure to L-arginine for 24 h. Protein and polyamine content was measured as described previously (Wallace *et al.*, 1988). Cell number was determined by MTT assay and NO was measured as nitrate and nitrite by Griess reaction.

L-Arginine increased the growth of HT115 cells in culture (Table 1). Intracellular arginine concentrations increased but, in contrast, the polyamine content decreased. There was no change in NO production in the presence of arginine.

Table 1: Effect of L-arginine on growth, polyamine and arginine content of HT115 cells

Conc of Arg (μM)	Cell No ($\times 10^4$)	Polyamine Content (nmol/mg protein)	Arginine Content (nmol/mg protein)
0	28.9 +/- 2.3	35.0 +/- 5.4	5.7 +/- 0.4
1	60.0 +/- 1.8*	24.3 +/- 2.8	6.4 +/- 1.2
100	53.3 +/- 2.0*	26.4 +/- 1.0	25.0 +/- 2.9*

Values are mean +/- SD (n=3) * $p < 0.01$ compared to control

The mechanism by which arginine increases cell growth is not clear but it does not appear to result from an increase in polyamine content or a decrease in NO content in these cells.

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246P DEVELOPMENTAL PROFILE OF IBMX-SENSITIVE CYCLIC GMP ACCUMULATION IN THE RAT CEREBELLUM

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The non-selective phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX), and the cyclic AMP elevating agent forskolin enhance basal and sodium nitroprusside (SNP)-elevated cyclic GMP levels in the guinea-pig cerebellum (Hémandez *et al.*, 1994). We suggested that the effect of IBMX might be due to direct PDE inhibition, and that elevation of cyclic AMP levels by forskolin prevents cGMP catabolism through direct competition at a common PDE. In the present study, we have investigated the effects of IBMX and forskolin on the accumulation of cyclic GMP in the rat cerebellum during different stages of development.

The accumulation of [^{3}H]-cyclic GMP was carried out at 37°C with rat (Wistar, male) cerebellar slices pre-incubated with [^{3}H]-guanosine, as previously described (Hernández *et al.*, 1994). Neonatal (8-10 PND) preparations consisted of 400μm parasagital slices (1-2 slices/tube in 300μL total volume). Cerebellar granule cells were cultured (DMEM + 25mM KCl and 2mM glutamine) from neonatal rats (8-10 PND) and assayed for [^{3}H]-cyclic GMP accumulation between 8 and 10 days *in vitro*. Data were expressed as a percentage conversion from the total [^{3}H]-guanine nucleotides with basal levels subtracted, from experiments carried out on at least 3 separate occasions.

Basal accumulation of [^{3}H]-cyclic GMP in cerebellar slices from neonatal, 200-220g, and 350-400g rats were 0.35 ± 0.10, 0.72 ± 0.13, 0.46 ± 0.07 % conversion, respectively. In the presence of 1mM IBMX, no significant change was observed (values were 0.35 ± 0.08, 1.01 ± 0.15 and 0.65 ± 0.10 % conversion, respectively). Similarly, the presence of 30 μM forskolin failed to alter basal values (0.26 ± 0.06, 0.76 ± 0.08, 0.47 ± 0.05% conversion, respectively).

The cyclic GMP response in the presence of 1mM SNP (10 mins) was greatest in slices derived from neonates, 4.41 ± 0.58 % conversion, compared with the 200-220g (1.09 ± 0.32 %) and 350-400g rats (1.14 ± 0.32 %). IBMX (1mM for 10 mins) had no significant effect on [^{3}H]-cyclic GMP accumulation evoked by 1 mM SNP in the neonatal cerebellum (5.49 ± 0.71 %, p = 28.8%). However, in more mature rats IBMX evoked a significant enhancement of SNP-stimulated [^{3}H]-cyclic GMP accumulation in 200-220g (5.93 ± 1.08 %, p=1.3%) and 350-400g rats (5.81 ± 0.50 %, p = 0.2%). The presence of 30μM forskolin was without effect on SNP-stimulated cGMP levels in all preparations (in order of increasing maturity) 3.90 ± 0.19 (p = 43.2%), 1.12 ± 0.38 (p=96.6%), and 1.02 ± 0.22 (p = 99.4%) % conversion. In rat cerebellar granule cells, a large response to SNP was evident, 6.12 ± 1.92 %, which increased to 15.67 ± 2.14 % in the presence of 1mM IBMX but was unaffected by 30 μM forskolin (6.66 ± 0.82 % conversion, n = 2).

These results indicate that PDE catabolism of cGMP in the rat cerebellum becomes more evident with increasing maturity both *in vivo* and *in vitro*. However, the forskolin-mediated enhancement of cGMP levels which we have previously observed in the guinea-pig cerebellum, appears not to occur in the rat cerebellum at any stage of development.

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247P ROLE OF PROTEIN KINASE C IN THE STIMULATION OF cAMP ACCUMULATION BY HISTAMINE IN CHO-K1 CELLS TRANSFECTED WITH THE BOVINE H_1 RECEPTOR

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In most cells, histamine H_1-receptors couple to inositol phospholipid (IP) hydrolysis, resulting in raised intracellular Ca^{2+} levels and activation of protein kinase C (PKC; Hill, 1990). In a previous communication, we have shown that histamine (HA) can stimulate cAMP accumulation on its own, and potentiate forskolin-stimulated cAMP accumulation in CHO-K1 (CHO-H_1) cells transfected with the bovine histamine H_1-receptor. Neither of the H_1-mediated effects on cAMP accumulation appear to involve the mobilisation of intracellular or extracellular Ca^{2+} (Sanderson *et al*, 1996). In this study, we have examined whether PKC activation is involved in histamine-stimulated cAMP accumulation, in CHO-H_1 cells, using the PKC inhibitor Ro 31-8220, and activator phorbol 12,13-dibutyrate (PDBu).

Accumulation of cAMP and IP hydrolysis in CHO-H_1 cells were measured as previously described (Megson *et al.*, 1995). Levels of IP hydrolysis are expressed as a percentage of the response to 300nM HA. All data are expressed as mean ± s.e.mean, n≥3, and were analysed by paired t-tests.

Ro 31-8220 (10μM) significantly reduced the levels of cAMP stimulated by 1mM HA alone, or in the presence of 1μM forskolin (decreases of 61±6% and 80±8%, $p<0.02$, respectively). However, IP hydrolysis stimulated by 10μM HA was not significantly decreased by the same concentration of this bisindolylmaleimide (Control: 134±6%, Ro 31-8220: 118±2). This observation confirms that the action of Ro 31-8220 on cAMP accumulation did occur predominantly through inhibition of PKC. Treating the CHO-H_1 cells for 30 min with 1μM PDBu did not augment basal or 1μM forskolin-stimulated cAMP levels (n=3), but did significantly inhibit the IP hydrolysis response to 300nM HA ($p<0.01$). This effect was reversed by co-treatment with 10 μM Ro 31-8220 or 24 h pre-incubation with 1μM PDBu. (Table 1). This same pre-incubation also reduced the augmentation of forskolin-stimulated cAMP accumulation by 1mM HA (decrease of 58±5%, $p<0.05$), though not the response to HA alone (n=3).

Table 1 Effect of PDBu on IP hydrolysis

	% Response 300nM HA		
	Control	Ro 31-8220	24h PDBu
Control (4α-phorbol)*	105 ± 3	118 ± 2	118 ± 8
PDBu	60 ± 3	120 ± 6	120 ± 4

(*4α-phorbol is an inactive phorbol ester).

The results of this study suggest that in CHO-H_1 cells, PKC activation is involved in the mechanisms linking H_1-receptors to changes in cAMP accumulation. Additional factors must also be involved, as the direct stimulation of PKC by PDBu failed to mimic the cAMP effects of HA; these factors remain to be defined.

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248P THE PARADOXICAL EFFECTS OF 8-(4-CHLORO-PHENYLTHIO)-cAMP AND FORSKOLIN ON RATES OF EXTRACELLULAR ACIDIFICATION IN RAT ISOLATED GASTRIC GLANDS

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Gastric acid secretion has been studied using various *in vivo* and *in vitro* techniques. Cellular responses to a variety of pharmacological interventions can be monitored by measuring changes in extracellular acidification rates (EAR) using microphysiometry (McConnell *et al.*, 1992). In the present study we have compared the effect of forskolin with that of 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) on EAR of rat isolated gastric glands.

Rat mucosae were isolated as described by Reeves and Stables (1985), and then digested in DMEM containing 1.4mgml^{-1} collagenase. Washed glands were settled in microphysiometer inserts (3μm pore) where they were perfused (120μlmin^{-1}) with bicarbonate free DMEM containing 0.02% bacitracin. Extracellular acidification rates were measured for 30s of a total cycle time of 2min 30s. Basal rates ranged between 70-250μV s^{-1} (0.07-0.25pH units min^{-1}). All values are mean ± s.e.mean or geometric mean with 95% confidence limits of at least 4 experiments.

Continuous perfusion with CPT-cAMP (100μM) caused a sustained increase in basal EAR of 26.2 ± 5.1% which was not modified by the cAMP phosphodiesterase inhibitor Ro20-1724 (4-[(3-Butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone). In the presence of Ro20-1724 (10μM), CPT-cAMP (100μM) increased EAR by 22.2 ± 1.8%. The K^+/H^+ ATPase inhibitor omeprazole (10μM) had little effect on basal EAR (max. change -3.3 ± 3.0%) but it attenuated the increase in EAR produced by CPT-cAMP to 12.7 ± 2.6%. The glucose transport inhibitor cytochalasin B potently decreased basal EAR (EC_{50} 0.51 [0.37-0.72]μM, max. inhibition 79.1 ± 2.4%). In the presence of cytochalasin B (10μM) responses to CPT-cAMP were abolished.

In marked contrast to CPT-cAMP, cumulative administration of forskolin (0.1-100μM) caused a sustained and concentration dependent decrease in EAR with an EC_{50} value of 6.9 (3.2-10.6)μM and a maximum inhibition of 75.1 ± 5.5%. Ro20-1724 (10μM) had no effect on the concentration response curve to forskolin (EPMR=1.3 ± 0.2). 1,9-dideoxyforskolin also decreased basal EAR but was approximately 10 fold weaker than forskolin (57.1 ± 5.8% inhibition at 100μM). Using a radioligand binding assay to quantify cAMP production in CHO cells (Williams *et al.*, 1996), we have confirmed that 1,9-dideoxyforskolin (100μM) has little effect on basal cAMP production whilst forskolin (10μM) caused marked increases (3.8 ± 0.4 and 68.1 ± 22.2pmol 200,000^{-1} cells respectively, basal 1.9 ± 0.1pmol).

We conclude that the cAMP analogue CPT-cAMP increased basal EAR in rat isolated gastric glands via a mechanism involving activation of K^+/H^+ATPase and which was dependent upon glucose uptake. Forskolin and 1,9-dideoxyforskolin decreased basal EAR with potencies which are similar to those reported for inhibiting glucose uptake and not for adenylyl cyclase activation (Laurenza *et al.*, 1989). Since cytochalasin B also decreased basal EAR in rat isolated gastric glands, it is tempting to speculate that forskolin and 1,9-dideoxyforskolin decreased basal EAR by inhibiting glucose uptake. This mechanism could potentially obscure responses due to stimulation of adenylyl cyclase.

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249P THE ENERGY REQUIREMENT FOR DEPLETION-ACTIVATED Ca^{2+} INFLUX IS NOT RATE-LIMITING

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These studies were conducted to determine if the energy requirement for Ca^{2+} influx through the depletion activated Ca^{2+} influx pathway is rate limiting. Patch-clamp electrophysiology was performed on rat basophilic leukemia cells of the 2H3 strain (RBL-2H3). Two approaches were used to isolate Ca^{2+} currents and reduce all other currents. First, K^+ was eliminated from pipette and bath solutions, and second by voltage clamping the cells at 0mV the contribution of voltage activated ion currents were minimized. Cells were placed into a Peltier chamber and perfused with solution containing 500 µM Ca^{2+} and 5 µM 2,5-di(t-butyl)-1,4-benzo - hydroquinone (BHQ). After a 5min period to deplete internal Ca^{2+} stores with BHQ (Moore et al., 1987), the cells were perfused with bath solution containing 2mM Ca^{2+} or higher. A Levenberg-Marquardt method was used to fit exponential curves to the influx current profile, and to allow comparison of the Ca^{2+} influx at various temperatures. The activation energy was calculated using the Arrhenius equation. An ANOVA and a paired Student's t-test were used for statistical analysis.

A concentration of 5 µM BHQ was responsible for an inward current of 42.0 ± 18.7 pA (n=7), and 10 µM BHQ activated an inward current of 376.5 ± 193.0 pA (n=6) carried by Ca^{2+}. By raising the $[Ca^{2+}]_o$ from 500 µM to 2 mM and 5 mM Ca^{2+}, and thus the driving force, an arithmetic increase in the inward current was observed ($P<0.01$, n=9).

The Ca^{2+} current could be completely blocked with 200µM La^{3+} ($P<0.05$, n=5). In contrast the outward current produced by BHQ was not affected by the Ca^{2+} driving force and could be completely blocked with 100 µM 4-acetamido -4'-isothiocyanostilbene - 2 ,2'-disulphonic acid (SITS) ($P<0.05$, n=7). The outward current is likely to be carried by Cl^- and was not studied further. Over the temperature range from 8.3 - 38.4 °C , the amplitude of the inward Ca^{2+} current increased with a Q_{10} of 1.15. Temperature also had an effect on the rate of the inward current, which increased with a Q_{10} of 1.20. The Arrenhius plot for the rate of the Ca^{2+} influx did not show a breakpoint indicating that the influx of Ca^{2+} does not have a rate limiting energy step.

The Ca^{2+} influx has a Q_{10} value which is similar to those for aqueous diffusion of ions, and unlike that which would be expected from a pump (Hille, 1992). Furthermore, unlike a pump the Ca^{2+} influx through the depletion-activated pathway does not have a rate limiting energy requirement. Supported by The Canadian Heart and Stroke Foundation.

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250P ATP STIMULATES INCREASES OF $[Ca^{2+}]$ IN SKATE (*RAJA*) ELECTRIC ORGAN SCHWANN CELLS VIA P_{2Y}-LIKE PURINOCEPTORS

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Schwann cells are integral cellular components of the dense cholinergic presynaptic plexus which innervates each electrocyte in skate electric organ. We have previously isolated this plexus ("nerve plate") from skate (*Raja brachyura* and *R. montagui)* electric organ as a functional preparation and monitored changes of $[Ca^{2+}]_i$ in its nerve terminals using the dye fluo-3 (Richardson *et al.*, 1995). Here we have used fura-2 loaded preparations and imaging technology to examine the effect of transmitter substances on $[Ca^{2+}]_i$ in the nerve plate Schwann cells.

