1P EFFECTS OF NICOTINE INFUSION ON OPERANT SELF-ADMINISTRATION OF ETHANOL

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

Male Lister rats, 200 - 250g at start were used; n = 14 (controls) and 13 (nicotine treatments),. They were trained to press levers to obtain alcohol, using the "sucrose fading" method of Grant and Sampson (1985), in which the animals learn to press a lever to obtain sucrose, then the solution is gradually changed to sucrose plus alcohol, then alcohol alone. The operant schedule used was a variable interval (15s) with 30 min access per day, 5 days per week. The rats were trained to respond for 5% ethanol, then osmotic minipumps were implanted, which provided 28 days of either 1.25 or 5 mg/kg/24h nicotine; controls were implanted with sham pumps. After two days recovery time, followed by two weeks self-administration of 5% ethanol, the ethanol was replaced by water for two weeks so that the extinction of responding for ethanol could be studied. Results were analysed by two way analysis of variance.

Nicotine infusions did not alter the self-administration of 5% ethanol (weeks 1 and 2 of nicotine infusion) but did delay the extinction of responding when the ethanol was replaced by water. During the third week after implantation of the minipumps (i.e. the first week of responding for water) the number of rewards attained by the group receiving 5 mg/kg/24h nicotine was significantly higher than that of control animals (Table 1; $F_{1,24}=10.77$, $P<0.01$ compared with corresponding control values compared over all 5 days of that week).

The results suggest that nicotine may alter the pharmacological effects of ethanol, resulting in greater persistence of responding for this drug, or may just affect the reactions to a change in the effects of the responses. Further work is in progress to study the effects of other concentrations of nicotine and to investigate the mechanisms involved.

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Table 1. Values are numbers of rewards attained in 30 min, mean ± s.e.m. Nic = nicotine, doses in mg/kg/24h; wk = week number after implantation of osmotic minipumps; week 1 = responding for 5% ethanol; week 3 = responding for water; d = day of the week.

Treatment	wk 2 d1	wk 2 d2	wk 2 d3	wk 2 d4	wk 2 d5	wk 3 d1	wk 3 d2	wk 3 d3	wk 3 d4	wk 3 d5
Controls	23.±2.4	26.8±3.0	19.3±2.2	21.2±2.7	20.2±2.9	18.1±2.5	15.0±2.4	15.9±1.7	14.3±1.4	11.8±0.9
Nic 1.25	23.9±3.3	20.7±3.2	16.7±2.1	15.6±2.2	16.5±2.5	16.8±1.7	12.8±1.6	12.5±1.5	13.1±1.1	12.1±2.2
Nic 5	28.2±3.2	25.0±2.9	25.3±3.0	25.3±3.2	22.5±2.9	22.7±2.5	19.6±2.1	18.2±2.5	21.1±2.7	21.1±2.7

2P EFFECT OF INTENSE METABOLIC STRESS UPON HEAT SHOCK PROTEIN 70i TRANSGENIC MICE: A [^{14}C]-2-DEOXYGLUCOSE AUTORADIOGRAPHY STUDY

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Heat shock protein 70 in its induced form (Hsp 70i), a member of the 70 kDa heat shock protein superfamily, is involved in the cellular stress response to insult or injury. Its activity as a molecular chaperone, as defined by Ellis and van der Vries (1981), has led several investigators to imply that hsp 70i may be a significant factor in cell survival following injury. Despite this, the precise mechanisms by which hsp 70i could facilitate cell survival remain unknown. The aim of this study was to ascertain whether there are any differences in function-related cerebral glucose utilisation between hsp 70i transgenic (Tg) mice and their wild type (WT) counterparts, using [^{14}C]-2-deoxyglucose autoradiography (Sokoloff *et al*, 1977) under basal conditions and during the intense metabolic activation of the limbic system produced by NMDA receptor blockade (Nehls *et al.*, 1988).

Tg mice over-expressing the human hsp 70i in the hippocampal CA 1, CA 2 and lateral caudate nucleus brain regions under the influence of promoter 1a of the LMO-1 gene, and their WT littermates, were studied. All animals used in the study were of the MF1 strain, male and weighed between 30 and 40 grams. The mice (14 Tg and 14 WT) were divided into groups of seven and injected intraperitoneally with a bolus of dizocilpine (1mg/kg) or saline at a volume of 0.1 ml/10g body weight, and again 10 minutes later with 5 µCi of [^{14}C]-2-deoxyglucose in 0.4ml saline. The animals were returned to their cages and decapitated 45 minutes post isotope administration. Regional isotope levels were then assessed quantitavely in 35 brain regions using a MicroComputer Imaging Device densitometer. These values were normalised to the cerebellar cortex and analysed using a one-way analysis of variance followed by a two-tailed t-test using a Bonferroni correction factor of four, for multiple comparison.

No significant alterations in glucose use were observed between Tg and WT saline treated animals in any of the 35 regions examined. Tg and WT groups treated with dizocilpine displayed 23 and 22 regions respectively, in which glucose use changed significantly when compared to their saline treated counterparts. These alterations in glucose use evoked by dizocilpine mirror previously reported findings in the rat in their anatomical distribution (Kurumaji *et al.*, 1989). When Tg animals treated with dizocilpine were compared to WT animals, five regions exhibited significant alterations in glucose use, namely: anterior thalamic nucleus (+37%) [in Tg relative to WT, both with dizocilpine treatement]; dorsal CA 1 *stratum lacunosum moleculare* (+27%); dorsal hippocampus CA 1 (+16%); superior olivary body (-22%); nucleus of the lateral lemniscus (-16%).

These observations show hsp 70i Tg mice exhibit demonstrable increases in functional limbic glucose use compared to their WT littermates in response to intense metabolic activation such as that produced by dizocilpine treatment.

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3P THE α_2 ADRENOCEPTOR CONTROLLING NORADRENALINE RELEASE IN THE RAT LOCUS COERULEUS IS OF THE α_{2A} SUBTYPE: VOLTAMMETRIC EVIDENCE

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Previous studies in this laboratory have shown that noradrenaline (NA) release in rat locus coeruleus (LC) slices may be detected by voltammetry and is under α_2-adrenoceptor control (Jorm & Stamford, 1993). However, there are currently at least three different subtypes of α_2-adrenoceptors: α_{2A}, α_{2B} and α_{2C} (Bylund, 1992). The aim of the present study was to determine, using fast cyclic voltammetry (FCV), the subtype of α_2-adrenoceptor involved in the control of NA release in LC.

350 μm thick slices containing LC, obtained from male Wistar rats (150-200g), were superfused with artificial CSF at 32°C for 1h before the first stimulation and throughout the experiment. Quantitative real-time NA release evoked by electrical pulses was measured using FCV at carbon fibre microelectrodes. In experiments with antagonist drugs a long stimulation train (40 pulses, 0.1 ms, 20 Hz, every 10 min) was used to allow activation of the autoreceptor. Conversely, a short stimulation train (20 pulses, 0.1 ms, 200 Hz, every 5 min) was needed in experiments studying agonist effects.

On long stimulus trains, the α_{2A}-selective antagonist BRL 44408 (2-[2H-(1-methyl-1,3-dihydroisoindole) methyl]-4,5-dihydroimidazole, 1μM) significantly increased stimulated NA release (+60 ± 2%, P<0.001, One Way ANOVA) whereas the $\alpha_{2B/C}$-selective antagonist ARC 239 (2-[2-[4-(o-methoxyphenyl)piperazin-1-yl] ethyl]-4,4-dimethyl-1,3-(2H,4H)-isoquinolinedione, 500nM) had no significant effect. On short stimulus trains, the α_2 agonist dexmedetomidine (Dex: 10nM) significantly decreased NA release (-47 ± 6%, P<0.001, figure 1). This decrease in NA release was antagonized by BRL 44408 (1μM), but not by ARC 239 (500nM).

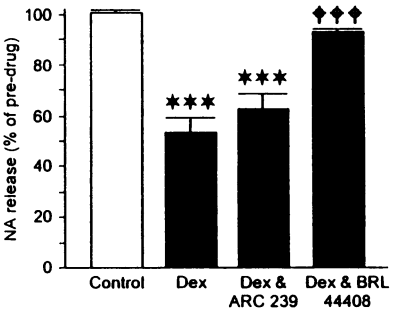

Figure 1: Reversal of the effect of Dex (10nM) by BRL 44408 (1μM) but not by ARC 239 (500nM): *** P<0.001 vs control; ♦♦♦ P<0.001 vs Dex (One Way ANOVA). All values are means ± s.e.m. (n=4 to 7)

We conclude that autoreceptor control of NA release in the LC is mediated by α_2-adrenoceptors of the α_{2A} subtype, as anticipated from the high α_{2A} adrenoceptor immunoreactivity in the LC (Talley et al, 1996).

LFC holds a Basque Government postdoctoral fellowship.

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4P COMPARISON OF REPEATED TREATMENT WITH L-DOPA, PERGOLIDE AND APOMORPHINE ON DYSKINESIA INDUCTION IN MPTP-TREATED COMMON MARMOSETS

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L-DOPA produces dyskinesias in patients with Parkinson's disease (PD) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated non-human primates (Bédard *et al* 1986). Bromocriptine and ropinirole also evoke dyskinesias in MPTP-treated primates previously primed with L-DOPA. However, *de novo* administration of these agonists is associated with very low levels of dyskinesia (Pearce *et al* 1998) which may be due to their long plasma half-lives - a property shared by pergolide. By contrast, *de novo* administration of short-acting agonists (e.g. (+)-PHNO and quinpirole) induces dyskinesias of a similar intensity to those produced by L-DOPA (Luquin *et al* 1992; Bédard *et al* 1993). We now compare the dyskinetic potential of pergolide (a D1/D2 agonist) and the short acting D1/D2 agonist apomorphine with that of L-DOPA in drug naive MPTP-lesioned common marmosets.

Adult common marmosets (*Callithrix jacchus*, n=12) were treated with MPTP (2mg/kg/day s.c. for 5 days) resulting in a stable akinetorigid state. After a recovery period of 15 weeks they were assigned to one of three groups (n=4) and treated with L-DOPA (carbidopa (12.5mg/kg po b.i.d.) 45 mins prior to LD (12.5 mg/kg po b.i.d.)); pergolide (0.5 mg/kg po gavage reduced to 0.4mg/kg od); or apomorphine (0.15 mg/kg s.c. b.i.d.) for 28 consecutive days. Animals were visually scored for dyskinesia using a scale of 0 (none) to 4 (severe, disabling) and disability (0 normal - 18 severely parkinsonian) on a daily basis and locomotor activity was assessed in computer linked observation cages equipped with infrared diode monitors, as previously described (Pearce *et al* 1995). The results were analysed using the Kruskall-Wallis test followed by the Mann-Whitney U test.

There was no difference in the locomotor and disability scores of the three treatment groups, indicating a comparable reversal of the motor deficits induced by acute MPTP treatment. L-DOPA rapidly induced severe dyskinesia. Dyskinesias exhibited by the apomorphine and pergolide groups were of a significantly lower intensity than those produced by L-DOPA (p<0.01), with pergolide showing a trend towards lower levels when compared with apomorphine although this did not reach significance (Figure 1).

As expected, pergolide, the longest-acting of these compounds, induced the fewest dyskinesias. However, although the plasma half-lives of L-DOPA and apomorphine are similar, far greater levels of dyskinesia were produced by L-DOPA. It is interesting to note that pergolide and apomorphine have similar profiles of receptor selectivity and induced similar levels of dyskinesia. These results indicate that the dyskinetic potential of a dopaminergic compound may not depend solely on its plasma half-life and that receptor profiles may play a role.

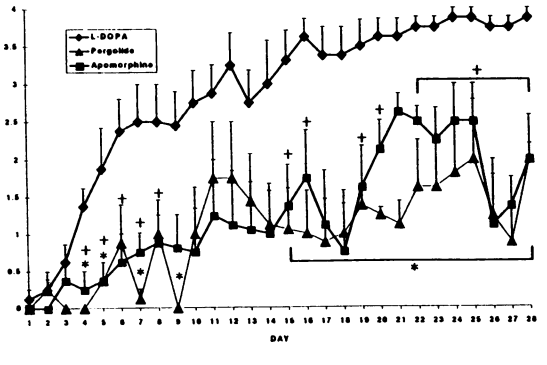

Figure 1 Mean daily dyskinesia scores in MPTP-treated common marmosets over 28 days. L-DOPA 8 CD (12.5mg/kg po b.i.d.); pergolide (0.5 mg/kg po gavage reduced to 0.4mg/kg od); or apomorphine (0.15 mg/kg s.c. b.i.d.)*,+p< 0.05 vs. L-DOPA group.

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5P THE EFFECT OF THE AGONIST Ac-RYYRWKNH$_2$ AND THE ANTAGONIST [Phe$^1\psi$(CH$_2$-NH)Gly^2]NOCICEPTIN (1-13)NH$_2$ AT THE ORL1 RECEPTOR OF CENTRAL AND PERIPHERAL SITES

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Recently identified ligands that are selective for the ORL1 receptor of central and peripheral sites, include the hexapeptide agonist Ac-RYYRWK-NH$_2$ (Dooley *et al.*, 1997) and the antagonist [Phe$^1\psi$(CH$_2$-NH)Gly^2]Nociceptin(1-13)NH$_2$ (Guerrini *et al.*, 1998).

We have shown that nociceptin, the endogenous agonist at the ORL1 receptor, and the hexapeptide Ac-RYYRWK-NH$_2$ are potent and efficacious agonists in the electrically-stimulated rat vas deferens (RVD) and that following intracerebroventricular (icv) administration to rats both will decrease locomotor activity (Nicholson *et al.*, 1997a) and stimulate feeding (Nicholson *et al.*, 1997b). We have now tested the ability of the antagonist [Phe$^1\psi$(CH$_2$-NH)Gly^2]Nociceptin(1-13)NH$_2$ to block the effects of nociceptin and Ac-RYYRWK-NH$_2$ in the RVD and *in vivo* in our locomotor test and feeding paradigm.

Male Hooded Lister rats (~250g) were implanted with cannulae into the right lateral ventricle under isoflurane anaesthesia (co-ordinates from Bregma: ventral 0.8, lateral 1.5 and caudal 3.5mm from the surface of the skull). Animals were housed individually and allowed 1 week to recover before use. Icv injections were in a volume of 2 µl, administered over 30 seconds. All peptides were administered in combination with a cocktail of peptidase inhibitors containing amastatin, bestatin, captopril and phosphoramidon (8mM). For locomotor studies, animals were placed in a novel environment, video monitored for one hour and then scored for horizontal locomotion and rearing behaviour. For feeding studies, animals were starved of food overnight. The following morning, free access to food was allowed for 30 minutes afterwhich the icv injection was administered. Animals were placed back into their home cages with food available. At 30 minutes post icv injection the remaining food was weighed. Data are presented as mean±s.e.mean. Statistical analysis was performed using the student's t-test.

In the RVD [Phe$^1\psi$(CH$_2$-NH)Gly^2]Nociceptin(1-13)NH$_2$ displayed a small amount of agonist-like activity (~30% of E_{max} for nociceptin) yet antagonised both the nociceptin and Ac-RYYRWK-NH$_2$ induced responses (pA$_2$ values of 7.29 and 7.20, slopes 0.82 and 0.86 respectively, n=3) with no depression of the E_{max}. In our locomotor studies, however, [Phe$^1\psi$(CH$_2$-NH)Gly^2]Nociceptin(1-13)NH$_2$ (5nmoles) caused a significant decrease in line crossings and no. rears ($P<0.05$) and so was not tested in combination with nociceptin or the hexapeptide agonist.

In our feeding paradigm, administration of 1nmole [Phe$^1\psi$(CH$_2$-NH)Gly^2]Nociceptin(1-13)NH$_2$ alone stimulated feeding (1.68±0.08g, n=5 compared with 0.70±0.31g, n=4 for vehicles) but when tested against the stimulatory effects of nociceptin (0.05nmoles) where 2.61±0.24g food was consumed (n=7), the effect was blocked (1.05±0.260g, n=10). In contrast, when the antagonist (1nmole) was tested against Ac-RYYRWK-NH$_2$ the stimulatory effect of the hexapeptide on feeding (with 0.03nmoles, 2.31±029g food eaten, n=8 compared with 1.27±0.29g for vehicles, n=8) was not blocked and food intake was increased further (3.89±0.44g, n=10).

Thus, our *in vitro* findings suggested a simple interaction between the antagonist [Phe$^1\psi$(CH2-NH)Gly^2]nociceptin(1-13)NH$_2$ and both of the agonists nociceptin and Ac-RYYRWK-NH$_2$. Our *in vivo* results, however, were less straightforward and suggested a more complex interaction.

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6P EVIDENCE THAT PINDOLOL LACKS THE ABILITY TO ENHANCE THE EFFECT OF SSRIs ON PRESYNAPTIC 5-HT FUNCTION

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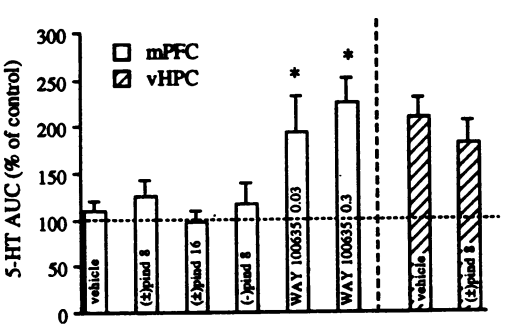
The ß-adrenoceptor and 5-HT$_{1A}$ antagonist, pindolol, is being tested in combination with selective 5-HT reuptake inhibitors (SSRIs) to improve the treatment of major depression. Clinical studies so far have produced contradictory results (e.g. Berman *et al.*, 1997 and Zanardi *et al.*, 1997). The rationale for these trials with pindolol is based on animal studies showing that 5-HT$_{1A}$ antagonists such as WAY 100635 potentiate the effect of an SSRI on extracellular 5-HT in the frontal cortex (Gartside *et al.*, 1995). Such antagonists are thought to act in this way by preventing SSRIs from activating 5-HT$_{1A}$ autoreceptors. Here we report the effect of pindolol in combination with the SSRI paroxetine on forebrain extracellular 5-HT as measured by microdialysis. WAY 100635 was used as a comparator.

Microdialysis probes were stereotaxically implanted in either the medial prefrontal cortex (mPFC) or the ventral hippocampus (vHPC) of male SD rats (280-310 g) under halothane anaesthesia. The following day, probes were perfused with artificial CSF (2 µl min^{-1}) and 20 min dialysates were collected from awake rats. The 5-HT content of dialysates was measured using HPLC-ECD. Once a stable baseline level of 5-HT was obtained, rats were pretreated with either vehicle or drug 30 min prior to paroxetine (5 mg kg^{-1} s.c.). The pretreatments tested were: vehicle (1 ml kg^{-1}), (±)-pindolol (8 or 16 mg kg^{-1} s.c.), (-)-pindolol (8 mg kg^{-1} s.c.), or WAY 100635 (0.03 or 0.3 mg kg^{-1} s.c.). The effects of paroxetine (5 mg kg^{-1} s.c) with either vehicle or (±)-pindolol (8 mg kg^{-1} s.c.) pretreatment were also studied in the vHPC. Dialysate levels of 5-HT were calculated as a % of the mean of the 3 samples preceding paroxetine. Area under the curves (AUCs) were constructed for the 3 h post-paroxetine period and between group comparisons were made using ANOVA and Dunnett's test.

Paroxetine did not increase extracellular 5-HT in the mPFC either alone (ie. after vehicle) or after pretreatment with (±)-pindolol or (-)-pindolol at any of the doses tested (Fig. 1). In contrast, pretreatment with WAY 100635 dose-dependently enhanced the effect of paroxetine on extracellular 5-HT. In the vHPC paroxetine alone caused a 2-fold increase in 5-HT but (±)-pindolol did not enhance this effect at the dose tested.

Figure 1. Effect of paroxetine in combination with vehicle, pindolol or WAY 100635 on extracellular 5-HT. Columns are mean ± s.e.mean AUC values (n=4-7). * $P<0.05$ versus corresponding vehicle.



In summary, at the doses tested pindolol did not enhance the effect of paroxetine on extracellular 5-HT in either the mPFC or vHPC. More importantly, our data indicate that pindolol compares poorly with WAY 100635 as an antagonist of the 5-HT$_{1A}$ autoreceptor. These data are in keeping with our recent finding that pindolol has partial agonist properties at 5-HT$_{1A}$ autoreceptors (Clifford *et al.*, 1998), and argue for the testing of alternative 5-HT$_{1A}$ antagonists in antidepressant trials.

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7P THERAPEUTIC ACTION OF HI-6 AGAINST SOMAN POISONING *IN VITRO*: AN INTERSPECIES COMPARISON

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Some oximes have beneficial actions in animals poisoned with nerve agents which are not related to their ability to reactivate inhibited cholinesterase (Van Helden *et al*, 1991). This study attempted to determine the significance of this direct action in man by comparing the effects of HI-6 in respiratory muscles of rodents, non-human primates and man following poisoning by soman. Open channel blocking by HI-6, believed to underlie the direct action (Tattersall, 1993), was also analysed in human muscle cells.

Diaphragms and intercostal muscles were removed from guinea pigs, marmosets and rhesus monkeys killed by stunning and exsanguination (guinea pigs), ip Euthatal (marmosets) or im ketamine followed by ip Euthatal (rhesus monkeys). Human intercostal muscle was obtained from four patients undergoing surgery for intrathoracic malignancy. All patients gave their informed consent and were anaesthetised using propofol and maintained with Isoflurane/N_2O/O_2. Muscle relaxation was induced with succinyl choline for intubation and maintained with vecuronium. Tissues were prepared for field stimulation (Wolthuis *et al*,1981) and suspended in Krebs solution (Edinburgh Staff, 1970) maintained at 37°C and gassed with carbogene throughout the experiments. Single channel recordings were made at 20°C in cell-attached patches from cultures of human skeletal muscle cells (SkMC 2859, Clonetics, San Diego, USA).

Soman (1μM) produced classical depolarisation tetanic blockade in all species within 5 to 10 min. HI-6 (600μM) partially restored neuromuscular function in all species. The recovery was analysed by washing out the HI-6 to remove direct action, and reinhibition of recovery due to cholinesterase (ChE) reactivation by addition of a second dose of soman. The relative contribution of direct oxime action, cholinesterase reactivation and adaptation are shown for the four species in figure 1.

ChE reactivation contributed to recovery only in guinea pigs. This is consistent with the slower rate of ageing of soman-inhibited ChE to a form resistant to reactivation in guinea pig (Smith and Wolthuis, 1983). Direct oxime action was similar in all tissues except guinea pig intercostal where the recovery was more pronounced. The consistent degree of direct action observed in diaphragms of all species, together with the presence of direct action in human intercostal muscle, indicates that this effect probably contributes to the protective action of HI-6 in man. Furthermore, single channel recordings showed that nicotinic channel blocking by HI-6 in human skeletal muscle cells was similar to that previously reported in mouse muscle (Tattersall, 1993). It has been shown that the channel blocking actions of oximes correlate strongly with their direct therapeutic action at the neuromuscular junction in rodents (Tattersall, 1993).

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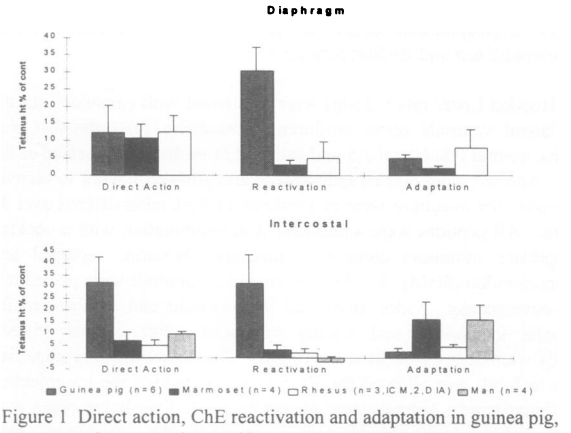

Figure 1 Direct action, ChE reactivation and adaptation in guinea pig, marmoset, rhesus monkey and human respiratory muscles exposed to soman. Mean ± sem (rhesus diaphragm: mean ± limits of variation).