Nerve plates were prepared and loaded with fura-2AM as described previously for fluo-3 loading (Richardson *et al.*, 1995). Preparations were perfused with skate Ringer (SR) (Richardson et al 1995) and all drugs were added by perfusion. Depolarisation was with 80mM KCl substituted in SR for equimolar NaCl. Nerve plates were monitored with a x100 objective and a Zeiss Axiovert 135TV. Fluorescence images (500-530nm) were captured during excitation at 340±12 and 380±12nm with an extended ISIS camera (Photonics Science) and were analysed with IonVision software (ImproVision). Ratios (340/380) were converted to $[Ca^{2+}]_i$ using the equation $[Ca^{2+}]=k_d(R-R_{min})/(R_{max}-R)$ where the constants (K_d=1686nM, R_{min}=0.53 R_{max}=11.27) were measured by imaging fura-2 in Ca^{2+}/EGTA buffers (Molecular Probes).

K^+ depolarisation produced a rapid and consistent increase of nerve terminal $[Ca^{2+}]_i$ (Richardson *et al.*, 1995). However, only occasional transient and delayed (by 5-10sec) responses were seen in Schwann cells. The possibility that these were triggered by mediators released from nerve terminals was tested by direct application of candidate substances. ACh induced a modest increase of $[Ca^{2+}]_i$ (to 93±43nM) in only 16 of 36 Schwann cells tested. In contrast ATP (50µM) produced consistent responses in all cells. These were composed of a rapid, transient, peak of $[Ca^{2+}]_i$ followed by a sustained plateau. The transient response was seen in the absence of $[Ca^{2+}]_o$ (Table) and could be blocked by preincubation with thapsigargin (500nM, 10min), indicating the release of Ca^{2+} from intracellular stores. The response to ATP was dose dependent (EC_{50} 2.8±1.2µM) and was mimicked by ADP (EC_{50} 2.8±1µM) and 2-methylthio-ATP (EC_{50} 1.7±1.1µM) (Table, EC_{50} = mean ± s.d. from ≥ 16 cells in ≥ 2 experiments). Adenosine, αβ-methylene-ATP and UTP were without effect (Table). These results suggest that ATP triggers release of Ca^{2+} from internal Schwann cell stores via a P_{2Y}-like purinoceptor. More importantly, they introduce the Schwann cell as an additional potential target for synaptically released ATP in the electric organ model of the cholinergic synapse.

Test compound	Resting or peak $[Ca^{2+}]_i$ (nM)	
No Addition	42±26	(n=232/33)
ATP	320±121*	(n=146/18)
ATP(Ca^{2+} free,1mM EGTA)	416±133*†	(n=36/4)
αβ-methylene-ATP	44±15	(n=9/2)
ADP	395±140*†	(n=31/5)
αβ-methylene-ADP	40±20	(n=7)
2-methylthio-ATP (30µM)	274±59*†	(n=24/3)
Adenosine	49±10	(n=13/2)
UTP	55±26	(n=19/3)

Additions were at 50µM unless indicated, n = no. of cells/no. of experiments. *Significantly different from basal levels ($p<0.001$ Students t-test), †Not significantly different from ATP alone.

Richardson,C.M., Dowdall,M.J., Green,A.C. *et al.* (1995) *J. Neurochem.* 64, 944-947.

251P AGONIST REGULATION OF N-METHYL-D-ASPARTATE (NMDA)-INDUCED INTRACELLULAR CALCIUM ELEVATIONS IN RAT CEREBELLAR GRANULE CELLS

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NMDA receptors experience prolonged agonist exposure during ischemic brain insults, when extracellular glutamate levels rise substantially. As NMDA receptors are widely accepted to play a central role in excitotoxic neuronal death following such insults, agonist regulation of their activity has important consequences.

We have investigated agonist regulation of NMDA-induced intracellular calcium ($[Ca^{2+}]_i$) increases using fura-2 ratiometric imaging of cultured rat cerebellar granule cells at 8 days in vitro. Cells were washed in Mg^{2+} free Locke buffer containing 1 µM tetrodotoxin (TTX) and then loaded with fura-2 AM (5 µM, 30 min). After a further wash cells were exposed to buffer with 10 µM glycine only (control) or 100 µM NMDA/10 µM glycine with and without 100 µM of the NMDA competitive antagonist D-aminophosphonopentanoic acid (D-AP5) for 30 min. The response to 1 mM NMDA/10 µM glycine was then tested. Data are expressed as the mean ± s.e. mean of cells from at least 3 separate cultures.

During 30 min continued exposure to 100 µM NMDA $[Ca^{2+}]_i$ levels recovered, so control and NMDA pretreated cells showed no difference in basal $[Ca^{2+}]_i$ (201 ± 9 nM n=82 and 183 ± 11 nM n=80, respectively). However, NMDA pretreatment reduced the peak $[Ca^{2+}]_i$ increase in response to 1 mM NMDA from 283 ± 15 nM (n=82) to 62 ± 4 nM (n=80). Co-incubation of 100 µM NMDA with 100 µM D-AP5 reduced the basal $[Ca^{2+}]_i$ to 63 ± 12 nM, and increased the peak NMDA response to 404 ± 37 nM (n=46). Five min following washout of NMDA the peak response to 1 mM NMDA recovered to 235 ± 33 nM (n=56). Including 1.2 mM Mg^{2+} in the buffer reduced basal $[Ca^{2+}]_i$ to 32 ± 5 nM (n=47). There was no increase in $[Ca^{2+}]_i$ in response to 1 mM NMDA in Mg^{2+}-containing buffer, indicating operation of the voltage dependent Mg^{2+}-block of the NMDA receptor. Cells pretreated for 30 min with 100 µM NMDA in Mg^{2+}-containing buffer still showed a peak increase in $[Ca^{2+}]_i$ of 278 ±21 nM (n=47) when perfused with 1 mM NMDA in Mg^{2+}-free buffer.

Raising basal $[Ca^{2+}]_i$ levels to 250 ± 20 nM (n=42) by exposing cells to 25 mM K^+ for 30 min did not reduce the response to 1 mM NMDA/10 µM glycine (283 ± 40 nM, no difference to controls).

Responses to 1mM NMDA in some cultures (5 out of 11) incubated in Mg^{2+} -free buffer with 10 µM glycine showed a peak $[Ca^{2+}]_i$ increase of only 69 ± 6.4 nM (n=70). The absence of glycine or addition of 100 µM D-AP5 resulted in a peak increases of 261 ± 24 nM (n=64) and 307 ± 13 nM (n=61) respectively. Even in the presence of TTX, it appears that in some cultures following addition of glycine endogenous glutamate causes substantial desensitisation of the NMDA response.

Since basal $[Ca^{2+}]_i$ levels in control and NMDA pretreated cells show no significant difference and exposing cells to 25 mM K^+ does not reduce the peak $[Ca^{2+}]_i$ increase induced by NMDA, the reduction in response is most likely due to NMDA receptor desensitisation rather than voltage gated Ca^{2+} channel inactivation or intracellular Ca^{2+} store depletion. This desensitisation requires ion flow through the receptor as Mg^{2+} blocks NMDA pretreatment effects. The decrease in basal and increase in peak NMDA $[Ca^{2+}]_i$ response in cells pretreated with D-AP5 suggests some tonic NMDA receptor activation and desensitisation by endogenous agonist in controls with added glycine.

252P CHANGES IN BEHAVIOUR AND C-Fos EXPRESSION FOLLOWING ADMINISTRATION OF DOMOIC ACID AT DIFFERENT STAGES OF RAT DEVELOPMENT

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This study was designed to determine dose-response curves for domoic acid (DOM) toxicity in rats at different stages of development, and further to determine if there were age-related changes in the pattern of central C-Fos expression following administration of equi-toxic doses of DOM.

Saline or different doses of DOM in saline were injected intraperitoneally in separate groups (N=6) of male and female Sprague-Dawley rats (Charles River, St. Constant, PQ) at post-natal days 0, 5, 14, 22 (weaning), 30 (females only) and 50 (males only). The last two ages were chosen to correspond to the time of sexual maturation. Behaviour was recorded each minute for 120 min using a 3 point scale of behavioural toxicity (max. score = 240). Supplemental heating was provided to prevent hypothermia. All other procedures were identical to those described by Tasker et al. (1991). Dose-response data were compared for potency and parallelism to determine equi-toxic doses. In a subsequent experiment groups of rats from P5 to adult were injected with saline or an equi-toxic but sub-convulsive dose of DOM. Following testing, animals were euthanized by pentobarbital overdose and perfused transcardially with 4% para-formaldehyde. Serial 40 µm sections of whole brain and spinal cord were washed, mounted and incubated in the presence of C-Fos antibody.

No significant differences were found between sexes at any of the ages tested prior to P30 and P50, so data at all earlier ages was collapsed across sex. Prior to weaning, pups were found to be significantly more susceptible to DOM-induced toxicity with interpolated ED50 values (± 95% Conf. Int.) of 0.12 ± 0.01, 0.15 ± 0.01, 0.30 ± 0.04, 1.06 ± 0.11, 3.35 ±1.71 and 2.87 ± 0.95 mg/kg for P0, P5, P14, P22, P30 and P50 respectively. Curves at P5 and P14 were statistically parallel and had different slopes from curves at P0, P22, P30 and P50. Comparisons of C-Fos data using image analysis revealed little or no label in P5 animals. There was widespread labelling relative to control at all other developmental stages, although expression was less robust in immature animals. Areas that showed a strong response included the nucleus gracilis, area postrema and NTS of the medulla, the parabrachial nucleus, certain hypothalamic nuclei, the amygdala, and various cortical regions.

We conclude that there are age-related differences in both behavioural toxicity and C-Fos expression in rats following systemic injections of DOM. It is not known at this time, however, whether the observed differences are due to maturation of the blood-brain barrier or upon the establishment and maturation of limbic system circuitry.

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253P CHARACTERIZATION OF ANTIBODIES DIRECTED AGAINST METABOTROPIC GLUTAMATE RECEPTOR mGluR5

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The activation of group 1 metabotropic glutamate receptors (mGluR's) has been implicated in synaptic plasticity. However, the two subtypes comprising the group 1 class of mGluR have a differential distribution. Whereas mGluR1 predominates in the cerebellum (Baude et al., 1993), in the hippocampus the major group 1 receptor subtype is mGluR5.

In order to investigate the cellular and sub-cellular localisation of mGluR5 on living cells peptides corresponding to the extracellular amino terminus of the mGluR5 receptor subtype were synthesized and purified. After conjugation to ovalbumin, they were then used as immunogens to generate anti-peptide antibodies.

The resulting antisera was purified on Affigel columns (BioRad) and assayed by immunoblotting and by immunofluorescence using Chinese Hamster Ovary (CHO) cells stably expressing either the mGluR1 or mGluR5 subtype (Aramori and Nakanishi, 1992).

The purified antibodies recognised a single band of 145 kDa in crude membranes derived from rat brain regions and also from CHO-mGluR5 cells. This is consistent with the size of the cloned cDNA (Abe et al., 1992) and also with that reported previously using an antibody directed against the intracellular carboxyl terminus (Romano et al, 1995). Prior incubation of the antibody with the appropriate peptide resulted in elimination of the immunoreactive band.

Confocal laser-scanning microscopy (MRC BioRad 1024) was performed on living CHO cells expressing the mGluR5 subtype. Immunofluorescence was restricted to the plasma membrane of these cells.

Further experiments are underway to determine if the mGluR5 subtype undergoes post-translational modification.

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254P PHOSPHOLIPASE A_2 AND IRRADIATION INACTIVATION TREATMENTS OF RAT BRAIN SECTIONS DISPLAY SIMILAR REGIONAL EFFECTS ON [^{3}H]AMPA BINDING

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We have described recently that pretreatment of rat cortical membranes with a 14kDa secretory group-I porcine pancreatic phospholipase A_2 ($sPLA_2$) modulates agonist [^{3}H]-AMPA binding (Dev *et al.*, 1995). The way by which $sPLA_2$ modulates [^{3}H]-AMPA binding is unclear, however possible mechanisms include the enzymatic actions of $sPLA_2$ working to cause a direct shift in AMPA receptor conformation by either altering the microlipid environment around the receptor (Tocco *et al.*, 1992), or modulating AMPA receptor-associated accessory proteins (Honoré and Neilsen 1985).

The presence of a putative modulatory protein, associated to the AMPA receptor has been reported (Honoré and Neilsen 1985). This study employed increasing levels of irradiation (MRad) on rat cortex which resulted in a curvilinear shape in [^{3}H]AMPA binding. The preferential destruction of large proteins, (e.g. the modulatory protein), before the destruction of smaller proteins, (e.g. AMPA receptor subunits) is thought to be the reason for the curvilinear relationship and the presence of an inhibitory modulatory unit attached to the AMPA receptor.

We have shown previously, a $sPLA_2$-evoked decrease in the affinity of [^{3}H]AMPA binding to cortex, shifting the K_i value from 81 ± 9nM to 266 ± 47nM (Dev *et al.*, 1995). Here we show that under the same experimental conditions, $sPLA_2$ causes an increase in the affinity of [^{3}H]AMPA binding to the hippocampus, shifting the K_i value from 99 ± 13nM to 14 ± 5nM.

Furthermore, in this study we used autoradiographical techniques in an attempt to 1) further define the specific regional effects of $sPLA_2$ and of irradiation treatments in [^{3}H]AMPA binding, 2) to show any regional similarities between $sPLA_2$ and irradiation treatment and 3) to elucidate the differential effects of $sPLA_2$ on [^{3}H]AMPA binding in the hippocampus and cortex.

We report that both $sPLA_2$ and irradiation treatments show an increase in [^{3}H]AMPA binding specifically in the CA1 and CA3 regions of the hippocampus and in the dentate gyrus. In agreement with radioligand homogenate binding studies there was no significant change seen in autoradiography in [^{3}H]AMPA binding to its high affinity site in the cortex (Dev *et al.*, 1995).

These results suggest that the presence of an inhibitory modulatory protein and its removal by $sPLA_2$ may explain some of the effects of $sPLA_2$ seen in the hippocampus, where [^{3}H]AMPA binding is increased. However, it fails to explain the $sPLA_2$-evoked decrease in [^{3}H]AMPA binding to the low affinity site seen in the cortex.