8P ACTIVATION OF CENTRAL 5-HT_{2B} RECEPTORS CAUSES RENAL SYMPATHOEXCITATION IN ANAESTHETIZED RATS

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It has been reported (Knowles *et al.*, 1997) that 5-HT_{2B} receptors may be involved in the modulation of renal sympathetic outflow in anaesthetized rats. Recently, BW 723C86 has been identified as an agonist (Kennett *et al.*, 1996) and SB 204741 (Baxter *et al.*,1995) as an antagonist with some selectivity for 5-HT_{2B} over 5-HT_{2C} and 5-HT_{2A} receptors. In addition, RS-102221 has been shown to be a selective antagonist for 5-HT_{2C} receptors (Bonhaus *et al.*, 1997) while ketanserin has been shown to be a selective antagonist for 5-HT_{2A} receptors (Bonhaus *et al.*, 1995). Therefore these ligands were used to investigate the role of central 5-HT_{2B} receptors in central cardiovascular regulation in anaesthetized rats.

In male Sprague-Dawley rats (250-375 g) anaesthesia was induced with isoflurane and maintained with α-chloralose (80 mg kg^{-1}; i.v.). Rats were artificially ventilated following neuromuscular blockade with decamethonium (3 mg kg^{-1}; i.v.). Simultaneous recordings were made of mean arterial pressure, heart rate and renal (RNA) and phrenic nerve activities (PNA; see Anderson et al., 1992). All experiments were carried out in the presence of 0.1 mg kg^{-1} i.v. of the peripherally acting 5-HT_2 receptor antagonist BW501C67 (Anderson *et al.*, 1992). Drugs were given i.c.v. in a volume of 5 μl over 15s. Changes were compared with vehicle controls that had been pretreated i.c.v. with antagonist vehicle, 10% polyethylene glycol 400, by two-way ANOVA and the least significant difference test was used to compare the means. All values are means ± s.e.mean.

Both 0.2 and 2 μmol kg^{-1} BW 723C86 (i.c.v.; n=4) caused a significant ($P < 0.05$) increase in renal nerve activity after 2 min of 31 ± 8 % and 60 ± 7%, respectively which was associated with no change in HR or PNA. However, the high dose was also associated, after 2 min, with a small and significant fall in MAP of –8 ± 3 mmHg. Pretreatment with SB 204741 (300 nmol kg^{-1}; n=3) blocked the effect of the low dose of BW 723C86 on RNA. However, pretreatment with either RS-102221 (300 nmol kg^{-1}; n = 5) or ketanserin (300 nmol kg^{-1}; n = 5) failed to affect the action of the low dose of BW 723C86 on RNA. Furthermore, the low dose of BW 723C86 in the presence of RS-102221 or ketanserin now caused a small and significant fall in MAP of -2 ± 1 mmHg after 5 min and -4 ± 2 mmHg after 3 min, respectively. Pretreatment (i.c.v.) with SB 204741 (n = 3), RS-102221 (n = 5) or ketanserin (n=5) had no effect on baseline variables.

As RS-102221 has a 500x higher affinity for 5-HT_{2C} receptors and ketanserin has over 1,000x higher affinity for 5-HT_{2A} receptors compared with SB 204741 (Bonhaus *et al.*, 1997) the present experiments indicate that the renal sympathoexcitation evoked by the low dose of i.c.v. BW 723C86 is mediated by activation of 5-HT_{2B} receptors. The present data indicates that the sympathoexcitatory action of BW 723C86 may be selective to the renal outflow and therefore it is suggested that central 5-HT_{2B} receptors may play a role in blood volume regulation.

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9P PHARMACOLOGICAL EVIDENCE FOR THE INVOLVEMENT OF IK_{Ca} AND SK_{Ca} IN EDHF-MEDIATED RELAXATION OF THE RAT HEPATIC ARTERY

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The combination of the potassium (K^+) channel inhibitors charybdotoxin (ChTx) and apamin prevents the action of endothelium-derived hyperpolarizing factor (EDHF) (Zygmunt & Högestätt, 1996). The target for these inhibitors is neither voltage-sensitive nor the large conductance Ca^{2+}-sensitive K^+-channel (Zygmunt *et al.*, 1997). We examined whether ChTx inhibits intermediate conductance Ca^{2+}-sensitive K^+-channels (IK_{Ca}) by replacing this toxin with clotrimazole (CLT) and its metabolite 2-chlorophenyl-bisphenyl-methanol (C23), inhibitors of IK_{Ca} (Brugnara *et al.*, 1995; Ishii *et al.*, 1997a). The effect of ketoconazole (KEC), an analogue of CLT which lacks effect on IK_{Ca} (Ishii *et al.*, 1997a), was also examined. To confirm the involvement of small conductance Ca^{2+}-sensitive K^+-channels (SK_{Ca}), apamin was substituted with scyllatoxin (ScTx) and d-tubocurarine (d-TC) (Auguste *et al.*, 1992; Ishii *et al.*, 1997b).

Hepatic arteries obtained from female Sprague-Dawley rats (250 g) were cut into ring segments and mounted in organ baths for recording of isometric tension. Relaxations induced by cumulative concentrations of acetylcholine (ACh) were studied in segments contracted with phenylephrine (0.1-10 μM). The effect of drugs on EDHF relaxations were recorded after inhibition of NO synthase by 0.3 mM N^G-nitro-L-arginine (L-NA) and cyclo-oxygenase by 10 μM indomethacin (Zygmunt *et al.*, 1997). Data are presented as mean ± s.e.mean and *n* indicates the number of vascular segments (animals) examined.

In the presence of apamin (0.3 μM), CLT caused a concentration-dependent inhibition of EDHF relaxations (Figure 1). The EDHF response was also reduced by C23 (3 μM) when combined with apamin (Figure 1) whereas KEC (10 μM) was without effect (pEC_{50}: 7.1±0.2, KEC *plus* apamin; 7.3±0.1, apamin and E_{max}: 94±3%, KEC *plus* apamin; 95±2%, apamin). CLT (0.3 μM) *plus* apamin had no effect on ACh-induced relaxations in the absence of L-NA (pEC_{50}: 7.4±0.1, CLT *plus* apamin; 7.7±0.1, control and E_{max}: 100±1%, CLT *plus* apamin; 100±1%, control, n=5).

The combination of ScTx (1 μM) and ChTx (0.3 μM) attenuated EDHF relaxations compared to ChTx alone (Figure 1). In the presence of ChTx, d-TC (0.1mM) partially inhibited the effect of EDHF (pEC_{50}: 6.4±0.1, d-TC *plus* ChTx; 7.5±0.1, ChTx; $P<0.05$, Student's t-test; and E_{max}: 98±1%, d-TC *plus* ChTx; 96±3%, ChTx n=6). The ACh-induced relaxation in the absence of L-NA was however unaffected by d-TC *plus* ChTx (pEC_{50}: 7.4±0.1, d-TC *plus* ChTx; 7.7±0.1, control and E_{max}: 100±1%, d-TC *plus* ChTx; 100±1%, control, n=5).

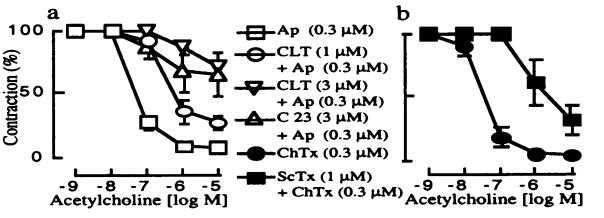

Figure 1 Effects of inhibitors of (a) IK_{Ca} and (b) SK_{Ca} on EDHF relaxations in the presence of (a) apamin (Ap) and (b) ChTx (n=6).

It is suggested that IK_{Ca} and SK_{Ca} are involved in the action of EDHF in the rat hepatic artery. The effect of CLT is not related to inhibition of cytochrome P450 mono-oxygenase since C23, which does not affect this enzyme system, also inhibited EDHF relaxations. Furthermore, the cytochrome P450 mono-oxygenase inhibitor KEC could not replace ChTx. An inhibitory effect of these drugs on muscarinic receptors seems unlikely since relaxations induced by ACh in the absence of L-NA were unaffected.

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10P MODULATION OF ACETYLCHOLINE RELEASE FROM CHOLINERGIC NERVES INNERVATING HUMAN AND GUINEA-PIG TRACHEA BY ENDOMORPHIN -1 AND -2

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Parasympathetic nerves play a dominant role in the control and regulation of airway tone in animals and humans. We have previously demonstrated that activation of opioid receptors results in inhibition of cholinergic neurotransmission and thus cholinergic contractile responses evoked by electrical field stimulation in human (Belvisi *et al.*, 1992) and guinea-pig (Belvisi *et al.*, 1990) airways. Recently, we confirmed the pre-junctional nature of this inhibitory response by demonstrating the inhibitory action of the selective OP_3 agonist [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) on acetylcholine (ACh) release from parasympathetic nerves innervating guinea-pig trachea (Patel *et al.*, 1997). Two endogenous peptides, endomorphin-1 and -2, with high affinity and specificity for the OP_3 opioid receptor have recently been isolated from bovine (Zadina *et al.*, 1997) and human (Hackler *et al.*, 1997) brain tissue. We have investigated the effect of these new putative ligands on cholinergic neurotransmission in epithelium-denuded guinea-pig and human tracheal strips. Data are expressed as mean ± s.e.m. and data obtained before and after drug treatment were compared by Wilcoxon's rank order test for paired data.

Male Dunkin-Hartley guinea-pigs (300-350g) were killed by cervical dislocation, the trachea removed and strips of smooth muscle mounted in chambers. Human tracheal smooth muscle was obtained from donors and for heart/heart-lung transplantation (n= 4 patients, aged 21- 48 years, 3 males). Tissues were superfused with oxygenated Krebs containing indomethacin (10 μM). ACh release was determined by measuring ^{3}H-overflow evoked by electrical field stimulation (EFS, 40 V, 0.5 ms, 4 Hz for 1 min) from tissues pre-loaded with [^{3}H]-choline. Endomorphins were added to the Krebs solution after one control EFS for 10 min, followed by a second EFS. One concentration of drug was tested per tissue.

Endomorphin-1 and -2 produced a concentration-dependent inhibition of EFS-induced ACh release in guinea pig trachea (table) compared to time matched vehicle control experiments (2.46 ± 6.5 % inhibition, n=7, N.S.).

Compound	10 nM	0.1 μM	1μM
Endomorphin 1	22.1 ± 9 (n=6)	41.8 ± 10.9* (n=7)	38.2 ± 5.2* (n=7)
Endomorphin 2	30.1 ± 5 (n=6)	40.7 ± 5.1* (n=7)	60.1 ± 6.3* (n=6)

Furthermore, endomorphin-1 and -2 potently inhibited the release of ACh from cholinergic nerves innervating the human trachea (at 1 μM, 64.7 ± 11.7 % inhibition, n=4, and 64.1 ± 15 % inhibition, n=3, respectively). The opioid receptor antagonist, naloxone (10 μM), had no effect on ACh release in guinea pig trachea (5.17 ± 10.3 % inhibition, n=8, NS). However, pretreatment of tissues with naloxone (10 μM for 20 min) abolished the inhibitory effect of endomorphin-1 (at 1 μM, 10.7 ± 32.4 % inhibition, n=7, NS) and endomorphin-2 (at 1μM, 12.08 ± 7.48 % inhibition, n=8, NS).

Endomorphin-1 and -2 inhibited ACh release in human and guinea pig trachea. These effects were concentration-dependent in the guinea pig trachea. Naloxone abolished the inhibitory effect of the endomorphins on EFS-induced ACh release in guinea pig airways. This data together with previous studies using DAMGO (Patel *et al.*, 1997) confirm a role for 'classical' (naloxone-sensitive) opioid receptors, presumably of the OP_3 subtype, in the control of ACh release from cholinergic nerves innervating guinea-pig trachea.

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11P NORMOXIC PERFUSION OF CHRONICALLY-HYPOXIC (CH) RAT ISOLATED LUNGS LEADS TO PULMONARY VASOCONSTRICTION MEDIATED BY ENDOGENOUS ENDOTHELINS VIA THE ACTIVATION OF ET_B RECEPTORS

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Recently, we have shown that acute hypoxia in lungs from normal rats causes pulmonary vasoconstriction associated with increased production of ET-1 (Smith *et al.*, 1997). However in CH lungs normoxic but not hypoxic perfusion, leads to pulmonary vasoconstriction. Therefore, we have examined the endogenous mediator/s involved in causing normoxia-induced pulmonary vasoconstriction in CH lungs.

Male Wistar rats (250-270g) designated for CH exposure were housed in a normobaric chamber at PO_2 10% 3 weeks prior to use. Control or CH rats were anaesthetised (Sagatal 60 mg kg^{-1},i.p) and heparinised (500 i.u, i.v.), 5min later lungs were isolated and perfused via the pulmonary artery at 5 ml min^{-1} (Krebs' solution gassed with 20% O_2/ 5% $C0_2$/ 75% N_2). After 15 min of single-pass perfusion lungs were perfused in a recirculating manner and were allowed 15 min to stabilise (recirculating volume 50 ml). Drugs were added to the perfusate 15 min after the start of recirculation. In other experiments lungs were perfused with hypoxic Krebs' solution (gassed with 95% N_2, 5% CO_2). The increases in PPP reported are taken as increases above the basal PPP in CH or control lungs.

Basal pulmonary perfusion pressure (PPP) was significantly higher in lungs from CH rats (10.1 ± 0.45 mmHg, n=6 $p< 0.01$) than in lungs from time-matched control animals (6.0 ± 0.35 mmHg, n=6). Normoxic perfusion significantly increased PPP in CH lungs compared to control rats (5.6 ± 1.1 mmHg vs. 0.36 ± 0.13 mmHg, $p< 0.01$) reaching maximum after 75 min of perfusion. In contrast hypoxic perfusion was associated with significantly lower increase in PPP (maximum increase was 0.15 ± 0.35 mmHg, n=3, $p< 0.01$) in CH lungs.

Indomethacin (10 μM) augmented the increase in PPP seen in CH lungs after 90 min of normoxic perfusion. The maximum increase in PPP in the presence of indomethacin was (9.2 ± 0.6 mmHg, n=4, $p< 0.05$) as compared in the absence of indomethacin (5.6 ± 1.1mmHg).

Prazosin (1μM) had no significant effect on normoxia-induced increase in PPP in indomethacin-treated CH lungs. The maximum increase in PPP in the presence of prazosin plus indomethacin (11.2 ± 2.3 mmHg, n=5) was not different to that in indomethacin-treated CH lungs (see above).

In contrast the mixed ET_A/ET_B receptor antagonist bosentan (5μM) inhibited the normoxia-induced increases in PPP in indomethacin-treated CH lungs. The maximum increase in PPP seen in CH lungs in the presence of bosentan was (3 ± 0.43 mmHg) significantly reduced when compared to indomethacin alone (9.2 ± 0.6 mmHg, n=4, $p<0.001$)

The ET_B receptor antagonist BQ788 (5μM) significantly attenuated the normoxia-induced increase in PPP seen in indomethacin-treated CH lungs. The maximum increase in PPP in the presence of BQ788 (1.83 ± 1.0 mmHg, n=4) was markedly lower than that in the presence of indomethacin alone ($p< 0.001$). In contrast the maximum increase in PPP in the presence of the selective ET_A receptor antagonist BQ123 (5μM) was 8.1 ± 0.43 mmHg. This was not significantly different from that seen in control indomethacin-treated CH lungs (n=4, $p< 0.05$).

In summary, normoxic perfusion of isolated lungs from CH rats produced pulmonary vasoconstriction which is augmented in the presence of indomethacin. The finding that normoxia-induced increases in PPP in indomethacin-treated CH lungs are inhibited by the mixed ET_A/ET_B receptor antagonist bosentan (Clozel *et al.*, 1994) or the selective ET_B receptor antagonist BQ788 (Ishikawa *et al.*, 1994) but not by ET_A antagonist BQ123 (Ihara *et al.*, 1992) suggests that normoxia-induced pulmonary vasoconstriction seen under these conditions is mediated by endothelins acting on ET_B receptors. Furthermore, from this and our previous study (Smith *et al.*, 1997) we provide evidence that ET-1 release from normal and CH lungs shows a differential sensitivity to the presence and absence of oxygen. In normal lungs hypoxia increases ET-1 release, while in CH lungs oxygenation of the perfusate elicits pulmonary vasoconstriction mediated by ET-1 release.

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12P INHIBITORY EFFECTS OF THE MUCOSA ON THE CONTRACTILE RESPONSES OF THE PIG DETRUSOR MUSCLE

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The bladder is a compliant organ, showing no significant rise in intravesical pressure during filling until voiding (Coolsaet 1985). The mechanisms underlying this compliance remain unknown although smooth muscle mechanical properties, supraspinal control of the micturition reflex and inhibitory innervation have all been proposed. More recently stretch induced relaxation factors have also been suggested to play a role (Andersson 1993). The present work was performed to determine if the epithelial mucosa has a role in the modification of detrusor muscle responses possibly by the release of a relaxation factor.

Strips of pig bladder were suspended in Krebs-bicarbonate solution under a resting tension of 1g at 37°C and gassed with 5% CO_2 in oxygen. Adjacent tissues of identical size were used in pairs with the mucosa being removed from one of the tissues (denuded). After 60 min a cumulative concentration-response curve to either carbachol or potassium chloride was constructed. After washing the tissues were equilibrated with antagonists for 30 min and a second carbachol curve constructed. Tension responses were expressed as a percentage of the maximal response in tissues without a mucosa.

Carbachol produced a concentration dependent contraction of the bladder tissue but with the response in the presence of the mucosa being only 45.9±4% of that in its absence ($P<0.01$). The mucosa also slightly decreased the sensitivity of the tissue with the geometric EC_{50} values increasing from $2.06x10^{-6}$M (95% C.L. $1.60x10^{-6}$M to $2.66x10^{-6}$M) in tissues without a mucosa to $6.81x10^{-6}$M (95% C.L. $5.07x10^{-6}$M to $9.19x10^{-6}$M) in tissues with a mucosa ($P<0.05$). Responses to potassium were also reduced by the mucosa being only 53±1.97% of the maximum response seen when the mucosa was removed ($P<0.05$).

To determine if the decrease in contraction was caused by the release of NO or other agents acting via soluble guanylate cyclase the effects of the NOS antagonist L-NOARG (50μM) and methylene blue (10μM) were examined. Neither agent however had any effect, with the maximal response to carbachol in tissues with mucosa being 32.1±8.0% with L-NOARG and 35.5±5.5% with methylene blue of the denuded maximal response. Prostaglandins, adenosine nucleotides and catecholamines, all agents known to relax detrusor muscle were not involved in mediating the effect of the mucosa as the maximal response to carbachol in tissues with mucosa was 32.2±6.1%, 40.9±11.7% and 50.2±6.8% of the denuded maximal response in the presence of indomethacin (5μM), suramin (100μM) and propranolol (1μM) respectively.

TEA was also without effect, with the maximal response to carbachol in tissues with mucosa being 51.2±6% of the denuded maximum in the presence of TEA (5mM), indicating an effect on potassium channels is not the mechanism by which the mucosa is inhibiting detrusor contractility.

The results show that the mucosa does inhibit the contraction of detrusor muscle and may well play a significant role in compliance. How the mucosa is causing relaxation remains unclear. If it is releasing a relaxing factor it is not nitric oxide or other agents affecting guanylate cyclase, prostaglandins, adenosine nucleotides or catecholamines nor is it having an action on potassium channels.

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13P α1-ADRENOCEPTOR SUBTYPES IN HUMAN PENILE TISSUE

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The mechanism of penile erection involves interaction between central and local factors. Research suggests that adrenergic activity is one of the most important modulators of erectile tissue. Current classification recognises three α1-adrenoceptor subtypes (α1A, α1B and α1D), characterised using pharmacological and molecular studies (Hieble et al, 1995). An α1-adrenoceptor with a low affinity for prazosin has been identified in functional studies (α1L). It is unclear how this receptor relates to the cloned receptors. α-adrenoceptor antagonists cause erection when injected intracavernosally (Brindley, 1986) and the aim of this study is to identify the α1-adrenoceptor subtypes involved.

Strips of erectile tissue from patients undergoing urethroplasty operations (mean age = 38 ±6 years) were suspended in gassed Krebs at 37°C under a resting tension of 1.5g. A 60min equilibration period was allowed before and after the first agonist dose-response curve with noradrenaline (NA). Tissues were then incubated with various antagonists for 60min (see Table 1), after which a second NA dose response curve was performed. All experiments were conducted in the presence of corticosterone (10μM), cocaine (10μM), propranolol (1μM) and yohimbine (0.5μM).

NA produced concentration-dependent contractions of isolated human penile tissue. Tamsulosin and 5-methyl-urapidil caused rightward shifts of the concentration-response curves giving high affinity estimates (pK_B values of 9.6 and 8.4 respectively) indicating the presence of the α1A-receptor. Prazosin, RS17053 and WB4101 had lower affinity estimates (pK_B values of 8.2, 7.4 and 8.3 respectively). The RS17053 and prazosin affinities are comparable to affinities published for the α1L-receptor with these drugs (Ford et al, 1996). Finally, 100nM BMY7378 failed to produce a shift, indicating that the α1D-receptor does not mediate contraction in this tissue. All the antagonists tested appeared to be acting competitively as maximum responses were not significantly reduced and hill slopes were similar to unity (Table 1).

Table1. Affinities for a range of antagonists in human penile tissue with noradrenaline as the agonist

Antagonist	n	pK_B [a b]	Control max. (g)[a]	Antagonist max. (g)[a c]	Hill[c]
Tamsulosin	5	9.61 ±0.25	3.45 ±0.94	3.54 ±1.00	0.83
5MeU	2	8.39 ±0.19	2.9 ±2.38	2.96 ±2.41	1.11
Prazosin	8	8.21 ±0.07	4.66 ±1.32	4.56 ±1.16	1.13
RS17053	3	7.37 ±0.24	3.09 ±1.67	3.06 ±1.54	1.31
WB4101	9	8.30 ±0.12	2.31 ±0.47	2.33 ±0.56	0.93

[a]Values are the mean ±sem of *n* experiments. [b]Values from individual shifts of dose response curves. [c]Hill slope and maximum response in the presence of the highest concentration of antagonist. Antagonist concentrations used: 30&100nM prazosin and WB4101, 30nM 5MeU and RS17053, 3&10nM tamsulosin.

The affinities of the various antagonists in human penile tissue suggests the involvement of the α1L-adrenoceptor subtype as the main mediator of contraction in this tissue.

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14P THE EFFECTS OF α- AND β-ADRENOCEPTOR ANTAGONISTS ON THE RABBIT CORPUS CAVERNOSUM RELAXATION MEDIATED BY *TITYUS SERRULATUS* SCORPION VENOM

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Tityus serrulatus scorpion venom (TSV) relaxes rabbit corpus cavernosum (RbCC) through activation of non-adrenergic non-cholinergic (NANC) nerve fibres with subsequent release of nitric oxide (Teixeira *et al.*, 1998). In this study, the effects of either α- (phentolamine, prazosin and yoimbine) or β- (propranolol, atenolol, butoxamine and ICI 118,551) adrenoceptor antagonists on the TSV-induced RbCC relaxations have been investigated.

Male New Zealand rabbits (2-2.5 kg) were anaesthetised with Sagatal (40 mg/kg, i.v.). Following penectomy, the RbCC was dissected and cleared of the tunica albuginea and surrounding tissues. The RbCC strips were mounted in a cascade system and superfused with warmed (37°C) and oxygenated (95% O_2 + 5% CO_2) Krebs solution. After a 60-90 min of equilibration, the tissues were precontracted with either noradrenaline (3 μM) or 5-hydroxytriptamine (5-HT; 3 μM - when α-adrenoceptor antagonists were assayed) in order to increase the basal tone, and continuously infused with indomethacin (5.6 μM) to inhibit generation of prostanoids.