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255P IMPORTANCE OF THE 2-(METHYLSUCCINIMIDO) BENZOYL MOIETY FOR THE POTENCY OF NORDITERPENOID LIGANDS AT α7-TYPE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

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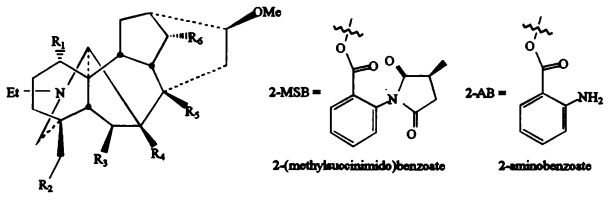
Methyllycaconitine (MLA, see Figure 1 for structure), from *sp.* of *Delphinium and Consolida,* is a potent and selective competitive antagonist at neuronal αbungarotoxin(αBgt)-sensitive nicotinic acetylcholine receptors (AChR; Wonnacott *et al.*, 1993), which correlate with the α7 subunit in mammalian brain. Hydrolysis of the C18 ester bond in MLA to yield lycoctonine greatly diminished nicotinic potency, whereas addition of the 2-(methylsuccinimido)benzoyl moiety to the norditerpenoid alkaloid, aconitine, converted it from a sodium channel activator into a nicotinic ligand, with potency comparable to that of MLA (Hardick *et al.*, 1995).

The aim of this study was to examine the importance of this ester moiety for nicotinic activity in an extended series of natural and semi-synthetic alkaloids. Compounds were tested for their ability to compete for [^{125}I]αBgt binding to rat brain P2 membranes (Table 1). Nudicauline and elatine, which also possess the 2-(methylsuccinimido)benzoyl moiety, inhibited [^{125}I]αBgt binding with nanomolar affinities, whereas all other compounds were at least two orders of potency weaker. Inuline (which retains the ester, but lacks the methyl-succinimido group) showed only modest potency.

These results support the importance of the 2-(methyl-succinimido)benzoyl moiety for potency at α7-type nicotinic AChR, and show that structural modifications to the norditerpenoid core are tolerated with little effect on activity.

Figure 1 Structure of MLA indicating positions of different substituents in the alkaloids examined.



Compound	R1	R2	R3	R4	R5	R6	IC_{50}±s.e.m. (n=3)
MLA	OMe	2-MSB	OMe	OH	OH	OMe	7.6±3.6nM
Nudicauline	OMe	2-MSB	OMe	OH	OH	OAc	1.7±0.7nM
Elatine	OMe	2-MSB	OMe	OCH_2O		OMe	6.1±1.5nM
Delsoline	OH	OMe	OMe	OH	OH	OMe	19±5.0μM
Condelphine	OH	OMe	H	H	OH	OAc	1.6±0.3μM
Delcorine	OMe	OMe	OH	OCH_2O		OMe	53±14μM
Inuline	OMe	2-AB	OMe	OH	OH	OMe	1.6±0.6μM

Table 1 Binding potencies of norditerpenoid alkaloids at rat brain [^{125}I]αBgt binding sites.

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256P BINDING STUDIES ON THE NICOTINIC ACETYLCHOLINE RECEPTOR OF *MANDUCA SEXTA*: A NICOTINE-INSENSITIVE, TOBACCO FEEDING INSECT

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Nicotine is a potent insecticide, causing a depolarising blockade of the nicotinic acetylcholine receptor (nicotinic AChR). However, the larvae of the tobacco hornworm, *Manduca sexta* suffer no detrimental effects from the ingestion of nicotine when feeding on tobacco. Trimmer & Weeks (1989) have postulated that this reduced nicotine sensitivity could reflect an altered nicotinic AChR, making it a natural, nicotine-insensitive, mutant.

We have investigated this in binding studies on brain membranes from adult *Manduca*. [^{125}I]α-Bungarotoxin (α-bgt) labels a specific binding site with Kd=7.2±2.4nM and B_{max}=186±14.5fmol/mg. Displacement curves and derived IC_{50} values are shown in figure 1. α-Bgt has the highest affinity for the site. The IC_{50} values for nicotine, epibatidine and imidacloprid (an insecticide with a postulated nicotinic cholinergic action) are similar, but one thousand-fold less than α-bgt. Acetylcholine and another tobacco alkaloid, anabasine, have lower binding affinities than nicotine for the [^{125}I]α-bgt binding site in *Manduca*. Comparisons with honeybee (table 1) show no major differences for α-bgt, imidacloprid or nicotine, indicating that a nicotine insensitive insect does not necessarily have a reduction in its nicotine binding affinity.

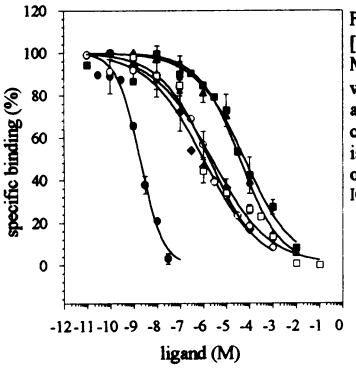

Figure 1. Displacement curves of [^{125}I]α-bgt by nicotinic ligands. Membranes (3.0mg/ml protein) were incubated with [^{125}I]α-bgt and varying concentrations of cold ligand and bound ligand isolated by vacuum filtration on GF/C filters.
IC_{50} ± s.e.mean; n=2-5
- α-bungarotoxin: 1.8 ± 0.75nM
- acetylcholine: 59 ± 14.3μM (in the presence of neostigmine)
- anabasine: 37.9± 5.8μM
- (±) epibatidine: 1.0 ± 0.23μM
- imidacloprid: 1.8 ± 0.3μM
- (-) nicotine: 2.3 ± 1μM

	α-bgt	imidacloprid	nicotine
Manduca	$1.8x10^{-9}$	$1.8x10^{-6}$	$2.3x10^{-6}$
Honeybee*	$7x10^{-9}$	$1.95x10^{-6}$	$1.55x10^{-6}$

Table 1. IC_{50} values (M) for displacement of [^{125}I] α-bgt. *Data from Tomizawa *et al.* (1995).

To see if an α-bgt-insensitive nicotinic AChR is present in *Manduca* brain we performed binding assays using [^{3}H]-epibatidine. At 1nM and 10nM concentrations, no specific [^{3}H]-epibatidine binding displaceable by excess nicotine or α-bgt was demonstrated. Thus, these data provide no evidence for a nicotinic AChR with an altered nicotine recognition site in *Manduca sexta*. This is consistent with our sequence data.

We thank the EC, BBSRC and Zeneca Agrochemicals for financial support.

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257P UPREGULATION BY CHRONIC AGONIST TREATMENT OF (±)-[^{3}H]EPIBATIDINE BINDING SITES ON M10 CELLS EXPRESSING THE α4β2 SUBTYPE OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

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Chronic treatment of animals with nicotine leads to upregulation of brain high affinity nicotinic agonist binding sites, corresponding to nicotinic acetylcholine receptors (AChR) comprised of α4 and β2 subunits (Wonnacott, 1990). *In vitro*, chronic treatment with nicotinic agonists of the M10 cell line expressing α4β2 nicotinic AChR results in similar upregulation (Peng *et al.*, 1994).

To exploit the M10 cell model for studies into upregulation mechanisms, this study investigated the use of the novel ligand (±)-[^{3}H]epibatidine to monitor cell surface nicotinic AChRs.

Nicotinic AChR expression in M10 cells was induced with dexamethasone and (±)-[^{3}H]epibatidine binding was examined 48 h later. Cells *in situ* were incubated with radioligand for 2 h at 20 °C in the presence and absence of 10^{-4} M (-)-nicotine to determine non-specific and total binding respectively. Saturable, specific binding was demonstrated, with a B_{max} of 237±6 fmol/mg protein. A K_d of 10.1±2.5pM was determined by analysis of (±)-[^{3}H]epibatidine association and dissociation rates. The (±)-[^{3}H]epibatidine binding site's nicotinic profile was confirmed in competition binding assays: K_i isoarecolone = 0.94 ± 0.29μM, (-)-nicotine = 11 ± 3nM, (±)-anatoxin-a = 2.5 ± 0.9nM, (±)-epibatidine = 12 ± 3 pM.

Chronic exposure (48h) of M10 cells to nicotinic agonists produced a concentration-dependent upregulation of surface (±)-[^{3}H]epibatidine binding sites. (-)-Cytisine, (±)-anatoxin-a, ABT418 and (-)-nicotine were equi-efficacious, producing maximum increases in (±)-[^{3}H]epibatidine binding sites of 205±35%, 196±34%, 180±9%, and 254±21% respectively. (±)-Epibatidine and isoarecolone were less effective with increases of 123±35% and 124±20% (Figure 1). A high (10μM) concentration of (±)-epibatidine blocked maximal upregulation by (-)-nicotine (50μM).

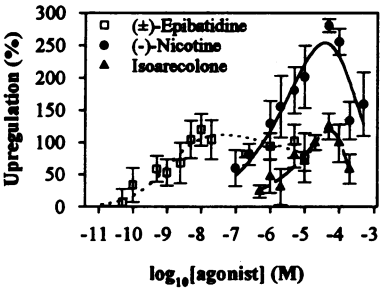

Figure 1. Upregulation of high affinity nicotinic agonist binding sites in M10 cells by chronic agonist exposure (48h). Sites determined by (±)-[^{3}H]epibatidine (500pM) binding *in situ*. Points are mean ± s.e. mean; n=3.

This study demonstrates that agonists other than (-)-nicotine upregulate nicotinic AChRs, with varying efficacy. EC_{50} values for upregulation are higher than K_i values for inhibition of agonist binding, suggesting that upregulation is not mediated by ligand binding to the high affinity desensitised receptor state.

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258P PHARMACOLOGICAL CHARACTERISATION OF THE UPREGULATION OF α7-TYPE NICOTINIC ACETYLCHOLINE RECEPTORS (AChR) IN RAT HIPPOCAMPAL CULTURES

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Nicotinic AChR's identified by the binding of [^{3}H] nicotine are upregulated in the brains of animals, following chronic treatment with nicotine *in vivo* (Wonnacott, 1990). Similar increases in the number of [^{3}H]nicotine binding sites have been found in the brains of human smokers. [^{3}H]nicotine labels the subtype of nicotinic AChR comprised of α4 and β2 subunits, and these receptor sites are also upregulated *in vitro*, in a stably transfected cell line exposed to agonist for a number of days (Peng *et al.*, 1995). The α7 nicotinic subunit is relatively abundant in the CNS, and has been correlated with [^{125}I]αbungarotoxin (αBgt) binding. Chronic nicotine treatment *in vivo* also increases the numbers of [^{125}I]αBgt binding sites, although higher drug concentrations appear to be required. We have recently characterised surface [^{125}I]αBgt binding sites in high density primary cultures of E18 rat hippocampal neurons and showed them to be upregulated by exposure to 10 μM nicotine for 7 days (Barrantes *et al.*, 1995).

To provide a comprehensive pharmacological characterisation of this response, hippocampal cultures were exposed to various nicotinic agonists and antagonists for 7 days prior to [^{125}I]αBgt (10nM) binding assays. In the absence of drugs, cultures expressed 59.5±6.3 (n=17) fmol [^{125}I]αBgt binding sites/mg protein. Table 1 shows that all agonists and competitive antagonists tested resulted in a statistically significant upregulation of [^{125}I]αBgt binding sites. However, the muscarinic agonist muscarine had no effect. Co-application of agonist (nicotine; 10μM) and antagonist (MLA; 100nM) resulted in an upregulation of [^{125}I]αBgt binding sites of 23.4±3.9% (n=3), similar to the upregulation seen with each drug alone. In contrast, the non-competitive antagonist mecamylamine failed to alter numbers of [^{125}I]αBgt binding sites.

Table 1. [^{125}I]αBgt binding to hippocampal cultures after 7 days treatment with nicotinic drugs (n=3-5)

Drug	Type	conc^n	(%control±sem)	
nicotine	agonist	10μM	133.9±4.2%	P<0.001
anatoxin-a	agonist	1μM	118.0±4.2%	P<0.05
anatoxin-a	agonist	10μM	160.8±8.0%	P<0.02
cytisine	agonist	20μM	128.0±9.3%	P<0.05
DMPP	agonist	10μM	138.1±11.9%	P<0.05
MLA	antagonist	100nM	122.7±7.2%	P<0.02
curare	antagonist	10μM	125.9±8.2%	P<0.01
mecamyl-amine	non-comp. antagonist	10μM	102.1±3.4%	-

These results demonstrate that both agonists and competitive antagonists can upregulate [^{125}I]αBgt-labelled nAChR. Thus, occupancy of the binding site may trigger this response.

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259P CONVERSION OF THE ION SELECTIVITY OF THE 5-HT$_3$ RECEPTOR FROM CATIONIC TO ANIONIC: A CONSERVED FEATURE OF THE LIGAND GATED ION CHANNEL SUPERFAMILY?

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The 5-HT$_3$ receptor is a ligand-gated ion channel which, together with the nicotinic acetylcholine (nACh), glycine and GABA$_A$ receptors, form an ion channel superfamily. These receptors are pentameric assemblies of subunits arranged around an axial channel. Each subunit is thought to consist of four transmembrane domains (M1 - M4) with M2 lining the wall of the channel. Galzi *et al.* (1992) identified regions of the channel that on mutation converted the ion selectivity of the α7nACh receptor from cationic to anionic. To investigate whether the 5-HT$_3$ receptor possesses similar determinants of ion selectivity, we generated equivalent mutations (Figure 1) and assessed changes of the properties of the mutant receptors using the whole cell patch clamp technique.

```
                    M2
                ________
WT 5-HT3R DSG ERVSFKITLLLGYSVFLIIVSDTLP
Mutant        ---PA----------T----------
```

Figure 1. Primary sequence of M2 and flanking regions of the 5-HT$_3$ receptor. Point mutations are indicated by the single letter code of the amino acid, dashes represent unchanged amino acids.

The coding sequence of the 5-HT$_3$ receptor was subcloned into the expression vector pRcCMV. Mutants were generated by site directed mutagenesis, stably expressed in HEK293 cells and characterised using the whole cell patch clamp technique. For ion selectivity experiments cells were voltage clamped over the range -80 to +40 mV and the peak current evoked by 10 μM 5-HT determined in the presence of normal extracellular solution (EC: 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Hepes, 30 mM Glucose, pH 7.2), 50% NaCl-Mannitol (0.5NaCl; as EC, except 65 mM NaCl, 130 mM Mannitol) and NaCl-Isethionate solutions (Na-Ise: as EC, except 15 mM NaCl, 115 mM Na-Isethionate). Current-voltage plots were constructed and the reversal potential (E_{rev}) for the wild type (WT) and mutant receptors determined in each solution.

The mutant receptors responded to 5-HT, although with a higher affinity (EC_{50} = 0.24 ± 0.03 μM, n=5) than the WT receptor (EC_{50} = 2.18 ± 0.13 μM, n=6) and most of the desensitisation in response to 5-HT was abolished. The reversal potential for the WT receptor was similar in both EC and Na-Ise solutions (E_{rev} was -4.75 ± 1.61 mV and -5.98 ± 1.33 mV, respectively, n=5) but was shifted to a more negative potential by the removal of 50% of the NaCl (E_{rev} = -22.40 ± 2.03 mV, n=5) indicating that WT receptors are predominantly cation-selective. In contrast, equivalent experiments for mutant receptors indicated that their reversal potential in EC solution (4.09 ± 1.81 mV) shifted to more positive values in both 0.5NaCl and Na-Ise solutions (E_{rev} respectively 18.49 ± 0.94 mV and 28.01 ± 2.88 mV, n=5) indicating that this mutant receptor had appreciable chloride permeability.

The results indicate that the changes in amino acids in this mutant were sufficient to switch the ion selectivity of the 5-HT$_3$ receptor. As the α7nACh and 5-HT$_3$ receptors appear to share common determinants of ion selectivity, this may be a conserved feature of the ligand-gated ion channel superfamily.

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Galzi, J.-L., Devilliers-Thiery, A., Hussy, N. *et al.*, (1992) *Nature* **359**, 500-505.

260P IMMUNOLOGICAL CHARACTERIZATION OF 5-HT$_3$ RECEPTOR TRANSMEMBRANE TOPOLOGY

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The 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor is a member of the ligand-gated ion channel superfamily which includes nicotinic acetylcholine, GABA$_A$ and glycine receptors. These receptors exhibit homology in electrophysiological and pharmacological properties as well as in primary structure. The best topological data for this superfamily are available for the nicotinic acetylcholine receptor, and suggests the N- and C-terminals are extracellular and four hydrophobic domains span the membrane (Changeux *et al.*, 1992). The 5-HT$_3$ receptor is most closely related to the nicotinic acetylcholine receptor with 27% sequence identity, and is predicted to have a similar structure (Maricq *et al.*, 1991). Presented here is the first experimental evidence that the N-terminal of the 5-HT$_3$ receptor is extracellular, thus confirming theoretical models.

HEK293 cells were grown in DMEM:F12 supplemented with 10% foetal calf serum on glass coverslips until 30% confluent. The cells were transiently transfected with plasmid (pRc/CMV) encoding 5-HT$_3$ receptor DNA using the calcium phosphate precipitation method and cultured for 72 h at 3% CO_2. The coverslips were then washed in tris-buffered saline (TBS) and fixed at 37°C for 20 min in Reagent A fixation medium (Harlan Sera-Lab). The antiserum used to label the N-terminal was generated against the peptide ^{23}GSRRATQARDTT^{36}Q. To label the predicted intracellular loop between transmembrane regions 3 and 4, an antiserum generated against a fusion protein corresponding to ^{301}T-^{429}V by Turton *et al.* (1993) was used. For the labelling of extracellular protein moieties antisera were diluted in TBS, for labelling those that are intracellular 0.3% Triton X-100 was added. Expressed 5-HT$_3$ receptors were visualized by indirect immunofluorescence using a biotinylated anti-rabbit secondary IgG and FITC fluorochrome.

A strong immunofluorescence signal in transfected permeabilised cells was found using both anti-N terminal and anti-intracellular loop antisera. This staining was localised mainly in the nuclear and plasma membranes, Golgi, endoplasmic reticulum and intracellular vesicles. The estimated efficiency of transfection was 10-20% of fixed cells. Without the presence of detergent the intracellular loop antiserum did not label cells, whereas the anti-N terminal antiserum labelled a distinctive ring around transfected cells (n>50 cells observed). Patches of intense fluorescence, corresponding to clustering of receptors, were observed in the plasma membrane.

The localisation of the N-terminal peptide on the outer side of the plasma membrane demonstrates that this region of the receptor is extracellular. This conclusion supports the ligand binding role of the N-terminal domain of the 5-HT$_3$ receptor.

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261P EFFECTS OF AGONISTS ON THE LONG AND SHORT SPLICE VARIANTS OF 5-HT$_3$ RECEPTOR STABLY EXPRESSED IN HEK 293 CELLS

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5-HT$_3$ receptors are unusual in the family of ligand-gated ion channels in that they form functional homopentameric receptors. To date only a single subunit has been cloned (Maricq *et al.*,1991) from a clonal cell line which expresses a high density of the receptor. In this and other cell lines, the subunit exists as two splice variants: a long form (5-HT$_3$-L) and a short form (5-HT$_3$-S) which lacks 6 amino acids in the intracellular loop between transmembrane regions M3 and M4.

We have previously shown that HEK 293 cells transiently transfected with the two forms of the receptors show different efficacy for the agonist 2-methyl-5-HT (2-Me-5-HT; Sepulveda & Lummis, 1994). Here we examine the effects of agonists on single HEK 293 cells stably transfected to express one or the other splice variant.

Full length 5-HT$_3$-S DNA was obtained from N1E-115 mRNA using PCR. Coding sequence for the additional 6 amino acids in the 5-HT$_3$-L subunit was inserted using site-directed mutagenesis. Sequences were inserted into the eukaryotic expression vector pRc/CMV and transfected into HEK 293 cells using calcium phosphate precipitation. Stable cell lines were selected using geneticin. 5-HT$_3$ receptor currents were examined using whole cell patch clamp.

EC_{50} values for 5-HT and 2-Me-5-HT were not significantly different for the two clones but mCPBG showed greater affinity for 5-HT$_3$-L than for 5-HT$_3$-S receptors (Table 1).

Table 1. EC_{50} values (μM) in the two 5-HT$_3$ receptor splice variants (Mean ± SEM, n>3; *sig. diff. p<0.05)

	5-HT	2-Me-5-HT	mCPBG
HEK/5-HT$_3$-L	2.98 ± 0.21	17.16 ± 2.87	1.20 ± 0.03
HEK/5-HT$_3$-S	2.68 ± 0.16	18.14 ± 4.91	2.30 ± 0.09*

The maximum current (Rmax) elicited by 5-HT compared to that elicited by 2-Me-5-HT and mCPBG differed significantly ($p<0.05$) in the two variants of the receptor: Rmax mCPBG/ Rmax 5-HT values were 0.68 ± 0.04 (n=3) and 0.91 ± 0.01 (n=3) in 5-HT$_3$-L and 5-HT$_3$-S receptors respectively; comparable values for 2-Me-5-HT were 0.30 ± 0.02 (n=10) and 0.23 ± 0.02 (n=8). Thus mCPBG can better discriminate between 5-HT$_3$-L and 5-HT$_3$-S receptors than 2-Me-5-HT.

We conclude that the six amino acid deletion in the 5-HT$_3$-S receptor results in differences between agonist potency and efficacy as compared to the 5-HT$_3$-L receptor and can be observed when the receptors are stably expressed in HEK 293 cells.

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262P CHARACTERISATION OF THE HUMAN 5-HT$_{2B}$ RECEPTOR EXPRESSED IN THE HUMAN NEUROBLASTOMA CELL-LINE SH-SY5Y

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Three subtypes of 5-HT$_2$ receptors, 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$, share a close sequence homology and have been shown to couple via $G_{q/11}$ to activate phospholipase C (Hoyer *et al.*, 1994). The 5-HT$_{2B}$ receptor is least well characterised member of this family. In order to investigate it's biochemical characteristics further we have stably transfected SH-SY5Y human neuroblastoma cells with the human 5-HT$_{2B}$ receptor.

pFUN2 vector containing cDNA encoding the human 5-HT$_{2B}$ receptor was transfected into SH-SY5Y cells using the polyamine lipofectant Transfectam. Selection was carried out by growth in Dulbeccos Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS), containing 654 μg/ml geneticin. Single colonies were established, grown to confluency and assayed for expression by saturation binding of [^{3}H]5-HT. Cells were cultured in DMEM and 5% dialysed fetal calf serum (dFCS) until confluent, then transferred into pure DMEM for 24 hours prior to binding assays. Cells were harvested by scraping and membranes prepared by homogenisation in 5mM Tris/EDTA (pH7.4) and washed twice by high speed centrifugation. Following re-suspension in incubation buffer (50mM Tris, 5mM $MgCl_2$, 1mM EGTA and 1mg/ml ascorbic acid), membranes were incubated with [^{3}H]5-HT (1-25nM) or [^{3}H]rauwolscine (0.5-10nM) at 37°C. After 60 minutes, reactions were terminated by rapid filtration through Whatman GF/C filters. Non-specific binding was determined using 10μM SB206553 (5-Methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo-[2,3-*f*]indole). To determine functional response, cells were labelled with [^{3}H]inositol (1μCi/ml) for 48 hours in inositol-free DMEM and 5% dFCS at 37°C. After washing and incubation for 30 minutes with 10mM LiCl, cells were stimulated by 5-HT (0.1nM-1μM) for 15 minutes at 37°C. The reaction was terminated with ice cold methanol, and [^{3}H]inositol phosphates extracted using Dowex AG1-X8 anion exchange columns.

[^{3}H]5-HT showed specific and saturable binding to cell membranes (Bmax=262 ± 44 fmol/mg protein, pK_d=7.99 ± 0.10, n=5). [^{3}H]Rauwolscine showed a higher level of binding (Bmax=349 ± 46 fmol/mg protein, pK_d=8.26 ± 0.22, n=3). This binding was inhibited by serotonergic ligands with the following order of potency:- methysergide = ritanserin > SB206553 > metergoline >yohimbine. The α-adrenoceptor antagonist phentolamine caused little inhibition of [^{3}H]rauwolscine binding at concentrations up to 1μM. Addition of 5-HT to cells caused a dose-dependent increase in inositol phosphate production (Emax=88 ± 8 % over basal, EC_{50}=12.0 ± 0.4 nM, n=4). Native SH-SY5Y cells showed no specific binding of [^{3}H]5-HT or [^{3}H]rauwolscine and no stimulation of PI hydrolysis by 10μM 5-HT.

We conclude that we have stably tranfected the human 5-HT$_{2B}$ receptor into SH-SY5Y cells. The antagonist [^{3}H]rauwolscine labels a larger population of these receptors than the agonist [^{3}H]5-HT. The inhibition profile is similar to that observed for the human 5-HT$_{2B}$ receptor expressed in AV12 cells (Kursar *et al.*, 1994). The functional assay demonstrates close coupling of the human 5-HT$_{2B}$ receptor to PI hydrolysis. This cell line will be useful for further investigation of 5-HT$_{2B}$ receptor modulation in a neuronal environment.

Alex Mitchell is an MRC Research Student.
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263P CHRONIC LITHIUM TREATMENT OF RATS ENHANCES 5-HT_{2A} RECEPTOR FUNCTION BUT NOT VIA AN INCREASE IN RECEPTOR DENSITY

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Lithium is used in the treatment of bipolar affective disorder, yet its mechanism of action is poorly understood. In addition, its use is associated with a number of unpleasant side effects and has a narrow therapeutic window. Knowledge of the primary effect or effects of lithium in the brain would thus provide important information in the development of alternative drugs with fewer side effects, but similar clinical efficacy. Our study was designed to investigate the effect of lithium on the 5-HT_{2A} receptor subtype, by comparing the consequence of chronic pretreatment of rats with lithium on 5-HT_{2A} receptor-mediated behavioural responses, Fos expression, and the density of this receptor subtype in the brain.

Rats received either control or lithium-containing (0.1% $LiCO_3$) chow (n=5) for 3 weeks prior to challenge with 8mg/kg of the 5-$HT_{2A/2C}$ agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). DOI-induced head shakes were then counted and locomotor activity was measured for 30 mins immediately after injection. Locomotor activity was monitored by an Opto-Varimex II Activity Monitor connected to an Apple II computer. Three hours after DOI injection, animals were killed with an overdose of sodium pentobarbitone (1ml/kg) then cardiac perfused with 4% paraformaldehye and their brains removed for immunohistochemical localisation of Fos protein. Fos protein was localised in 100μM-thick Vibratome sections using an antiserum (Cambridge Research Biochemicals) to the N-terminal region of the Fos molecule. Another group of rats, also receiving either control or lithium-containing chow (n=5), had their brains analysed for the distribution and density of 5-HT_{2A} receptor binding sites by quantitative [^{3}H]ketanserin autoradiography. 12μM-thick cryostat sections were incubated with 2nM [^{3}Hketanserin (± 500nM unlabelled spiperone) to define binding of the ligand to 5-HT_{2A} receptor sites. Blood samples were taken from all animals for analysis of plasma lithium levels by atomic absorption spectroscopy. Results from behavioural experiments are expressed as means ± s.e.m.

Treatment with lithium was found to have no effect on DOI-induced head shakes (8.3 ± 2.9 head shakes per 30 min in lithium treated animals vs. 10.2 ± 2.3 in animals from the control diet group), but it significantly enhanced DOI-induced locomotor activity ($p<0.05$; Students *t*-test), recorded in a 30 min period directly after DOI administration (1068.5 ± 72.5 in lithium treated animals vs. 495.8 ± 34.8 in control animals). Furthermore, such treatment dramatically enhanced DOI-induced Fos expression throughout the cerebral cortex, particularly in caudal piriform cortex and also in claustrum. In contrast, such treatment had no effect on the density of [^{3}H]ketanserin binding to 5-HT_{2A} receptors in any brain region examined. The distribution of 5-HT_{2A} receptor binding sites was similar to that reported in other studies. Plasma lithium levels were between 0.4 and 0.6 mEq/l in each study.

The results of these studies indicate that the enhancing effect of lithium on DOI-induced locomotor activity and Fos expression are not mediated by a change in the density of 5-HT_{2A} receptor binding sites; therefore another mechanism, such as an alteration in second messenger function (eg. phosphatidyl inositol turnover) must be considered.