The non-selective β-adrenoceptor antagonist propranolol (1 μM, n=8) virtually abolished the isoproterenol (ISO; 100 nmol)- and TSV (30 μg)-induced RbCC relaxations. Similarly, the β_2-adrenoceptor antagonists butoxamine (3 μM, n=8) and ICI 118,551 (1 μM, n=6) significantly inhibited the RbCC relaxations induced by TSV (70±11% and 46±7% inhibition, respectively) and ISO (65±8% and 42±7% inhibition, respectively). The inhibition by propranolol, butoxamine and ICI 118,551 of the TSV-induced relaxations were reversible since relaxations were restored 25 min after stopping the infusion of these antagonists.

In contrast, the β_1-adrenoceptor antagonist atenolol (1 μM, n=4) failed to affect the relaxations induced by TSV (69±22% before and 60±20% during atenolol infusion) and ISO (62±6% before and 49±14% during atenolol infusion).

The non-selective α-adrenoceptor antagonist phentolamine (10 μM, n=6) reversibly abolished the TSV (30 μg) -induced relaxations. As opposed, the α_1-adrenoceptor antagonist prazosin (1 μM, n=9) significantly potentiated the TSV-induced relaxations (49±7% before and 70±8% during prazosin infusion; $p<0.05$). At the concentration used above, prazosin abolished the RbCC contractions evoked by noradrenaline (50 nmol) without affecting those evoked by the α_2-agonist clonidine (100 nmol). The infusion of the α_2-adrenoceptor antagonist yoimbine (1 μM, n=9) markedly inhibited the TSV-induced relaxations (70±8% before and 22±6% during yoimbine infusion). After stopping yoimbine infusion, the relaxations evoked by the venom were restored. The concentration of yoimbine was effective since it abolished the clonidine-induced RbCC contractions.

Our results demonstrated that both selective β_2- (butoxamine, ICI 118,551) and α_2- (yoimbine) adrenoceptor antagonists inhibit the TSV-induced RbCC relaxations whereas α_1- (prazosin) adrenoceptor antagonist potentiates the relaxations. Since the relaxation by TSV is entirely due to nitric oxide release (Teixeira *et al.*, 1998), our results suggest the existence of a co-transmission between adrenergic and nitrergic nervous fibres in cavernosal tissue.

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15P EFFECTS OF AM 404, A CANNABINOID REUPTAKE INHIBITOR, ON EDHF-MEDIATED RELAXATIONS IN THE RAT ISOLATED MESENTERY

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We have recently proposed that an endocannabinoid may be an endothelium-derived hyperpolarising factor (EDHF; Randall *et al.*, 1996). We have now examined the effects of AM404 (N-(4-hydroxyphenyl) arachidonylethanolamide), an inhibitor of the neuronal cannabinoid reuptake transporter (Beltramo *et al.*, 1997), on EDHF-mediated relaxations.

Male Wistar rats (300-550g) were anaesthetised with sodium pentobarbitone (60mg kg^{-1}, i.p.) and the mesenteric arterial bed was isolated (Randall *et al.*, 1996) and perfused with oxygenated Krebs-Henseleit solution, containing indomethacin (3μM). Following 20 min equilibriation, methoxamine (1-2μM) was added in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) to increase perfusion pressure (80-100mmHg). The vasorelaxant effects of carbachol (acting via EDHF) were assessed in the absence and presence of AM404 (3μM or 10μM). Addition of AM404 caused reductions in tone which were restored by supplements of methoxamine. Concentration-response curves were also constructed for the relaxant effects of AM404 (10nM-10μM) in the absence and presence of the CB_1 cannabinoid receptor antagonist, SR141716A (1μM), and also L-NAME (300μM) and their combination.

Carbachol induced dose-related relaxations (ED_{50}=3.26±0.57nmol, mean±s.e.mean; maximum relaxation (R_{max}) =87.0±2.5%, n=16). 3μM AM404 (n=7) caused a significant inhibition of EDHF-mediated relaxations, with an ED_{50} value of 10.3±1.9nmol, ($P<0.01$; ANOVA) and an R_{max} value=43.6±9.3% ($P<0.001$). In the presence of 10μM AM404 (n=7), the relaxant effects of carbachol were comparable (ED_{50}=10.7±0.6nmol; R_{max}=51.6±3.9%) to those in the presence of 3μM. AM404, under control conditions, caused relaxations of tone, which were L-NAME and SR141716A insenstive. However, the relaxations to AM404 (10μM) were sensitive to the combination of L-NAME and SR141716A (75.0±5.0% v 50.1±7.7%; $P<0.05$; n=6). In the presence of 3μM AM404, vasorelaxation to the K-channel activator levcromakalim was unaffected (R_{max}=89.7±3.8% v 99.7±1.5%, n=10), but at 10μM AM404 there was significant inhibition of relaxation to levcromakalim (62.1±12.37% v 99.7±1.5%; $P<0.05$; n=4).

The results of the present study show that AM404 selectively inhibits EDHF-mediated relaxations at 3μM. On the basis of this finding, we propose that the cannabinoid transporter may be involved in the actions of EDHF. In this respect, there are two possibilities: (1) released EDHF is rapidly taken up by the cannabinoid reuptake transporter and recycled, such that in the presence of AM404, EDHF becomes depleted as this is interrupted; (2) the cannabinoid transporter is essential for EDHF release from the endothelium. The additional effects of AM404 at 10μM, on K-channels and CB_1 receptors, question the specificity of AM404 at this concentration. Nevertheless, at 3μM, AM404 inhibits EDHF-mediated relaxation; the underlying mechanisms are currently being investigated.

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16P ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR MEDIATES TO A LARGE EXTENT ACETYLCHOLINE-INDUCED RELAXATION OF HUMAN SUBCUTANEOUS RESISTANCE ARTERIES

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Endothelium-dependent relaxations are achieved by a combination of prostacyclin (PGI_2), nitric oxide (NO) and an endothelium-derived hyperpolarizing factor (EDHF). Inhibition of these responses using cycloxygenase inhibitors (indomethacin), nitric oxide synthase inhibitors (L-N^G-nitroarginine; L-NNA) and potassium channel blockers (charybdotoxin (ChTx) and apamin), respectively, has shown that EDHF assumes a greater functional role than NO as arteries decrease in size (Shimokawa *et al.*, 1996). In this study, the contributions of PGI_2, NO and EDHF to acetylcholine (ACh)-mediated relaxation were assessed in human subcutaneous resistance arteries.

Gluteal subcutaneous fat biopsies were taken under local anaesthesia (2% lignocaine hydrochloride) from normotensive male volunteers (age 56.9±3.3yrs, n=14). The study was approved by the local ethics committee, and all subjects gave their informed written consent. Resistance arteries (mean internal diameter 182±15μm, n=16) were dissected from the biopsies and mounted as ring preparations in a small vessel myograph, containing PSS maintained at 37°C and continuously gassed with 95% O_2/ 5% CO_2, for measurement of isometric force. The vessels were set to their optimum resting force (Mulvany & Halpern, 1977), viability was assessed using a standard start procedure (Aalkjaer *et al.*, 1987) and the integrity of the endothelium was confirmed by adding ACh (0.1-10μM) to vessels submaximally contracted with noradrenaline (NA; 3μM). Cumulative concentration-response curves (CCRCs) were obtained to ACh and repeated following incubation with either (1) indomethacin (10μM for 45min), (2) L-NNA (100μM for 45min) or (3) L-NNA, with a combination of ChTx (50nM for 10min) and apamin (30nM for 10min). Results are mean ± s.e.mean (n=6 for each group) and were compared using Student's paired t-test.

Human subcutaneous resistance arteries, precontracted with NA, relaxed in a concentration-dependent manner to the endothelium-dependent vasodilator ACh. Incubation of the arteries with indomethacin, L-NNA or L-NNA plus ChTx and apamin had no effect on the resting tone. The magnitude and sensitivity of the ACh-induced relaxation were not altered following incubation with indomethacin (maximum relaxation (%) 90.80±4.69 vs 97.56±1.83, P=0.18 and -logIC_{50} 7.23±0.25 vs 7.24±0.20, P=0.96). However, following exposure to L-NNA, ACh-induced relaxation was significantly reduced, but not totally attenuated, (maximum relaxation (%) 91.55±3.95 vs 61.68±3.38, $P<0.0001$) with a corresponding rightward shift of the CCRC (-logIC_{50} 7.19±0.13 vs 6.41±0.10, $P<0.005$). The L-NNA resistant component of ACh-induced relaxation was further attenuated by the combination of ChTx and apamin (maximum relaxation (%) 92.59±3.65 vs 14.93±10.36, $P<0.002$. -logIC_{50} values could not be calculated).

These results demonstrate that endothelium-derived vasodilators do not contribute to the resting tone of human resistance arteries under isometric conditions *in vitro*. The endothelium-dependent relaxation of these arteries in response to ACh is partially mediated by NO but a greater part of the response was independent of NO production. These results compare with those reported for rat mesenteric resistance arteries (Shimokawa *et al.*, 1996) and the inhibition of this NO-independent relaxation with potassium channel blockers indicate that this may be mediated by the as yet unidentified EDHF.

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17P A SUPEROXIDE DISMUTASE MIMETIC IMPAIRS BASAL NITRIC OXIDE ACTIVITY IN RAT AORTA

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A superoxide dismutase (SOD) mimetic Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) restores nitric oxide (NO)-dependent relaxations following impairment by oxidant stress in rabbit aorta (MacKenzie & Martin, 1998). The aim of this study was to examine further the properties of MnTMPyP by assessing if it mimics the ability of authentic SOD to relax precontracted endothelium-containing rings of rat aorta by protecting basal NO from destruction by superoxide anion (O_2^-) (Mian & Martin, 1995).

Female Wistar rats were killed by stunning and exsanguination. The thoracic aorta was removed and cut into rings which were suspended in tissue baths containing oxygenated Krebs solution at 37°C. They were then contracted with phenylephrine (PE, 0.1 – 0.3 μM) and cumulative concentration-response curves to SOD (0.1 – 300 u ml^{-1}) or MnTMPyP (10 nM – 30 μM) constructed. The effects of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) and of endothelial removal were examined on the responses to these agents. The effects of SOD (250 u ml^{-1}) were also examined on MnTMPyP-induced changes in tone. Data are expressed as mean ± s.e. mean of ≥ 6 observations and differences determined by ANOVA followed by the Bonferroni post test.

SOD (0.1 – 300 u ml^{-1}) produced concentration-dependent relaxation of rat aortic rings (maximal relaxation 74.01 ± 5.9 % of initial tone). This relaxation was abolished by L-NAME or endothelial removal ($P<0.001$ for both). In contrast, MnTMPyP (10 nM – 30 μM) produced an enhancement of PE-induced tone (maximum contraction attained 156 ± 9.2 % of initial tone). Pretreatment with L-NAME or endothelial removal abolished the enhancement of PE-induced tone seen with MnTMPyP (maximum contraction 113.7 ± 4.5 and 106.2 ± 4.7 %, respectively. $P<0.001$ for both). Pretreatment with SOD also resulted in blockade: it produced an 3-fold rightward shift but did not depress the maximum enhancement of PE-induced tone seen with MnTMPyP (137.6 ± 9.9 % of initial tone).

Authentic SOD induces endothelium-dependent relaxation of rat aortic rings by protecting basal NO from destruction by O_2^- (Mian & Martin, 1995). In contrast, the SOD mimetic, MnTMPyP, induces an endothelium-dependent enhancement of PE-induced tone in aortic rings. The abolition of this contractile action by treatment with SOD or L-NAME or by removal of the endothelium suggests that MnTMPyP destroys basal NO activity by the generation of O_2^-. Thus, in contrast to its ability to protect NO from an applied oxidant stress (MacKenzie & Martin, 1998), MnTMPyP can paradoxically destroy basal NO through generation of O_2^-. This ability of MnTMPyP to be either a net scavenger or generator of O_2^- depending on the redox environment has been previously described (Gardner *et al.*, 1995). The ability of MnTMPyP to destroy basal NO activity thus compromises its therapeutic potential in the treatment of vascular pathologies associated with oxidant stress.