264P NOVEL FLUORINATED SURFACTANTS FOR PERFLUOROCHEMICAL EMULSIFICATION: INHIBITORY EFFECTS ON HUMAN PLATELET AGGREGATION *IN VITRO*

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Emulsified perfluorochemicals have been used clinically for myocardial oxygenation and research is focused on developing more concentrated formulations which, in principle, have improved oxygen-carrying characteristics (Lowe, 1994). One approach is to incorporate "fluorophilic" surfactants to enhance emulsion stability. This study has assessed the bio-compatibility of a range of novel, non-ionic, glycosidic or polyol fluoro-surfactants, in a human platelet bioassay validated previously with the co-polymer surfactant, *Pluronic*® F-68 (PF-68; Edwards *et al.*, 1995, 1996). Novel fluoro-surfactants, containing glycosidic ('S' series) or polyol ('P' series) derivatives, were synthesised via simple routes using highly fluorinated isocyanates with amino alcohols, polyethoxylated alcohols and unprotected or partially protected sugars (at anomeric carbon) (Jouani *et al.*, 1994); yields of compounds were 88-95%. Blood (9.0 ml) from volunteers was placed into tubes containing 50 μg ml^{-1} hirudin (*Revasc*™; Ciba, U.K.) and incubated at 37°C (30 min). Aliquots (460 μl) of blood were placed in plastic tubes in a water bath (37°C) and pre-incubated for 2 min with 20 μl of 0.001-10.0% (w/v) of fluoro-surfactant or PF-68. Tubes were sampled (15 μl), 20 μl of saline added and stirred continuously (1000 rpm). Sampling (15 μl) was performed at 4, 6 and 8 min and samples placed in 36 μl of formaldehyde fixing solution. Thirty six μl of fixed sample was mixed with 9.1 ml of saline and transferred to an Ultra-Flo 100 Platelet Counter for counting. Platelet counts were calculated as % fall from time 0 and expressed as % aggregation. Statistical comparisons were made by the paired Wilcoxon two-tailed test. The mean (± s.e. mean) spontaneous platelet aggregation in normal whole blood after 8 min was 15 ± 2% (n = 15). The polyol ('P') fluoro-surfactants significantly ($P < 0.05$) reduced platelet aggregation in a dose-dependent manner, comparable to that caused by PF-68. For example, the mean aggregation with 0.32% (w/v) of fluoro-surfactant P1 or P4 (8 min) was 8 ± 1% or 10 ± 1% (n = 6), respectively, compared to 6 ± 1% (n = 15) with 0.32% of PF-68. Inhibition of platelet aggregation by the 'S' compounds was much less pronounced than with PF-68. The mean platelet aggregation (8 min) with compounds S2 or S4 was 13 ± 1% or 12 ± 2% (n = 6), respectively ($P < 0.05$), which was *ca.* 2-fold less than with PF-68. These results show that the polyol fluoro-surfactants can inhibit spontaneous human platelet aggregation and mimic the anti-thrombotic effects of PF-68 more effectively than their sugar-derived counterparts.

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265P EFFECTS OF PARTHENOLIDE AND FEVERFEW (*TANACETUM PARTHENIUM*) EXTRACTS ON HUMAN NEUTROPHILS *IN VITRO*

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The medicinal herb, feverfew (*Tanacetum parthenium* L.), has clinically proven efficacy in migraine prophylaxis, and parthenolide, a sesquiterpene lactone, is considered the principal active component responsible for its therapeutic effects (Groenewegen *et al.*, 1992; Knight, 1995). Measurements of total parthenolide by HPLC depend both on extraction procedures and solvents used (Brown *et al.*, 1996) and may not necessarily give a precise assessment of the total pharmacological activity of plant material. Thus, the present study has (i) evaluated the biological activity of commercial parthenolide and that of feverfew extracts on human blood neutrophils *in vitro*, as assessed by inhibition of cellular chemiluminescence, and (ii) compared bioassay data with total parthenolide content of extracts, as measured by HPLC. Blood (4.5 ml) from 8 volunteers (m:f 4:4) was placed into tubes containing 3.13% (w/v) trisodium citrate dihydrate (0.5 ml) in a water bath (37°C); samples were incubated for 30 min before use. Whole blood was used to avoid artefacts caused by pre-activation of neutrophils by the separation procedure (Neilson *et al.*, 1992). Fifty μl of blood was added to 10 μl of either (i) extracts of glasshouse-grown feverfew plants, or (ii) commercial parthenolide (Sigma, U.K.), and 50 μl of this mixture was added to 1.0 ml of phosphate-buffered saline (PBS) containing *Luminol*® (5-amino-2,3-dihydro-1,4-phtha-lazine-dione; Sigma-Aldrich, U.K.; cat. no. A8511; 100 μg ml^{-1}). Twenty μl of phorbol 12-myristate 13-acetate (PMA; Sigma; 100 μg ml^{-1}) was added to each sample to stimulate oxidative burst and the chemiluminescence recorded every 2 min for 20 min. The total parthenolide content of plant material extracted with different solvents was also measured by HPLC (Brown *et al.*, 1996). Statistical comparisons were made by the paired Wilcoxon two-tailed test. Mean (± s.e. mean; n = 6) parthenolide in acetone-ethanol feverfew extracts (1.29 ± 0.18% leaf dry weight) was greater ($P < 0.05$) than in PBS (0.50 ± 0.10%) or chloroform-PBS extracts (0.05 ± 0.04%), as determined by HPLC. Parthenolide-like activity in acetone-ethanol extracts, as measured by inhibition of neutrophil chemiluminescence (2.18 ± 0.48%), was greater ($P < 0.05$) than that for extracts prepared in PBS (0.85 ± 0.09%) or chloroform-PBS (0.20 ± 0.08%). Parthenolide-like activity, as assessed by the neutrophil bioassay, was consistently greater (maximum 1.7 times) than parthenolide measured by HPLC. The greater bioactivity of extracts suggests that components additional to parthenolide may contribute to the pharmacological effects of feverfew by suppressing neutrophil-induced inflammation which may underpin migraine pathology.

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266P SENSITIVITY OF T CELL ACTIVATION TO WORTMANNIN, RAPAMYCIN AND CHLOROQUINE

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It is well established that T cells require two signals for their full activation (Schwartz, 1992). The presentation of an antigen to the T cell receptor (TCR) leads to signals that include PKC activation and elevation of calcium levels. However, these signals are not sufficient to induce T cell proliferation and cytokine production. The engagement of the CD28 receptor is also required. CD28-induced signals are less well defined but are thought to involve phosphatidylinositol-3-kinase (PI3K)(Ward et al., 1995) and acidic sphingomylinase (Boucher et al., 1995). In this study the influence of wortmannin, a PI3K inhibitor, rapamycin, which inhibits the activation of p70 S6-kinase, a proposed downstream target of PI3K, and chloroquine, an acidic sphingomylinase inhibitor, on TCR and CD28 activation in resting T cell and T cell blasts were investigated.

Resting T cells purified from both male and female healthy donors were stimulated and proliferation was measured as the incorporation of [^{3}H]-thymidine during the final 18 hours of a 72 hour incubation (all experiments were done in triplicate). The presence of an anti-CD3 antibody (anti-TCR) (10ug/ml) and CHO cells transfected with the CD28 ligand, B7-1, for 72h led to a 50-fold increase in proliferation (n=6). This was dose-dependently inhibited by wortmannin at 1nM (28 ± 12%, n=4), 10nM (61 ± 9%, n=5) and 100nM (74 ± 13%, n=5). Rapamycin at 1nM had no effect (n=4), but inhibition was clearly apparent at 10nM (68 ± 12%, n=5) and 100nM (76 ± 8%, n=5). T cell proliferation was also reduced in the presence of 25 and 50μM chloroquine (87 ± 7% and 91 ± 6%, n=5), although little effect (15±28%) was seen at 12.5μM (n=3). The generation of the proliferative cytokine, IL-2, was simultaneously measured using the CTLL cytotoxicity assay[1] and followed a similar pattern as observed with [^{3}H]-thymidine incorporation.

T cell blasts were generated by stimulating peripheral blood mononuclear cells with superantigen (SEA) (10ng/ml). Eight days post stimulation the blasts were incubated with anti-CD3 and CHO (B7-1) transfectants, in order to activate both the TCR and CD28 receptors. This treatment resulted in an increase in proliferation (300-fold, n=4) which was inhibited in the presence of 10 and 100nM wortmannin (59 ± 4% and 78 ± 8%, n=3, respectively). The proliferative response of the T cell blasts was blocked following incubation with rapamycin (10 and 100nM; 96 ± 0.3% and 97 ± 0.6%, respectively) and chloroquine (25 and 50μM; 80 ± 3% and 98± 0.3%, n=3, respectively). However, in contrast to the cytokine profile seen with resting T cells the proliferative response induced in T cell blasts did not generate any detectable levels of IL-2. It is possible that a different proliferative cytokine (e.g. IL-4) may be important in these cells.

These results provide evidence for the involvement of PI3K and acidic sphingomylinase in activation of both resting and T cell blasts.

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267P EFFECT OF ORALLY ADMINISTERED BAY X7195 UPON LEUKOTRIENE C_4-INDUCED EOSINOPHILIA IN THE GUINEA-PIG

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In human asthma pulmonary eosinophil infiltration and activation are believed to be involved in the pathogenesis of the disease (Walker *et al.*, 1991). In guinea-pigs cysteinyl leukotrienes (LT) have been shown to induce pulmonary eosinophilia (Crean *et al.*, 1989). The aim of this study was to investigate the time-course of LTC_4-induced pulmonary recruitment of eosinophils in the guinea-pig. In addition, we have investigated the effect of BAY x7195, a cysteinyl-LT receptor antagonist (Abram *et al.*, 1993) against the maximal eosinophil recruitment seen 24 hours (h) post LTC_4 challenge.

Male guinea-pigs (450-630g) were restrict fed overnight and pretreated with mepyramine (10mg/kg i.p.) 55 minutes prior to a 10 minute challenge with vehicle or inhaled LTC_4 (1µg/ml). Animals were killed either 3, 8, 24 or 72h after challenge and bronchoalveolar lavage (BAL) performed. Eosinophil peroxidase (EPO) activity, as an additional marker of eosinophilia, was determined in BAL for the time course study (Gulbenkian *et al.*, 1992). EPO levels in lung tissue were also measured in the drug study (Yeadon *et al.*, 1993).

Statistically significant BAL eosinophilia was seen 3, 8, 24 and 72h post LTC_4 inhalation, with BAL neutrophilia observed at 3 and 24h after LTC_4 inhalation (Table 1). Maximum eosinophilia was observed at 24h after LTC_4 inhalation and since BAL EPO activity was statistically significantly correlated to BAL eosinophil number at this time ($r=0.78$ $p = 0.01$, $n = 10$) it was used as a marker of BAL eosinophilia. BAY x7195 (10mg/kg p.o.; 1h pre and 6h post LTC_4 challenge) caused a statistically significant inhibition of LTC_4-induced eosinophilia 24h post challenge (BAL EPO 76% inhibition, $p = 0.0004$, $n = 9$-12; lung tissue EPO 74% inhibition, $p = 0.0073$, $n= 9$-12).

In conclusion, inhalation of LTC_4 by conscious guinea-pigs results in BAL eosinophilia and neutrophilia, both of which were maximal 24h after challenge. Oral pretreatment with BAY x7195 significantly attenuated both the BAL and lung tissue eosinophilia. BAY x7195 could prove to be an effective therapeutic agent for the treatment of asthma where cysteinyl-LT's may play a role in the pulmonary recruitment of eosinophils associated with this disease.

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TABLE 1	Total Eosinophils x 10^5		Total Neutrophils x 10^5		BAL EPO (dA/min @ 340 nm)	
Time Post Challenge (h)	Vehicle	LTC_4	Vehicle	LTC_4	Vehicle	LTC_4
3	5.3 ± 3.2	16.0 ± 8.5 *	1.6 ± 0.9	3.8 ± 1.5 *	93.9 ± 57.9	187.5 ±129.4 ns
8	4.3 ± 1.2	13.5 ± 9.7 *	3.8 ± 2.6	7.2 ± 2.0 ns	136.9 ± 36.1	462.1 ± 129.8 ***
24	5.3 ± 3.9	25.4 ± 16.5 **	1.5 ± 1.1	9.6 ± 2.6 ***	91.3 ± 60.3	368.2 ±162.0 ***
72	3.8 ± 1.9	15.3 ± 6.3 ***	1.3 ± 0.6	1.2 ± 1.0 ns	29.3 ± 17.0	153.1 ± 23.0 ***

Data represents median ± semi-interquartile range, n = 10 * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, ns = not significant from time matched control Mann-Whitney statistics

268P NEUROLEPTIC DRUGS BEHAVE AS INVERSE AGONISTS AT THE SHORT ISOFORM OF THE HUMAN D_2 DOPAMINE RECEPTOR HETEROLOGOUSLY EXPRESSED IN CHO CELLS

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We have previously shown (Hall & Strange, 1996) that (+)-butaclamol behaves as an inverse agonist, rather than a simple neutral antagonist, at the short isoform of the human D_2-dopamine receptor ($D_{2S}R$) heterologously expressed in Chinese hamster ovary (CHO) cells. This effect showed the stereoselectivity expected for a D_2-like dopamine receptor as (-)-butaclamol was essentially inactive over a similar concentration range. We now present data which suggest that a number of other D_2 'antagonists', many of which are used clinically as antipsychotic drugs, also exhibit inverse agonism at $D_{2S}R$ in this cell line.

Pre-incubation of cells for 40 min with D_2 'antagonists' resulted in a marked concentration-dependent increase in the amount of cAMP that these cells produced in response to 10 µM forskolin (in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (1 mM)). All of the compounds tested induced increases in cAMP accumulation which were not significantly different from that induced by a maximally active concentration of (+)-butaclamol which was included with each dose-response curve ($P>0.05$, paired *t*-test) (Table 1). The inverse agonist effect showed strong stereoselectivity for (-)-sulpiride and *cis*-flupenthixol over (+)-sulpiride and *trans*-flupenthixol as expected for a D_2 dopamine receptor (Seeman, 1980). The EC_{50} values of the compounds used for this increase in cAMP accumulation are shown in Table 1.

Thus, a number of compounds previously thought to be neutral antagonists at D_2 dopamine receptors appear in fact to be inverse agonists at this receptor. A number of these compounds are widely used as neuroleptic drugs and these findings may therefore have implications as to their mechanism of action and, possibly, their side effects.