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18P ROLE OF ENDOTHELIUM IN CLASSICAL AND ATYPICAL β-ADRENOCEPTOR-MEDIATED VASORELAXATION IN RAT ISOLATED AORTA

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Vascular endothelium is involved in modulation of β-adrenoceptor-mediated relaxation. In addition to β_2-adrenoceptors and a small population of β_1-adrenoceptors (O'Donnel & Wanstall, 1985) rat aorta is reported to contain atypical β-adrenoceptors (Oriowo, 1995). The aim of the present study was to investigate the role of endothelium in classical and atypical β-adrenoceptor-mediated relaxation in rat isolated thoracic aorta.

Male Wistar rats were stunned and killed by cervical dislocation. Thoracic aortae were removed and prepared as described previously (Brawley *et al.*, 1997). After an equilibration period of 1 h the artery rings were constricted with noradrenaline (1 μM) and the contraction allowed to stabilise over a period of 10 min. The integrity of the endothelium was tested with acetylcholine (1 and 10 μM). Preparations with intact endothelium produced greater than 50% relaxation while successful endothelial denudation was confirmed by lack of acetylcholine-induced relaxation. After washout, some tissues were incubated with L-NAME (100 μM) or propranolol (0.3 μM) for 30 minutes with control tissues receiving no treatment. The rings were then contracted again with noradrenaline and cumulative concentration-response curves (CRCs) to isoprenaline or the atypical β-adrenoceptor agonist CGP 12177A (Mohell & Dicker, 1989) carried out. After washing, tissues were contracted with noradrenaline for a third time before challenging with acetylcholine to check endothelial function.

Propranolol (0.3 μM) shifted the isoprenaline CRC to the right with an estimated pA_2 of 7.9. As reported previously (Brawley *et al.*, 1997), L-NAME (100 μM) or removal of endothelium significantly reduced isoprenaline-induced relaxation. After these treatments propranolol produced little or no further shift of the isoprenaline CRC (e.g. % relaxation to 100 μM isoprenaline, mean±s.e. mean (n)): (a) control, 94±3 (8); L-NAME, 42±6 (8); L-NAME + propranolol, 54±15 (5): (b) control, 90±5 (7); endothelium removal, 40±6 (6); endothelium removal + propranolol, 42±7 (7).

CGP 12177A produced concentration-dependent vasorelaxation in rat aorta which was unaffected by propranolol (0.3 μM). L-NAME (100 μM) reduced relaxant reponses induced by CGP 12177 although the reduction was less than obtained with isoprenaline (% relaxation to 300 μM CGP 12177 lowered from 91±3, n=19, to 72±8, n=8, $P<0.05$).

The relatively poor antagonism of isoprenaline by propranolol and the agonist effects of CGP 12177A support previous findings of atypical β-adrenoceptors in this preparation (Oriowo, 1995). After removal of endothelium or pretreatment with L-NAME responses to isoprenaline appear to be mediated only by atypical β-adrenoceptors, suggesting that there may be differential modulation of classical and atypical β-adrenoceptor-mediated responses by endothelium.

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19P INHIBITION OF ENDOTHELIUM-DEPENDENT RELAXATION OF RAT ISOLATED AORTIC RINGS BY A LOW MOLECULAR MASS COMPLEX OF COPPER

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Low molecular mass complexes of essential metalloelements such as $Cu(II)_2(3,5\text{-Diisopropylsalicyclate})_4$ (CuDIPS), are effective scavengers of reactive oxygen species such as superoxide ($O_2^{\cdot}$), hydroperoxyl radical and hydroxyl radical, and may be therapeutically useful in the treatment of a number of inflammatory disorders (Sorenson, 1989). CuDIPS has also been shown to down-regulate the activity of nitric oxide synthase (NOS) *in vitro* (Baquail & Sorenson, 1995), but the effect of this metallocomplex on NO-mediated vasorelaxation has yet to be investigated.

Male Wistar rats (250-300 g) were stunned and then killed by cervical dislocation. Segments of aorta (2 mm wide) were mounted in organ baths for recording of isometric tension as previously described (Plane *et al.*, 1997). In some experiments, the endothelial cell layer was removed mechanically by gently rubbing the intima with a wire. Tissues were maintained at 37°C in oxygenated Krebs buffer, containing indomethacin (2.8 μM). All data are expressed as mean ± s.e. mean and differences between mean values were calculated using the Students' t-test.

Acetylcholine (ACh; 0.01-3 μM) and A23187 (0.01-3 μM) each caused concentration-dependent relaxation of endothelium-intact arterial segments pre-constricted with phenylephrine (PE; 3 μM). The maximal reversal of PE-induced tone by ACh and A23187 tone was 103 ± 7 % (n=10) and 92 ± 4 %, (n=9), respectively. Relaxation to either ACh or A23187 was abolished by pre-incubation with the NOS inhibitor L-N^G-nitro arginine (100 μM; 30 mins; n=4 in each case). Following pre-incubation with CuDIPS (50-100 μM; 30 mins), relaxation of aortic rings to ACh and A23187 was significantly attenuated and the maximum responses were reduced to 36 ± 8 % (n=6; P<0.01) and 32 ± 12 % (n=5; P<0.01), respectively. Following washout of CuDIPS, the inhibition of ACh-evoked relaxation persisted and the maximum reversal of PE-induced tone was 65 ± 12 % (n=6; P<0.05). The copper-free carrier molecule 3,5-Diisopropylsalicyclate$_4$ (DIPS; 100 μM; 30 mins), caused a small attenuation of relaxation to ACh and A23187 which was reversed on washout. In the presence of DIPS, the maximum responses to ACh and A23187 were reduced to 70 ± 7 % (n=5; P<0.05) and 84 ± 6 % (n=6; P<0.05), respectively. A lower concentration of either CuDIPS or DIPS (10 μM; 30 mins) did not significantly alter relaxation to ACh (n=4 in each case).

The NO donor 3-morpholinosydnonimine (SIN-1; 0.01-10 μM) evoked concentration-dependent relaxation of endothelium-denuded aortic rings pre-contracted with PE (3 μM). The maximal reversal of PE-induced tone to SIN-1 was 95 ± 2.7 %, (n=16). Pre-incubation with CuDIPS (100 μM; 30 mins) caused a significant, reversible potentiation of relaxation to lower concentrations of SIN-1 but did not alter the maximal response (98 ± 1.0 %; n=4; P>0.05) or the concentration at which this was achieved. In contrast, DIPS (100 μM; 30 mins) did not significantly alter relaxation to SIN-1 (n=4).

These data show that the essential metalloelement complex CuDIPS inhibits arterial relaxation to endothelium-derived NO but potentiates relaxation to exogenously applied NO. These observations indicate that the inhibitory effects of this complex may be due to an action on the vascular endothelium, possibly involving down-regulation of the activity of endothelial NOS (Baquail & Sorenson, 1995). In contrast, the potentiation of relaxation to the NO donor SIN-1 observed in the presence of CuDIPS may be due to the ability of this complex to scavenge reactive oxygen species such as $O_2^{\cdot}$, which inactivate NO (Sorenson, 1989).

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20P GM-CSF RELEASE FROM HUMAN VASCULAR SMOOTH MUSCLE CELLS IS SUPPRESSED BY CO-INDUCED COX-2

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Neutrophil recruitment and activation are primary events in the development of a number of vascular diseases. Neutrophils survival can be promoted by the cytokine granulocyte macrophage-colony stimulating factor (GM-CSF). GM-CSF is thought to be released primarily from endothelial cells and activated leukocytes. We have recently shown that human arterial and venous smooth muscle cells can be induced to release GM-CSF and to express cyclo-oxygenase-2 (COX-2) when stimulated with inflammatory cytokines such as IL-1β and TNFα (Mitchell *et al.*, 1998). Furthermore, we have shown that GM-CSF release is further increased when the COX inhibitor indomethacin is included together with the cytokines (Mitchell *et al.*, 1998). In order to establish more directly a role for COX in these studies we have investigated the effects of a range of inhibitors, including a selective COX-2 inhibitor, on GM-CSF release by human venous and arterial cells.

Samples of saphenous vein (SV) and internal mammary artery (IMA) were dissected clean, cut into small pieces and placed in supplemented culture medium as described previously (Bishop-Bailey *et al.*, 1997). Following explantation, cultured venous and arterial cells were plated onto 96 well plates. When cells reached confluence culture medium was replaced with new medium containing increasing concentrations ($1x10^{-7}$ to $1x10^{-3}$M) of different COX inhibitors including the COX-2 selective inhibitor, L-745,337 (Chan *et al.*, 1995), and COX-1/COX-2 inhibitors aspirin, meloxicam or nimesulide. COX inhibitors were added to venous or arterial cells in the presence of IL-1β (1ng/ml). After 24-hours the medium was removed. Prostaglandin (PG) E_2 release was measured by RIA (Mitchell *et al.*, 1993) and GM-CSF release by ELISA (Saunders *et al.*, 1997). In both venous and arterial cells stimulated with IL-1β, L-745,337 caused concentration-dependent reductions in PGE_2 release and increases in GM-CSF release (Figure 1).

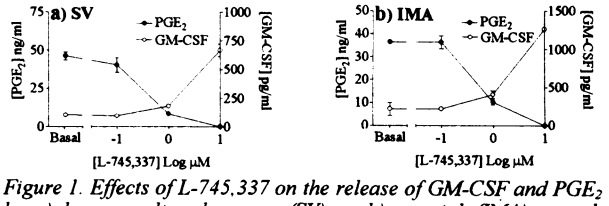

Figure 1. Effects of L-745,337 on the release of GM-CSF and PGE_2 by a) human cultured venous (SV) or b) arterial (IMA) smooth muscle cells stimulated with IL-1β. Data represents mean ± s.e.m. for 3 experiments using cells cultured from 1 patient. Similar results were obtained using cells from 3 other patients.

Similarly GM-CSF release was increased (% increased above basal; E-max) when COX activity was blocked with aspirin (267.1±22.5% in venous; 106.4±12.0% in arterial cells) nimesulide (493.8±5.5% in venous; 112.3±8.5% in arterial cells) or meloxicam (473.6±22.9% in venous; 122.1±9.5% in arterial cells).

In this study GM-CSF release by human vascular smooth muscle cells was increased by a number of chemically distinct inhibitors of COX, including the COX-2 selective compound L-745,337. Thus, we suggest that the inhibition of COX (probably COX-2), and not some other effect of the drugs is the mechanism by which GM-CSF release is increased. Therefore the supression of GM-CSF release by COX-2 may serve to limit the inflammatory response at the level of neutrophil survival.

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21P INTERACTIONS BETWEEN INOSITOL TRISPHOSPHATE RECEPTORS AND FLUORESCENT Ca^{2+} INDICATORS

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Fluorescent Ca^{2+} indicators are extensively used to record changes in free cytosolic Ca^{2+} concentration. We previously reported that Fura 2 and BAPTA were competitive antagonists of cerebellar inositol trisphosphate ($InsP_3$) receptors with half-maximal effects (IC_{50}) occurring at 120μM and 340μM, respectively (Richardson and Taylor, 1993). Subsequent studies have both confirmed our results and their potential to confuse analyses of Ca^{2+} signalling pathways (Combettes and Champeil, 1994). In order to maximise ^{3}H-$InsP_3$ binding, the previous analysis was performed in media buffered at pH 8.3. We recently developed a scintillation proximity assay (SPA) for $InsP_3$ receptors (Patel *et al.*, 1996) and in the present study we use it to re-examine the effects of fluorescent Ca^{2+} indicators on ^{3}H-$InsP_3$ binding to pure cerebellar $InsP_3$ receptors under more physiological conditions (pH 7.2). $InsP_3$ receptors were purified from rat cerebella as previously described (Richardson and Taylor, 1993) and then coupled to wheat germ agglutinin-coated SPA beads (Patel *et al.*, 1996) during a 2.5h incubation at 4°C in medium containing 20mM Tris, 5mM EDTA, 0.1% Surf-Actamps X-100, pH 8.3. The beads were then washed, resuspended in Ca^{2+}-free incubation medium (20mM Hepes, 5mM KH_2PO_4, 0.1% Surf-Actamps X-100, pH 7.2), and incubated with ^{3}H-$InsP_3$ (3nM, 60Ci/mmol) on ice for 10min before recording the level of total ^{3}H-$InsP_3$ binding. Appropriate concentrations of the potassium salts of the fluorescent Ca^{2+} indicators (Molecular Probes) were then added and the level of ^{3}H-$InsP_3$ binding determined after a further 10min incubation. Non-specific binding was determined and the results analysed as previously described (Patel *et al.*, 1996).