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Table 1 Functional parameters of antipsychotic agents for inverse agonism at $D_{2S}R$ expressed in CHO cells (values are mean±s.e.mean of 3 - 4 separate determinations in duplicate)

Drug	pEC_{50}	EC_{50} (nM)	Maximal Effect*
Chlorpromazine	7.89±0.11	13	0.88±0.08
Clozapine	6.52±0.18	297	0.86±0.11
Domperidone	8.75±0.05	1.8	0.95±0.05
cis-Flupenthixol	8.65±0.11	2.2	0.77±0.11
trans-Flupenthixol	6.78±0.20	163	1.10±0.10
Haloperidol	8.69±0.08	2.1	0.95±0.03
Nemonapride	9.34±0.01	0.46	0.92±0.04
Spiperone	9.26±0.19	0.55	1.03±0.05
(-)-Sulpiride	7.34±0.10	45.5	1.01±0.05
(+)-Sulpiride	6.01±0.13	977	1.05±0.25

*Relative to a maximally effective concentration of (+)-butaclamol (1 µM).

269P CHARACTERISATION OF [^{3}H]-PROSTAGLANDIN E_2 BINDING TO PROSTAGLANDIN EP_4 RECEPTORS EXPRESSED USING SEMLIKI FOREST VIRUS

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In this study we have used a [^{3}H]-PGE_2 radioligand binding assay to pharmacologically characterise prostaglandin EP_4 receptors which have been expressed in CHO cells using the Semliki Forest Virus (SFV) expression system (Lundstrom et al, 1994).

[^{3}H]-PGE_2 (0.4nM for competition studies, 0.2-25nM for saturation studies), membranes (5µg protein per tube) and test compounds were incubated in assay buffer (10mM MES, 10mM $MnCl_2$, 1mM EDTA, pH6/NaOH), for 45 mins at room temperature. The reaction was terminated by filtration through Whatman GF/B filters using a Brandel cell harvester. Filters were washed 4 times with 1ml of ice cold wash buffer (10mM MES, 0.01% BSA, pH6/NaOH) and filter bound [^{3}H]-PGE_2 determined by liquid scintillation spectrometry. 1µM PGE_2 was used to define non-specific binding. Data was analysed by computer-assisted non-linear analysis (LIGAND for saturation data, ALLFIT for competition studies). Results are given as mean ± S.E.M.

Saturation analysis of [^{3}H]-PGE_2 binding to EP_4 receptors showed that binding was saturable and to a single site of high affinity, Kd = 1.12 ± 0.3nM and Bmax = 3.1 ± 0.3 pmolmg^{-1} protein. Specific [^{3}H]-PGE_2 binding was >80% of total [^{3}H]-PGE_2 binding at the Kd value. The association time course for [^{3}H]-PGE_2 specific binding to EP_4 receptors was rapid ($t_{1/2}$ = 12.5 ± 1.29 mins), increased with time, reached equilibrium after 45 mins at room temperature and remained stable for 90 mins. In competition studies the affinities of various prostanoids were determined. These are listed in table 1.

Table 1: Affinity values of compounds for the inhibition of [^{3}H]-PGE_2 binding to EP_4 receptors.

Compound	pKi	n_H	n
PGE_2	8.6 ± 0.06	1.01	8
PGE_1	8.5 ± 0.1	0.97	4
$PGF_{2\alpha}$	6.1 ± 0.2	0.68	4
Butaprost	5.3 ± 0.3	0.99	3
Sulprostone	4.9 ± 0.1	0.65	3
AH23834	5.4 ± 0.2	0.65	3

These compounds show the same rank order of potency as previously determined for EP_4 receptors in the pig saphenous vein (Coleman et al, 1992).
In conclusion, the use of the SFV receptor expression system has provided a high enough level of EP_4 receptor expression to allow a detailed pharmacological characterisation of this receptor.

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270P THE ABILITY OF SPERMINE TO REDUCE THE PROTECTIVE ACTION OF CYCLOSPORIN A WITHIN RAT HEPATIC MITOCHONDRIA

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There has been increasing interest in the involvement of heavy metals in the aetiology of various neurological disorders. For example, iron and manganese have been implicated in Parkinson's disease (Liccione and Maines, 1988) and we have previously shown that manganese uncouples energy metabolism in brain mitochondria (Menton and Markham, 1994). This effect coupled with an interference of mitochondrial calcium (Ca^{2+}) homeostasis severly compromises mitochondrial energy production (Gavin et al., 1990). We have previously reported the protective action of cyclosporin A (Cyc A) upon disrupted energy metabolism associated with pore opening by calcium (Ca^{2+}) and [P_i] in rat hepatic mitochondria (Menton and Markham, 1995). In this investigation the ability of the polyamine spermine to modify Mn^{2+} stimulated respiration and the potential protective action of Cyc A was determined.

Rat liver mitochondria were prepared from female Wistar rats (200-225g). Mitochondria incubated in the presence of 5mM glutamate plus 5mM malate, displayed respiratory control index (RCI) of 8.64 ± 0.69 (n=4). Oxygen consumption was measured using a Clark type O_2 electrode (Rank Bros, Bottisham, Cambridge).

Mn^{2+} (150 µM) increased state 4 respiration (ADP absent) from 4.32 ± 0.93 to 8.86 ± 0.98 ng atoms O min^{-1} mg protein^{-1} (n=4; P<0.05). This resulted in a decrease in RCI from 8.64 ± 0.69 to 3.70 ± 0.46 (n=4; P<0.05) even though there was no effect upon state 3 respiration (ADP present). Cyc A (3 µM) had no effect upon this stimulation of respiration and as such provided no protection to energy metabolism. Increasing the concentration of Mn^{2+} (1.5 mM) resulted in a similar increase in state 4 respiration from 4.32 ± 0.93 to 48.21 ± 9.24 ng atoms O min^{-1} mg protein^{-1} (n=4; P<0.05) with no significant change in state 3 respiration. Cyc A (3 µM) produced significant reductions in state 4 respiration. Spermine (0.5 mM) produced a response at both Mn^{2+} concentrations which was unaffected by Cyc A (Table 1).

	Control State 4	Spermine 0.5mM	Cyc A (3µM)	Spermine plus Cyc A
Mn^{2+} 150µM	8.86 ± 0.98	29.93 ± 2.98	10.67 ± 0.55	29.73 ± 3.85
Mn^{2+} 1.5 mM	48.21 ± 9.24	92.68 ± 9.96	17.36 ± 2.76*	83.95 ± 6.30

Table 1. Effect of spermine, Cyc A and a combination of both upon Mn^{2+} stimulated state 4 respiration in liver mitochondria, measured in ng atoms O min^{-1} mg protein^{-1},compared to Mn^{2+} alone; mean ± s.e. mean; n=4; *P<0.05. Unpaired student 't' test.

Data shows that in rat liver mitohondria, at relatively low Mn^{2+} concentrations (150 µM), Cyc A cannot prevent uncoupling as was found for uncoupling concentrations of Ca^{2+} (Menton and Markham, 1995). High concentrations of Mn^{2+} (1.5 mM) produced stimulation of respiration which could be reduced by incubation with Cyc A. This reduction in respiration by Cyc A was inhibited by spermine. The ability of spermine to reduce the potential protective action of Cyc A may therefore result from a direct action on the pore or indirect actions involving either changes in uniporter activity or interaction with mitochondrial membrane lipids.

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271P (-)-DEPRENYL PREVENTS APOPTOSIS INDUCED BY TROPHIC WITHDRAWAL IN PRIMARY CULTURES OF RAT RETINAL GANGLION CELLS

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(-)-Deprenyl, a monoamine oxidase (MAO) inhibitor, has been shown to increase neuronal survival in *in vivo* models of cell death and PC12 cells *in vitro* independently of MAO inhibition (Ansai et al. 1993; Tatton, 1994;1996). We used serum and NGF withdrawal to induce apoptotic death in primary cultures of mixed retinal cells (40% glia and 60% neurons) obtained from 3-5 day postnatal Long Evans rat pups. Increasing concentrations of (-)-deprenyl from 10^{-11} M to 10^{-5} M were used in serum/NGF-deprived media in order to determine whether (-)-deprenyl could increase cell survival following withdrawal of trophic support. Cell viability was determined by "blinded" observers counting the total number of viable cell nuclei after Zapoglobin (Coulter Electronics, Burlington, Ontario) induced cell lysis at 24 hours post treatment. Cells were assayed for internucleosomal DNA cleavage characteristic of apoptosis by *in situ* end labelling. Our results indicate that (-)-deprenyl reduced cell death in a dose-dependent manner. Increased cell survival occurred at concentrations too low to inhibit MAO ($<10^{-9}$M). Trophic withdrawal decreased cell survival from $6.75 \times 10^4 \pm 1.22$ in the serum/NGF group to $2.49 \times 10^4 \pm 0.18$ cells in the serum deprived group (Student's paired t-test, $p<0.001$) in two seperate experiments (n=8 ± SEM). Serum/NGF deprived media supplemented with (-)-deprenyl increased cell survival at 24 hours from $2.49 \times 10^4 \pm 0.18$ cells in the minus serum group to $4.89 \times 10^4 \pm 0.48$ and $6.69 \times 10^4 \pm 0.66$ ($p<0.001$) in the treatment groups with 10^{-9} M and 10^{-11}M (-)-deprenyl, respectively. Preliminary data using the protein synthesis inhibitor cyclohexamide (1ng/ml), given concomitantly with (-)-deprenyl resulted in a 1.3 fold (cyclo. + 10^{-9} M deprenyl, $p<0.05$) and 2.26 fold (cyclo. + 10^{-11} M deprenyl, $p<0.001$) decrease in cell survival compared to (-)-deprenyl alone. These findings demonstrate that the increase in rat retinal ganglion cell survival following trophic withdrawal required the induction of protein synthesis. This effect produces cell rescue via a decrease in apoptosis.

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272P POTENCY OF ANTAGONISTS AT THE CALCITONIN GENE-RELATED PEPTIDE RECEPTOR OF RAT L6 CELLS

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Receptors for calcitonin gene-related peptide (CGRP) and amylin exist on rat soleus muscle (Beaumont *et al.*, 1995). $CGRP_{8-37}$ is a widely used antagonist against CGRP (Mimeault *et al.*, 1992), and both $amylin_{8-37}$ and AC187 (acetyl-[Asn^{30},Tyr^{32}]salmon $calcitonin_{8-32}$) are active against amylin (Beaumont *et al.*, 1995). However, the selectivity of these agents is largely unknown. The rat L6 cell line has CGRP receptors with a high affinity for $CGRP_{8-37}$ (pA_2 =8.1), but no amylin receptors (Poyner *et al.*, 1992). Thus it provides a simple system at which to investigate the pharmacology of agents on CGRP receptors. In this study the relative potency of a variety of antagonists on the CGRP receptor were determined.

L6 cells were grown as described previously (Poyner *et al.*, 1992). Human αCGRP caused a concentration-dependant stimulation of cAMP production, ($-Log_{10}EC_{50}$ of 9.5±0.15 (n=3): maximum stimulation of 150pmol cyclic AMP per 10^6 cells). Cells were pretreated with antagonists (10nM to 10μM) for 10 min, before challenge with 10nM human αCGRP (a supramaximal concentration) for 5 min. Cyclic AMP was extracted and measured by a radioreceptor assay as described previously (Poyner *et al.*, 1992). Concentration-response curves were constructed for each antagonist for inhibition of CGRP-stimulated cyclic AMP production, and $-Log_{10}(IC_{50})$s (pIC_{50}s) were measured.

CGRP-stimulated cyclic AMP production was potently inhibited by $CGRP_{8-37}$ (Table 1). AC187 was significantly less potent than $CGRP_{8-37}$, but was almost equipotent with $amylin_{8-37}$. This latter compound was significantly more potent than $CGRP_{19-37}$ (Table 1). $Amylin_{19-37}$ and [Tyr^0]-$CGRP_{28-37}$ caused only slight antagonism at concentrations of 1μM (inhibitions of 22±10% and 17±10% of CGRP stimulation of cyclic AMP production, n=5 for both agents).

These results confirm that there is a CGRP1-like receptor subtype expressed on rat L6 cells, and establish the relative potency order for the above antagonists at this receptor. From the data of Beaumont *et al.* (1995), it would appear that AC187 is 10-fold selective for amylin receptors over CGRP receptors . The potent antagonists can all form an amphipathic helix involving residues 8-18 (Mimeault *et al.*, 1992), which thus appears to be an important determinant for high affinity binding to this receptor.

Table 1 pIC_{50} values for antagonists against CGRP stimulation of cyclic AMP production.

Antagonist	pIC_{50}
$CGRP_{8-37}$	7.8±0.19 (n=5)
AC187	6.8±0.10 (n=5) †
$amylin_{8-37}$	6.6±0.16 (n=5)
$CGRP_{19-37}$	6.1±0.24 (n=6) *

Values are means ± s.e.means. † significantly different from $CGRP_{8-37}$, $P<0.05$, Students t-test. * significantly different from $amylin_{8-37}$, $P<0.05$, Students t-test.

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273P EXPRESSION, PURIFICATION, REFOLDING AND CHARACTERISATION OF THE IMMUNOGLOBULIN-LIKE SUB-DOMAINS OF THE NERVE GROWTH FACTOR RECEPTOR (TrkA)

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Nerve growth factor (NGF) is one of a family of neurotrophic factors which are necessary for the development and maintenance of the central nervous system. In Alzheimer's disease (AD) one of the early symptoms is loss of memory which is related to the degeneration of basal forebrain cholinergic neurons. NGF acts as a trophic factor for these neurons and has been shown to increase cholinergic function both *in vitro* and *in vivo*. The development of small molecule NGF agonists may be useful for the treatment of the memory impairments observed in patients with AD.

NGF binds with high affinity to the extracellular region of the tyrosine kinase receptor TrkA. Sequence homology and secondary structure prediction studies have shown that the extracellular domain of the TrkA receptor comprises a leucine rich region flanked by two cysteine cluster regions, followed by two immunoglobulin like (Ig-like) domains (Schneider and Schweiger, 1991). It has been shown recently by mutagenesis studies that the Ig domain closest to the transmembrane spanning region not only confers specificity but provides the main contacts for binding of TrkA receptors to NGF (Urfer et al., 1995: Perez et al., 1995).

The cDNA encoding the two TrkA Ig-like domains has been subcloned into the vector pET-15b (Novagen) which has been used to transform the *E.coli* strain BL21(DE3). Recombinant Ig-like domains were produced as inclusion bodies and purified by ultracenrifugation over discontinuous sucrose gradients. These were then solubilised in 6M urea containing β-mercaptoethanol and refolded by dialysing out the urea and β-mercaptoethanol. Further purification on a Resource Q column (Pharmacia) and by nickel chelation chromatography (since the recombinant protein is produced with six histidines on the N-terminus) produces a protein which is greater than 99% pure, as shown by SDS-PAGE analysis. The yield of purified protein is 16mg from a one litre culture. Circular dichroism studies have shown that the protein is mainly β-sheet as would be expected for Ig-like domains. We have also shown that the protein binds to NGF in a competitive binding assay using [I^{125}]NGF and A875 cells (human melanoma cell line expressing the p75 NGF receptor) to give a K_d of 3.3nM. Nanomolar concentrations of the recombinant protein was shown to inhibit NGF induced neurite outgrowth in rat PC12 cells. Future structural studies of the Ig-like domains both on their own and bound to NGF may be useful for the rational design of NGF receptor agonists.

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274P INHIBITION OF THE INFLAMMATORY RESPONSE TO ANTIGEN CHALLENGE IN THE HAMSTER CHEEK POUCH BY THE TACHYKININ NK1 RECEPTOR ANTAGONIST SR140333

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Tachykinins are contained, along with calcitonin gene-related peptide, in primary afferent neurons that innervate the hamster cheek pouch (Svensjö. *et al*, 1980); and tachykinins acting via NK_1 receptors have been shown to cause vasodilatation (Hall & Brain, 1994) and increase plasma extravasation (Gao *et al*, 1993) in this preparation. Topical antigen challenge in cheek pouches of immunized hamsters leads to an acute inflammatory reaction involving arteriolar vasodilatation and increased plasma extravasation from post-capillary venules. The aim of this study was to determine whether the release of tachykinins contributes to these effects. Hamsters (male, Golden Syrian 60-100g) were immunized with 10 μg ovalbumin (OVA) and boosted 4 weeks later with 1 μg OVA (both doses in 0.2 ml saline containing 10 mg aluminium hydroxide; i.p.; see Raud *et al.*, 1989). Experiments were carried out 7-10 days after the second injection. Single arterioles (20 - 40 μm diameter) in the cheek pouch were visualised by intravital microscopy with video-recording, and vasodilatation was determined by measuring vessel diameters by image analysis (see Hall & Brain, 1994). Plasma extravasation was quantified by fluorescence spectrometry of superfusate, collected by fraction collection, following i.v. fluorescein isothiocyanate dextran (FITC-dextran; 70,000 mw). Topical antigen challenge was carried out by superfusion of the whole pouch with 10 μg ml^{-1} OVA (5 min). Antigen challenge in the cheek pouch of immunized hamsters evoked a rapid inflammatory reaction consisting of an immediate short-lasting arteriolar vasoconstriction followed by prolonged vasodilatation and plasma extravasation (n=10). In vehicle ($Al(OH)_3$) treated hamsters antigen challenge was without significant effect (unpaired t-test) on arteriole diameter or leakage of FITC-dextran (P>0.05;n=6).

In the cheek pouch of immunized hamsters the arteriole vasodilator response to antigen was maximal 8 min after challenge (arteriole diameter increased by 33.8 ±9.3%; P<0.05; n=5). This vasodilator response was partially inhibited (to 12.8 ± 8.5%) in cheek pouches where the NK_1 receptor selective antagonist SR140333 (1-[2-{3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azonia-bicyclo[2.2.2]octane, chloride; Emonds-Alt *et al*, 1993) (10 nM) was superfused over the pouch 15 min prior to antigen challenge (n=5). Plasma extravasation increased immediately following, (maximum 150% increase at 15 min) and continued for up to 60 min after, OVA challenge. The increase in FITC-dextran in superfusate collected in the 10 min following challenge was significantly inhibited in cheek pouches where the NK_1 receptor antagonist SR140333 (10 nM) was superfused 15 min prior to challenge (P<0.05; n=5). Basal arteriole diameter and leakage of FITC-dextran were unaffected by SR140333 (10 nM; P>0.05; n=6). We conclude that in the cheek pouch of immunized hamsters, tachykinins are released following antigen challenge and, via activation of NK_1 receptors, contribute to the resultant increased plasma extravasation and vasodilatation.

Acknowledgements
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275P CHARACTERIZATION OF THE ACTIONS OF NALOXONE BENZOYLHYDRAZONE AT μ-OPIOID, κ-OPIOID AND ORL1 RECEPTORS IN ISOLATED TISSUES FROM RAT AND GUINEA-PIG

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Molecular cloning has revealed that the putative murine κ3 opioid receptor (Pan *et al.*, 1995) is in fact 95% homologous with the human orphan opioid receptor (ORL1) for which an endogenous ligand, nociceptin or orphanin FQ, has been isolated (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). It has been suggested that naloxone benzoylhydrazone (Nalbzoh) produces its analgesia by an agonist action at κ3 receptors (Paul *et al.*, 1990). The endogenous agonist nociceptin, however, is hyperalgesic when given i.c.v. to mice. We have sought to characterize the pharmacological profile of nalbzoh at μ, κ and ORL1 receptors. All data presented are from 3-5 experiments.

Vasa deferentia from Wistar rats (250g) were mounted in tissue baths and stimulated electrically at a frequency of 0.2Hz (Henderson *et al.*, 1982). Nociceptin (in the presence of the peptidase inhibitors thiorphan (10μM) and bestatin (10μM)) and D-Ala^2, MePhe^4-Glyol enkephalin (DAMGO) applied in single doses at 10min intervals produced concentration-dependent inhibitions of the nerve-evoked contractions. The IC_{50} values for nociceptin and DAMGO were 25nM (95% confidence limits 14-46nM) and 200nM (95% confidence limits 174-234nM) respectively. Responses to DAMGO (300nM) were abolished by naloxone (1μM) whereas those to nociceptin (100nM) were unaffected. Nociceptin (100-300nM) did not reduce contractions of vasa deferentia induced by α,βmethyleneATP (10μM). Nalbzoh (0.03-3 μM) was without agonist action but when applied for at least 15min before the agonist produced a parallel shift to the right of the concentration-response curve for nociceptin. A plot of log(dose ratio -1) against log[nociceptin] gave a straight line (slope equal to unity) from which a Kd of 275nM was calculated. Nalbzoh (30nM) produced a parallel shift to the right of the concentration-response curve for DAMGO with an apparent Kd of 3nM.

The ileum myenteric plexus-longitudinal muscle preparation was isolated from guinea-pigs (300g) of either sex, set-up and stimulated electrically as described by Kosterlitz *et al.* (1970). Both DAMGO (1-300nM) and nalbzoh (3-300nM) applied as single doses at 10min intervals depressed the nerve-evoked contractions in a concentration-dependent manner. The maximum inhibition produced by nalbzoh (around 50%) was less than that produced by DAMGO (>90%). The IC_{50} values for DAMGO and nalbzoh were 8nM (95% confidence limits 6-10nM) and 6nM (95% confidence limits 2-15nM) respectively. Nociceptin (0.01-3μM) in the presence of the peptidase inhibitors showed only very weak inhibition in the ileum preparation (maximum inhibition <20%). The inhibition produced by nalbzoh (30-300nM) was abolished by the κ-opioid receptor antagonist norbinaltorphimine (norBNI; 30nM) whereas that by DAMGO was unaffected by norBNI (30nM). In the presence of norBNI (30nM), nalbzoh (30nM) applied for at least 15min before the agonist produced a parallel shift to the right of the concentration-response curve for DAMGO with an apparent Kd of 2nM.

These data indicate that nalbzoh is a potent μ-opioid receptor antagonist, a κ-opioid receptor agonist and a competitive ORL1 receptor antagonist.

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276P CHARACTERISATION OF [^{3}H]CGP 54626A BINDING TO GABA$_B$ RECEPTORS IN RAT CEREBELLAR SYNAPTIC MEMBRANES

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The pharmacological properties of the rat GABA$_B$ receptor have been investigated in binding studies using [^{3}H]GABA as a radioligand (Bowery et. al., 1983). The solubilisation of GABA$_B$ receptor from porcine brain synaptic membranes has also been investigated, using [^{3}H]CGP 54626A, a GABA$_B$ selective antagonist (Facklam & Bowery, 1993). We have examined the binding of [^{3}H]CGP 54626A to rat cerebellar membranes, and carried out preliminary investigations into the optimal conditions for solubilisation.

Cerebella from female Wistar rats (approx. 200g) were homogenised in 0.32 M ice-cold sucrose. The homogenate was centrifuged for 10 min at 1,000 g, the supernatant layer collected and recentrifuged for 30 min at 48,000 g. The resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.4) followed by 3 freeze-thaw-wash steps. The final crude synaptic membranes were resuspended and stored at -80°C for at least 18 hours.

Membranes were then resuspended in 50 mM Tris-HCl, pH 7.4 at 0°C and incubated with [^{3}H]CGP 54626A, routinely 1.5 nM, and varying concentrations of unlabelled test compound in a final reaction volume of 0.1 ml. Non-specific binding was detected by addition of 100 μM GABA. The binding reaction was for 1 hour at 4°C and was terminated by rapid filtration through GF/C filters pre-soaked in 0.3 % polyethylenimine.

[^{3}H]CGP 54626A bound to rat cerebellar membranes with a Kd of 0.97 ± 0.19 nM, a B_{max} of 2.18 ± 0.06 pmol.mg protein^{-1} and a Hill coefficient of 0.98 ± 0.04 (mean ± s.e.mean, n=3). Specific binding represented > 90 % of total binding. Association of [^{3}H]CGP 54626A was rapid, with greater than 80% of maximal binding occurring within 20 minutes at 4°C. Dissociation of [^{3}H]CGP 54626A was relatively slow with approximately 50 % of specific binding still associated 90 min after addition of excess unlabelled GABA. [^{3}H]CGP 54626A was displaced by a variety of GABA$_B$ selective ligands, but was not displaced by the GABA$_A$ selective agonist isoguvacine. The rank order of effective competitors was CGP 54626 (IC_{50}=5.6nM) ≥ CGP 55845 (IC_{50}=8.9nM) > GABA > baclofen > CGP 35348 = CGP 36742 (IC_{50}=20μM). Initial solubilisation studies indicated that CHAPS or Triton X-100, used at concentrations of 0.5 % and 1 %, respectively, provided the most efficient conditions for solubilisation of the GABA$_B$ receptor. We are currently investigating the effects of high salt solution and inclusion of glycerol on the yield of solubilised receptors.

In conclusion, [^{3}H]CGP 54626A has proved a useful radioligand for investigating the binding characteristics of GABA$_B$ receptors in the rat cerebellum. Future work will be aimed at using affinity chromatography to purify the solubilised GABA$_B$ receptor.

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277P [^{3}H]-DOXAZOSIN AS A RADIOLIGAND FOR THE LABELLING OF α_{1A}, α_{1B} AND α_{1L} ADRENOCEPTOR SITES USING RAT SALIVARY GLAND, LIVER AND BRAIN

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Doxazosin is a selective α_1-adrenoceptor antagonist (Davey, 1989) and is soon to be introduced by Amersham in a tritiated form. The aim of this study was to assess the suitability of this radioligand for the study of α_1-adrenoceptor subtypes.

Salivary gland, brain and liver membranes from male wistar rats (250-300g) were prepared and incubated with appropriate concentrations of [^{3}H]doxazosin and displacing drugs. Non-specific binding was defined by 10μM phentolamine. Displacement curves were carried out using 12 concentrations of competing drug at a [^{3}H]doxazosin concentration of 1.3nM.

Table 1. Affinities of α_1-selective compounds for [^{3}H]doxazosin in rat tissue. (Values represent mean pK_i values ± s.d; n=3-4).

	Salivary Gland	Liver	α_{1A}/α_{1B}
5-Methylurapidil	8.57 ± 0.36	7.03 ± 0.24	34.60
WB 4101	8.95 ± 0.30	7.78 ± 0.04	14.79
Spiperone	7.26 ± 0.07	8.31 ± 0.08	0.089

70% of the total [^{3}H]doxazosin binding to membranes prepared from rat liver was specific and of a high affinity (pK_d=9.1±0.08). In competition experiments, prazosin displaced from a single binding site (pK_i =9.4±0.2) with an affinity some 5000-fold greater than the α_2-antagonist, rauwolscine (pK_i =5.7±0.1). The affinity of unlabelled doxazosin (pK_i =8.7±0.2) was similar to that of [^{3}H]doxazosin. The affinities of a range of α_1-subtype selective antagonists were also determined at membranes prepared from rat salivary gland and liver, tissues which possess homogenous populations of α_{1A}- and α_{1B}-receptors, respectively

5-Methylurapidil and WB4101 were found to have 35- and 15-fold greater affinities at α_{1A}- than at α_{1B}-adrenoceptors respectively, whilst spiperone was found to be 12-fold selective for α_{1B}-sites (see table 1). This confirms previous reports of the selectivity of these drugs (Ford *et al.*, 1994). In rat brain, the displacement of binding by all three antagonists was associated with Hill coefficients significantly less than unity and each drug identified two affinity sites corresponding to 30-40% α_{1A} and 60-70% α_{1B} adrenoceptors. For prazosin displacement of [^{3}H]doxazosin in the brain, Hill coefficients were again significantly less than unity (0.55 ± 0.18, $p<0.01$) and the data were best described by a two-site fit. A receptor population consisting of 71% high affinity sites (pK_i = 9.30) and 29% low affinity sites (pK_i = 7.07) was identified. This observation cannot be accounted for by the existence of a heterogeneous population of α_{1A} and α_{1B} sites, since prazosin exhibited a high affinity for both of these receptors in the salivary gland (pK_i = 8.8 ± 0.1) and liver (pK_i = 9.4 ± 0.2) respectively. These two differing affinity sites may correspond to the α_{1H} and α_{1L} adrenoreceptors identified in functional studies (Flavahan & Vanhoutte, 1986).

In conclusion, these results indicate that [^{3}H]doxazosin binds to α_{1A}, α_{1B} and α_{1L}-receptor sites in rat tissue.