Each of the indicators completely displaced specifically bound ^{3}H-$InsP_3$ from its receptor, although they differed in their affinities. The IC_{50} values (means±SEM, n=3) were as follows (μM): Fura 2 = 137±14, Indo 1 = 76±19, Rhod 2 = 66±7, Quin 2 = 20±4, Fluo 3 = 13±3, Ca Green-5N = 6.5±0.5. KCl itself inhibited ^{3}H-$InsP_3$ binding, but only at much higher concentrations (IC_{50}=100mM) than were added with the indicators. Full length recombinant rat type 1 and 3 $InsP_3$ receptors expressed in insect Sf9 cells were used to examine ^{3}H-$InsP_3$ binding in Ca^{2+}-free cytosol-like medium (Cardy *et al.*, 1997). Both Fura 2 (IC_{50} = 782±73μM, n=3) and Ca Green-5N (IC_{50} = 66±4μM, n=3) completely inhibited binding, but only in their Ca^{2+}-free forms.
We conclude that under physiological conditions, Fura 2 and Ca Green-5N in their Ca^{2+}-free forms compete with $InsP_3$ for binding to its receptor, that Ca Green-5N binds with appreciably higher affinity than Fura 2, and that types 1 and 3 $InsP_3$ receptors are similarly affected by fluorescent Ca^{2+} indicators. The interaction between fluorescent Ca^{2+} indicators and $InsP_3$ receptors may significantly perturb measurements of receptor-evoked intracellular Ca^{2+} signals.

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22P Ca^{2+}-INDEPENDENT INHIBITION OF TYPE 1 INOSITOL TRISPHOSPHATE RECEPTORS BY CALMODULIN

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The receptors for inositol trisphosphate (IP_3) are intracellular Ca^{2+} channels that are stimulated by Ca^{2+} and IP_3 and responsible for initiating and propagating intracellular Ca^{2+} signals. Calmodulin, a ubiquitously expressed Ca^{2+}-binding protein, mediates many effects of cytosolic [Ca^{2+}] and contributes to feedback regulation of many Ca^{2+} channels and pumps. We recently demonstrated that calmodulin, irrespective of whether it has Ca^{2+} bound, inhibits IP_3 binding to purified cerebellar IP_3 receptors (Patel *et al.*, 1997). In the present work we have examined the effects of calmodulin on [^{3}H]-IP_3 binding to recombinant rat type 1 and 3 IP_3 receptors expressed in insect Sf9 cells (Cardy *et al.* 1997).
In Ca^{2+}-free cytosol like medium, calmodulin caused a concentration-dependent (IC_{50}= 818±124nM, n=4; mean ± s.e.mean) and reversible inhibition of [^{3}H]-IP_3 binding to type 1 IP_3 receptors. A maximally effective calmodulin concentration (50μM) inhibited [^{3}H]-IP_3 binding by 44±3% (n=6) by decreasing the affinity (K_d) of the receptor for IP_3 from 15.9±0.5nM to 24.4±0.7nM (n=6), without affecting the maximal binding or the Hill slope (all Hill slopes were not significantly different froom unity). The effect was not reproduced by the related Ca^{2+}-binding proteins, troponin C, parvalbumin or S-100 (200μM). Increasing the free [Ca^{2+}] (<2nM-1.1μM) inhibited [^{3}H]-IP_3 binding to type 1 receptors by 46±4% (n=3), but the further inhibition caused by calmodulin (1μM) was similar (32±4%, n=3) at all [Ca^{2+}] indicating that the effect of calmodulin was Ca^{2+}-independent. In the absence of Ca^{2+}, [^{125}I]-calmodulin bound to a single site (K_d= 960nM, n=2) on each type 1 receptor subunit and to an additional site in the presence of Ca^{2+} (K_d= 695nM, n=2). There was no detectable binding of [^{125}I]-calmodulin to type 3 receptors and [^{3}H]-IP_3 binding was insensitive to calmodulin at all [Ca^{2+}]. The Ca^{2+}-independence of the calmodulin effect was examined using the Ca^{2+}-calmodulin antagonists W7 (20μM) and trifluoperazine (20μM), and peptides (10μM) from the Ca^{2+}-calmodulin-binding domain of the type 1 IP_3 receptor (KSHNIVQKTALNWRLSARNAAR) (Yamada *et al.*, 1995) and Ca^{2+}-calmodulin-dependent protein kinase II (LKKFNARRKLKGAILTTMLA). The antagonists and peptides fully reversed the inhibition caused by calmodulin (1μM), but only when the free [Ca^{2+}] exceeded 100nM; they were ineffective in the absence of Ca^{2+}. A mutated form of the IP_3 receptor peptide (W to A; 10μM) that fails to bind Ca^{2+}-calmodulin had no effect on the inhibition of IP_3 binding. Camstatin (APETERAAVAIQAQFRKFQKKKAGS) is derived from the Ca^{2+}-independent calmodulin-binding domain of PEP19 (Slemmon *et al.*, 1996). In Ca^{2+}-free medium, camstatin (50μM) inhibited the effect of calmodulin (1μM) by 93±6% (n=3) and a submaximal concentration (10μM) reduced the effect of calmodulin (1μM) on [^{3}H]-IP_3 binding to an identical extent (72±6%, n=4) at all [Ca^{2+}]. We conclude that calmodulin specifically inhibits [^{3}H]-IP_3 binding to type 1 IP_3 receptors in both its Ca^{2+}-bound and Ca^{2+}-free forms. This may be the first example of a protein regulated by calmodulin in a Ca^{2+}-independent manner. Inhibition of type 1 IP_3 receptors by calmodulin may regulate their sensitivity to IP_3 in response to the changes in cytosolic free calmodulin concentration proposed to accompany stimulation of neurones.
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23P ANTAGONISM OF RECEPTOR-MEDIATED INS(1,4,5)P_3-INDUCED Ca^{2+} SIGNALLING BY THE PARTIAL AGONIST 3F-Ins(1)P,(4,5)PS_2

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We have recently characterised endogenous P2Y-purinoceptor signalling in a mouse L-fibroblast cell-line, transfected either with blank vector (Lvec) or a plasmid allowing 7-9 fold overexpression of the type 1 inositol 1,4,5-trisphosphate receptor ($InsP_3R$) (L15). Lvec and L15 cells exhibited significantly different profiles with respect to purinoceptor activation by UTP, causing Ins(1,4,5)P_3 generation and Ca^{2+} mobilization from intracellular stores, (Davis *et al.*, 1997). To date very few competitive $InsP_3R$ antagonists have been identified for the study of $InsP_3R$ function. In this study we have examined how microinjection of single, intact cells with 3-fluoro-inositol-1-phosphate-4,5-bisphosphorothioate (3F-Ins(1)P, (4,5)PS_2), an $InsP_3R$ partial agonist (Wilcox *et al.*, 1997), can influence agonist-stimulated Ca^{2+} signalling in Lvec and L15 cells.

Cell culture and steady-state $^{45}Ca^{2+}$ release from Lvec and L15 permeabilised cell populations were performed as previously described (Mackrill *et al.*, 1996). Microinjection of single fura-2/AM-loaded cells (2 μM, 60 min) was performed using an Eppendorf micro-manipulator and transjector system. Initial experiments determined that the injection volume was 1.9 ± 0.4 pl, and pipette dilution factor was 13.2 ± 2.8. Injections were performed in the absence of extracellular Ca^{2+} and in the presence of 2 U ml^{-1} of apyrase (to remove any ATP which might be released during injection). Changes in $[Ca^{2+}]_i$ were determined from ratiometric fura-2 fluorescence changes using video imaging.

Heparin, an established $InsP_3R$ antagonist, caused a concentration-dependent inhibition of $^{45}Ca^{2+}$ release induced by 1μM Ins(1,4,5)P_3 in permeabilised Lvec and L15 cells (maximal inhibition at 300 μg ml^{-1}; K_i, 19 ± 4; 44 ± 9 μg ml^{-1} respectively, n =3). Microinjection of heparin into intact Lvec cells (pipette concn. 4 mg ml^{-1}) abolished agonist-mediated Ca^{2+} signalling at all concentrations of UTP (up to 1 mM). Injection of lower doses of heparin abolished Ca^{2+}-release at sub-maximal, and attenuated release at maximal, concentrations of UTP (n=3). 3F-Ins(1)P,(4,5)PS_2 was initially confirmed as a partial agonist, releasing 26.1 ± 0.7 and 30.8 ± 3.0 % of $^{45}Ca^{2+}$ from the ionomycin-sensitive pool in saponin-permeabilised Lvec and L15 cells compared to 67.3 ± 1.4 and 85.0 ± 2.5 % release by Ins(1,4,5)P_3 respectively, in these cell-lines. Microinjection of 3F-Ins(1)P, (4,5)PS_2 also provided evidence of partial agonism. This agent increased $[Ca^{2+}]_i$ from basal values of < 60 nM, to peak responses of 156 ± 11 and 199 ± 29 nM in Lvec and L15 cells: these values compare to respective peak responses of 355 ± 18 and 414 ± 17 nM to a maximal concentration of Ins(1,4,5)P_3 (pipette concn. 10 μM), (n=10).

The ability of the partial agonist to antagonise agonist-stimulated Ca^{2+}-signalling was also studied. Cells within a field were injected (3F-Ins(1)P,(4,5)PS_2 pipette concn. 1 mM), in the absence of extracellular Ca^{2+} and stimulated with 3 μM followed by 300 μM UTP. Cells injected with 3F-Ins(1)P,(4,5)PS_2 (n = 15) did not exhibit significant increases in $[Ca^{2+}]_i$ in response to either concentration of UTP. Subsequent challenge with ionomycin (1 μM) revealed that UTP-stimulated cells exhibited minimal subsequent increases in $[Ca^{2+}]_i$, whereas 3F-Ins(1)P,(4,5)PS_2-injected cells possessed a substantially intact intacellular Ca^{2+}-store.

These data provide the first direct evidence of antagonism of receptor-mediated Ca^{2+} signalling by an $InsP_3R$ -based analogue.

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24P ANALYSIS OF M_1, M_2, M_3, M_4–MUSCARINIC CHOLINOCEPTOR–G PROTEIN COUPLING USING [^{35}S]-GTPγS AND Gα-SPECIFIC IMMUNOPRECIPITATION.

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Muscarinic acetylcholine receptors (mAChRs) belong to the 7-transmembrane receptor superfamily and initiate their signalling pathways by activating heterotrimeric G proteins, with the M_1, M_3 and M_5 subtypes traditionally believed to mediate signalling by interaction with $G\alpha_{q/11}$ and M_2 and M_4 interacting with the isoforms of G_i (Caulfield, 1993). Here we present evidence that immunoprecipitation of [^{35}S]-GTPγS-bound Gα-subunits can be used as a quantitative measure of agonist activity at mAChRs, and that human mAChR subtypes expressed in Chinese hamster ovary cells display differing G protein activation profiles.

The binding of radiolabelled GTPγS to the G-protein α-subunit has been used as a measure of the activation of a number of G protein-coupled receptors (Friedman *et al.*, 1993; Burford *et al.*, 1998). This method was adapted to investigate mAChR-G protein interactions. Optimum assay conditions were found to be 2 min incubations at 30°C in the presence of 1 nM [^{35}S]-GTPγS, 10 mM $MgCl_2$, 100 mM NaCl and 75μg membrane protein ml^{-1}, with inclusion of 1 μM or 10 μM GDP for the M_1/M_3, and M_2/M_4 subtypes respectively. Results are shown as means ± s.e.mean for at least 3 separate membrane preparations.

Expression levels (pmol mg^{-1} protein; n=5), assessed using [^{3}H]-NMS saturation binding, were 2.39 ± 0.19 and 2.52 ± 0.10 for CHO-m1 and -m3, and 0.91 ± 0.02 and 1.51 ± 0.10 for CHO-m2 and -4 cell-lines, respectively.