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278P DEMONSTRATION OF SUB-NANOMOLAR BINDING OF DOMOIC ACID TO THE [^{3}H]KAINIC ACID BINDING SITE IN RAT BRAIN CORTEX

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Kainic acid (KA) is the prototypical agonist for the KA class of excitatory amino acid receptors. Previous studies of [^{3}H]KA binding to rat brain homogenate binding sites have reported two sites with dissociation constants for KA of 5-20 nM for the high affinity site and 50-300 nM for the low affinity binding site while domoic acid (Dom) has reported IC50 values in the range 4-50 nM (Johansen *et al.* 1993). In the process of describing the Dom interaction with [^{3}H]KA binding, we had cause to re-evaluate the binding conditions and properties of this radioligand.

[^{3}H]KA (58 Ci/mmol) binding to a rat (male, Sprague-Dawley, 200 - 250 g) brain cortex lysed synaptosomal preparation was performed at low radioligand (0.05 - 0.1 nM) and receptor concentrations, an assay volume of 5 ml with a 60 min incubation at 23 °C terminated by vacuum filtration. These assay conditions revealed IC50 values for Dom, KA, glutamate and glutamine of 0.38 ± 0.03 nM (mean ± s.e.mean, n = 4), 1.1 ± 0.3 nM (n = 4), 89 ± 7 (n = 3) and 1500 ± 500 nM (n = 3), respectively. Non-linear analysis of the binding parameters indicated a single interaction with dissociation constants of 0.50 ± 0.14, 1.5 ± 0.3, 80 ± 13 and 1900 ± 700 nM, respectively. For these 14 experiments the concentration of receptors was a value of 0.0080 ± 0.0004 nM (receptor density, 4.0 ± 0.5 fmoles/mg tissue. Data from either individual or pooled experiments, did not argue for the presence of more than one binding site.

Previous studies with [^{3}H]KA have been limited by low specific activity and a desire to simultaneously characterise high and low affinity binding sites. Filtration binding assays are inappropriate for studying these low affinity interactions (Bennett and Yamamura, 1985). Indeed, when concentrations of radioligand and receptor are kept low, there is no need to invoke a low affinity site for [^{3}H]KA. These results may help to explain the potency of KA and Dom as neurotoxins and illustrate the necessity of performing pharmacological assays at concentrations of drug and receptor which do not preclude observing high affinity interactions.

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279P INTRASTRIATAL INJECTION OF 6-HYDROXYDOPAMINE DOES NOT INDUCE LIPID PEROXIDATION IN THE RAT: HISTOCHEMICAL ANALYSIS

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Biochemical analysis using the thiobarbituric acid reactive substances (TBARS) assay has shown that 6-hydroxydopamine (6-OHDA) can induce lipid peroxidation in mouse striatum (Ogawa *et al.* 1994). We further attempted to identify histochemically the substructures within the rat basal ganglia which are affected by 6-OHDA-induced peroxidation, and to correlate this to dopaminergic cell loss.

In vitro experiments: Brains from normal male Wistar rats (200-250g) were sectioned at the level of the straitum, globus pallidus (GP) and substantia nigra (SN). Each area was incubated with ascorbic acid (150μM), and either $FeCl_3$ (1.5-150μM) or EDTA (150μM). Sections were fixed in 5% trichloroacetic acid (TCA), and washed four times in 0.9% saline. Incubation in 0.1% (w/v) 3-hydroxynaphthoic acid hydrazide (NAH) dissolved in 10% DMSO, and 0.9% saline containing 5% acetic acid followed for 14h. The reaction was stopped using 15% DMSO in 1mM HCl after which the slides were reacted with Fast Blue B dye in PBS pH 6.5, for 20 min. Slides were washed and mounted wet in glycerol jelly.

Animal experiments: Male Wistar rats (200-250g) were anaesthetised with 30mg/kg sodium pentobarbitone i.p. and placed in a stereotaxic frame. 6-OHDA (16μg in 4μl 0.9% saline, containing 0.1%w/v ascorbic acid) or vehicle was injected unilaterally into the left striatum, co-ordinates A/P:+0.7mm from bregma, L: +2.8mm, V:-4.6mm, incisor bar: -3.3mm (Paxinos & Watson 1986). Animals were allowed to recover then killed by exsanguination at 2h, 1, 2, 3, 5, 7 or 14 days. Each group contained 3 or 4 animals. TH (tyrosine hydroxylase) immunohistochemistry, cresyl violet staining and Fast Blue B staining were performed on adjacent sections (12μm) from the striatum, GP and SN. Two further groups of lesioned animals were transcardially-perfused with TBA, 3 days post-lesion. Sections of 30μm were visualised for TBARS using fluorescence microscopy (White *et al.* 1993).

In vitro experiments demonstrated a concentration-dependent staining with NAH/Fast Blue B in all the tissues tested after induction of lipid peroxidation using $FeCl_3$/ascorbate. Therefore, this method could be used for further *in vivo* experiments to identify sites of lipid peroxidation. 6-OHDA caused a large loss of TH staining in the striatum, maximum at 3 days, without retrograde neuronal cell loss in the SN, even at 2 weeks. Cresyl violet reflected the loss of dopaminergic nerve terminals by loss of staining up to 3 days post-lesion, after which, proliferation (probably of glial cells) was seen in the lesioned area. At no time was staining with Fast Blue B seen in the striatum, GP or SN, compared to normal right-side controls or sham-lesioned animals. In TBA-perfused animals, staining was identical in both 6-OHDA and sham-lesioned animals, where fluorescence was only seen along the needle tract of the striatum. No staining was seen in control tissue.

In conclusion, 6-OHDA does not induce lipid peroxidation in rat basal ganglia over a short (2h) or long (1 day-2 weeks) time period. If the neurotoxicity of 6-OHDA to dopaminergic neurones is indeed mediated by free radicals, other biomolecules such as proteins may be attacked and their function altered.

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280P IONOTROPIC GLUTAMATE RECEPTOR STIMULATION INCREASES ENDOGENOUS LEVELS OF ADENOSINE IN RAT STRIATUM

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Brain levels of endogenous adenosine increase under conditions of increased energy demand and decreased energy supply, e.g. ischaemia, hypoglycaemia, and seizures. Adenosine is thought to be neuroprotective. An important therapeutic objective continues to be the identification of agents that increase the levels and actions of endogenous adenosine. We found previously in rat that unilateral intrastriatal injections of the glutamate receptor agonist N-methyl-D-aspartate (NMDA) dose-dependently increased levels of endogenous adenosine. Coadministration of inhibitors of adenosine transport (dilazep, DLZP) and metabolism (deoxycoformycin, DCF) enhanced these NMDA-induced increases (Delaney & Geiger, 1995). Here, we determined the time course for the NMDA-induced increases in adenosine, the dose dependence of the effects of DCF and DLZP, and tested the hypothesis that non-NMDA receptor activation could also increase levels of endogenous adenosine.

Male Sprague-Dawley rats (170-220 g), anaesthetized with sodium pentobarbital (74 mg/kg i.p.), received unilateral intrastriatal injections (0.5 μL) of drug in 50 mM TRIS-HCl pH 7.4. Unless otherwise stated, rats were killed 15 min post-injection by high energy focused microwave irradiation (Delaney and Geiger, 1996). Striatal adenosine levels (pmol/mg protein), determined by HPLC, were expressed as a percentage of those in uninjected contralateral striatum; levels were unchanged in buffer injected striata. Data represent mean ± s.e.mean.

A time course of changes in adenosine levels after injection of 25 nmol NMDA revealed a maximal increase at 15 min (284 ± 56 %; n = 8), returning to control values (106 ± 33%; n = 4) by 45 min. The glutamate receptor agonists, NMDA, kainic acid (KA), and α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA) each dose-dependently increased adenosine levels. Maximal increases in adenosine levels for NMDA were 613 ± 191 % at 150 nmol (n = 4), for KA were 569 ± 29 % at 5 nmol (n = 3), and for AMPA were 192 ± 30 % at 19 nmol (n = 4). The apparent EC_{50} values were 25 nmol for NMDA, 0.25 nmol for KA, and 16 nmol for AMPA. The effects of NMDA were completely blocked by MK801 (dizocilpine) ($p < 0.01$; Student's *t*-test) and were enhanced by co-injections of DCF or DLZP alone or in combination. KA (0.25 nmol) increased adenosine levels to 350 ± 61 % (n = 5). This was reduced to 154 ± 5 % (n = 4) by the KA/AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione ($p < 0.05$; Student's *t*-test).

This *in vivo* model of ionotropic glutamate receptor-induced increases in endogenous adenosine levels may serve as a means to determine the neuroprotective role(s) of adenosine in excitotoxicity.

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281P THE EFFECT OF DIADENOSINE POLYPHOSPHATES ON ENZYMES INVOLVED IN ADENOSINE METABOLISM AND ON ENDOGENOUS ADENOSINE LEVELS IN RAT BRAIN

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A series of adenosine containing compounds, diadenosine polyphosphates (Ap_nA, where n = 2 to 6), have been found in a variety of mammalian tissues. Ap_4A and Ap_5A have been found to be released from rat brain (Pintor et al., 1993). In addition to actions at purinergic receptors, Ap_nAs have been found to affect the activity of enzymes involved in the metabolism of adenosine in peripheral tissues. These effects include inhibition of adenosine kinase (AK) and stimulation of 5'-nucleotidase (5'-N) suggesting that Ap_nAs may act to increase adenosine. Such increases could be beneficial in the CNS where adenosine is thought to be neuroprotective against such pathogenic conditions as ischaemia and hypoxia. In this study, we determined the effects of Ap_nAs on 5 enzymes involved in brain purine metabolism and tested the hypothesis that Ap_nAs could increase levels of endogenous adenosine in rat brain.

Male Sprague Dawley rats (170-250 g) were used in these studies. Brain homogenates were assayed for adenosine deaminase (ADA), adenosine monophosphate deaminase (AMPDA), AK, extracellular (ecto) 5'-N, and intracellular (endo) 5'-N activity and the effect of 100 μM Ap_nAs (n = 2-6) upon enzyme activity was determined. In order to examine the effect of Ap_nA's on endogenous adenosine levels, anaesthetized rats (74 mg/kg i.p. sodium pentobarbital), received unilateral intrastriatal injections (0.5 μL Ap_nA in 50 mM TRIS-HCl buffer pH 7.4). Rats were killed 15 min post-injection by high energy focused microwave irradiation (10 kW, 1.25 s), and striatal adenosine levels (pmol/mg protein) were measured by HPLC, and expressed as % of those in the uninjected contralateral striatum. Data represent mean ± s.e.mean.

ADA and ecto 5'-N activity were unaffected by Ap_nAs. AMPDA activity (nmol/mg protein/15 min) was increased from 493 ± 27 to 593 ± 43 ($p < 0.05$; Student's *t*-test) by Ap_5A and to 726 ± 36 ($p < 0.001$; Student's *t*-test) by Ap_6A. AK activity (nmol/mg protein/5 min) was significantly decreased ($p < 0.001$; Student's *t*-test) from 9.7 ± 1.1 to 0.46 ± 0.24 by Ap_4A, to 0.5 ± 0.12 by Ap_5A, and to 5.9 ± 1.4 by Ap_6A. Ap_4A decreased endo 5'-N activity (nmol/15 min/mg protein) from 269 ± 38 to 172 ± 16 ($p < 0.05$; Student's *t*-test). Intrastriatal injection of 10 nmol Ap_4A and Ap_5A decreased adenosine levels to 52 ± 11 % ($n = 6$; $p < 0.05$; Student's *t*-test) and 88 ± 28 % (n = 9), respectively. Injection of 5 nmoles of a metabolically stable analogue of Ap_4A, $Ap_2CH_2p_2A$, also decreased adenosine levels (60 ± 23 %; n = 3). Adenosine levels were unchanged in vehicle-injected striata.

Despite the potent inhibition of AK activity by ApnAs, these compounds did not increase levels of endogenous adenosine in striatum. The mechanism of action by which ApnAs decrease adenosine levels remains to be determined.

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282P THE INHIBITORY EFFECTS OF 5-HT ON LH RELEASE IN THE RAT MAY BE MEDIATED VIA 5-HT_{1A} AND 5-HT_7 RECEPTORS

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Ovariectomised Wistar rats (200 - 250 g) primed with 5μg/rat oestradiol benzoate (OB) followed 48 hours later by 0.5 mg progesterone (P) exhibit a rise in plasma LH concentration, 4 hours after the P, as measured by radioimmunoassay in samples collected from the tail vein at 30 minute intervals for a 5 hour period following P. Administration of 2 μg/0.4 μl/side 5-HT into the zona incerta (ZI; an area in the dorsal hypothalamus) 2 hours after P, significantly inhibits the LH rise (Siddiqui et al 1994).

Using the same experimental protocol, we have now investigated the receptor type mediating the 5-HT-induced inhibition of LH release. The effect of 5HT was mimicked by 8-OH-DPAT (5-HT_{1A} agonist), but not by DOI (5-HT_2 agonist), BMY7378 (5HT_{1A} presynaptic agonist) or MCPP (5-$HT_{1B/2C}$ agonist) all given bilaterally into the ZI at 2 μg 0.4 μl and DOI also at 10 μg. The rise in LH seen in this model after steroid treatment was calculated from the difference in LH concentration taken at a time of basal release (2 hours after P) and the time of maximum release (3.5 to 4.5 after P). Differences between basal and peak LH (ng/ml ± SE) after saline was 8.5 ± 0.86 (8), after 5-HT was 4.7 ± 0.7* (16); 8-OH-DPAT 2.2 ± 0.5* (7); DOI (10 μg) 9.9 ± 0.99 (7); BMY 7378 7.6 ± 1.4 (13); MCPP 10.6 ± 1.8 (6). An asterisk indicates that the change in LH was significantly ($P<0.05$) different from that seen after saline (ANOVA and Gabriel's test). The effect of 5-HT and 8-OH-DPAT was antagonised by both WAY100135 (2mg/kg I/P; 5-HT_{1A} antagonist) and ritanserin (0.25 mg/kg I/P; 5-HT_2 antagonist) given 1 hour before the agonist. (Difference between basal and peak LH (ng/ml ± SE); 8-OH-DPAT 2.2 ± 0.5 (7); 8-OH-DPAT + WAY100135 8.1 ± 0.9* (7); 8-OH-DPAT + ritanserin 7.0 ± 1.3* (8); *$P<0.05$ compared to 8-OH-DPAT alone). Since 8-OH-DPAT and ritanserin both have a moderate affinity for the 5-HT_7 receptor and WAY100135 does not (To *et al.*, 1995), our findings indicate that 5-HT in the ZI may exert an inhibitory effect on LH release via both 5-HT_{1A} and 5HT_7 receptors.

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