Upon stimulation with a maximally effective concentration of methacholine (MCh, 1 mM), large increases in [^{35}S]-GTPγS binding above basal levels were observed for all four subtypes in both the presence and absence of GDP. Under optimal assay conditions M_1-mAChR activation caused a greater increase in $G\alpha_{q/11}$-[^{35}S]-GTPγS binding compared to M_3-mAChR (M_1, 37359 ± 3208; M_3, 12527 ± 2318 d.p.m. over basal, respectively). The M_1-mAChR activation also appeared to occur more rapidly (response at 1 min: M_1, 36383 ± 1625; M_1, 6746 ± 883 d.p.m. over basal). The EC_{50} values for activation by MCh were also markedly different (4.5 ± 1.2 and 23.8 ± 2.6 μM for the M_1- and M_3-mAChRs respectively (n=3)).

In contrast M_2 and M_4 receptors showed little difference in their abilities to activate G_i isoforms under optimal assay conditions. At 1, 2 and 5 min the M_2 receptor caused increases of 6089 ±815, 9510 ± 1142 and 6129 ± 1697 d.p.m. over basal respectively. For the M_4 receptor similar stimulations were observed at these time-points (3924 ± 261, 5408 ± 873 and 5238 ± 419 d.p.m. over basal).

We conclude that the M_1-mAChR subtype seems to interact with $G\alpha_{q/11}$ more efficiently, with faster kinetics and more efficaciously when compared to the M_3 subtype expressed at an identical receptor density, whilst M_2 and M_4 subtypes appear to display equal coupling efficiency to $G\alpha_i$.

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25P COMPARATIVE STUDY OF EXTRACELLULAR SIGNAL REGULATED PROTEIN KINASE (ERK) AND C-Jun NH_2-TERMINAL KINASE (JNK) IN CHO CELLS EXPRESSING M_2- AND M_3-MUSCARINIC RECEPTORS

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ERK and JNK are activated by G-protein-coupled receptors via parallel protein kinase cascades. Upon sustained stimulation, ERK and JNK translocate to the nucleus (Chen *et al.*, 1995) where each can phosphorylate and regulate subsets of transcription factors. Previous studies have demonstrated that ERK and JNK activation occur with different time-courses in cells expressing muscarinic receptor subtypes (Coso *et al.*, 1995; Mitchell *et al.*, 1995). In the present study, we have examined the time- and concentration-dependencies of ERK and JNK activation in CHO cells recombinantly expressing M_2- or M_3- muscarinic receptors.

CHO-m2 and -m3 cells (B_{max}, 1521 ± 111; 2231 ± 172 fmol mg^{-1} respectively) were cultured in 6-well dishes. Agonist stimulations were performed in Krebs-Henseleit buffer. ERK was immunoprecipitated with an ERK 1-selective antiserum and kinase activity assessed from cleared lysates by EGFR-peptide phosphorylation; JNK activity was determined using a GST-c-Jun fusion protein coupled to glutathione-Sepharose (Deacon & Blank, 1997).

Time-courses of ERK and JNK activitation were assessed following addition of methacholine (100 μM). Pre-stimulation activities of ERK were similar in the two cell-lines (m2, 98 ± 17; m3, 165 ± 50 fmol mg^{-1} protein min^{-1}; n > 3). In both CHO-m2 and -m3 cells, significant increases in activity were observed by 2 min, and maximal kinase activities were achieved by 5 min after agonist challenge (m2, 3123 ± 573; m3, 3814 ± 1038 fmol mg^{-1} protein min^{-1}; n >3). Pre-stimulation activities of JNK were also similar in the two cell lines (m2, 212 ± 44; m3, 136 ± 55 fmol mg^{-1} protein min^{-1}; n > 3). Agonist challenge in CHO-m2 caused only a modest (2 fold) maximal increase in JNK activity at 10 min which decreased over the subsequent 30 min. In contrast, methacholine stimulated a large (10-fold), but delayed increase in JNK in CHO-m3 (peak at 30 min, 1488 ± 307 fmol mg^{-1} protein min^{-1}; n > 3), and this response was sustained for at least 80 min.

The concentration-dependencies for the ERK and JNK responses stimulated by methacholine (0.01-100 μM) were also studied. EC_{50} values for these responses (for ERK at 5 min and JNK at 30 min) are shown in Table 1.

Table 1. Summary of the time- and dose- dependencies of the ERK, and JNK activation in CHO m2 and CHO m3 cells. (± S.D n=3-9)

CHO-slm2	ERK	JNK
-log EC_{50} (M)	5.47 ± 0.17	Not Determined
Maximal Fold Activity Over Basal	31.8 ± 5.9	2.0 ± 0.6

CHO-m3	ERK	JNK
-log EC_{50} (M)	5.32± 0.13	5.84 ± 0.12
Maximal Fold Activity Over Basal	23.2 ± 6.11	9.6 ± 2.0

Therefore, we report that there are differences in the time-courses and fold activations of ERK and JNK with respect to the CHO-m2 and -m3 receptor. These data compare to those data reported for m1 and m2 receptors stably expressed in Rat 1a fibroblasts (Mitchell *et al.*, 1995). However, in contrast, to the m1 receptor that is not coupled to ERK activation in Rat 1a cells, the m3 receptor can mediate dramatic activation of ERK in CHO cells and cause more sustained activation of JNK.

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26P SUBUNIT SELECTIVE MODULATION OF $GABA_A$ RECEPTORS BY THE NSAID MEFENAMIC ACID

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Mefenamic acid (MFA) is the fifth most widely used non-steroidal anti-inflammatory drug (NSAID) and a common source of self-poisoning; coma and convulsions have been reported in over one third of all cases of overdose (Smolinske, *et al.*, 1990). We have previously reported that MFA modulates $GABA_A$ mediated currents recorded from rat hippocampal neurones (Halliwell & Davey, 1994) and Xenopus *laevis* oocytes injected with rat cortex poly$^+$ RNA (Woodward *et al.*, 1994). In the present study we have further investigated the site(s) of action of MFA on human recombinant $GABA_A$ receptors expressed in Xenopus oocytes.

Stage V or VI Xenopus *laevis* oocytes were nuclear injected with cDNAs encoding for $\alpha_1\beta_2\gamma_{2s}$, $\alpha_1\beta_2$, $\alpha_1\beta_1\gamma_{2s}$, or $\alpha_1\beta_1$ $GABA_A$ receptor subunits. 2-7 days later conventional two electrode voltage clamp techniques were used to record GABA-activated currents from injected cells voltage clamped at -60mV (unless otherwise stated). All drugs were perfused on to cells; agonist activated currents were measured at their peak. Responses in the presence of drugs are expressed as a percentage of control (±s.e.mean of n experiments); EC_{20}, EC_{50} and IC_{50} values were interpolated from sigmoidal curves fitted to agonist and drug concentration effect data. Experiments were undertaken at ambient room temperature (20-25°C).

GABA EC_{20} (10μM)-evoked currents recorded from $\alpha_1\beta_2\gamma_{2s}$ receptor isoforms were potentiated by MFA (0.3-300μM) with a bell-shaped concentration-effect curve; the EC_{50} (and 95% Confidence Interval, [C.I.]) for this effect was 2.2μM (1.9-2.5μM, n=6) and a maximal potentiation to 355±40% (n=6) of control was observed with 30μM MFA. Similarly, GABA EC_{20} (3μM)-evoked currents recorded from cells expressing $\alpha_1\beta_2$ receptor subunits were also potentiated by MFA (1-100μM) with a bell-shaped concentration-effect curve; the EC_{50} (and 95% C.I.) for this effect was 9.5μM (3-29μM) with a maximal potentiation to 593±90% (n=4) of control observed with 30μM MFA. Potentiation of GABA (3μM) evoked currents in $\alpha_1\beta_2$ receptor isoforms was approximately four fold greater in cells voltage clamped at -120mV than in cells voltage clamped at +30mV, indicating a weak voltage dependent effect on $GABA_A$ receptors by MFA. In contrast, MFA had little or no effect on EC_{20} (10μM) GABA-evoked currents recorded from cells expressing $\alpha_1\beta_1\gamma_{2s}$ receptor subunits *and* concentration-dependently inhibited EC_{50} (10μM) currents recorded from $\alpha_1\beta_1$ receptor isoforms; the IC_{50} for this action was 47μM (38-58μM, n=6). In contrast to the subunit sensitive actions of MFA, sodium pentobarbitone (10-100μM) potentiated GABA-evoked currents in all 4 receptor isoforms investigated in this study.

These data demonstrate for the first time that mefenamic acid modulates human recombinant $GABA_A$ receptors in a highly β receptor subunit dependent fashion and in manner similar to that reported for the anticonvulsant, loreclezole (Wafford *et al.*, 1994) and the general anaesthetic, etomidate (Hill-Venning *et al.*, 1997). Additional experiments have investigated the molecular site of action of MFA on recombinant $GABA_A$ receptors with single point mutations critical for the effects of loreclezole and etomidate (Thomas *et al.*, this meeting). Our experiments provide a molecular mechanism for the complex central nervous system effects of mefenamic acid in humans.

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27P BINDING OF THE ANTAGONIST RADIOLIGAND [^{3}H]-ZM241385 to A_{2A} ADENOSINE RECEPTORS IN RAT BRAIN HOMOGENATES

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A_{2A} adenosine receptors show a limited distribution in the central nervous system, being most dense in the neostriatum, nucleus accumbens and olfactory tubercle (Ongini & Fredholm, 1996). In the main, these receptors have been localised using agonist radioligands (e.g. [^{3}H]-CGS21680; Jarvis *et al.* 1989), that also bind to other sites that are incompletely defined. A number of A_{2A}-selective antagonists have been developed, many of which have been radiolabelled, but with limited availability (Ongini & Fredholm, 1996). In this report, I describe a preliminary characterization of binding of the tritium-labelled form of the A_{2A}-selective antagonist ZM241385 (Poucher *et al.* 1995).

Binding of [^{3}H]-ZM241385 was conducted over 30 minutes at room temperature, in TE buffer (50 mM Tris, 1 mM EDTA, pH 7.4) containing adenosine deaminase (1 U.mL^{-1}) and Triton X-100 (0.01 %), harvesting bound ligand by rapid filtration over GF/B filters (Alexander *et al.*, 1994). Membranes from rat neostriatum or cerebellum were prepared by repeated homogenisation and centrifugation, in 10 volumes of TE buffer and at 36 000 g for 15 minutes. Saturation analysis was conducted over the nominal radioligand concentration range of 0.25 - 16 nM, while competition curves were conducted at 1 nM. Non-specific binding was defined by 5 mM theophylline. Data reported are means ± SEM of at least three separate experiments.

Analysis of saturation isotherms in neostriatal membranes for [^{3}H]-ZM241385 showed the radioligand to have a K_d of 0.84 ± 0:05 nM, with a B_{max} of 1680 ± 66 fmol.mg protein^{-1}. At 1 nM radioligand, non-specific binding was 7 ± 1 % total binding. Analysis of saturation data conducted using cerebellar membranes showed that [^{3}H]-ZM241385 bound specifically, but a K_d could not be calculated since binding failed to saturate. At 1 nM [^{3}H]-ZM241385, binding to a density of 19 ± 8 fmol.mg protein^{-1} could be calculated. At this radioligand concentration, non-specific binding represented 67 ± 12 % of total binding.

Analysis of antagonist competition (over the range 10^{-10} – 10^{-6} M) for [^{3}H]-ZM241385 binding to neostriatal membranes showed monophasic profiles with Hill slopes not significantly different from unity. The A_{2A}-selective antagonist SCH58261 (Ongin & Fredholm, 1996) bound with much greater affinity than the A_1-selective antagonist DPCPX (Ongini & Fredholm, 1996) with pK_i values [Hill slope] of 8.93 ± 0.46 [-0.92 ± 0.27] and 6.56 ± 0.05 [-1.23 ± 0.24], respectively.

Competition curves constructed with the A_{2A}-selective agonist CGS21680 (Ongini & Fredholm, 1996), over the concentration range 10^{-10} to 10^{-4} M, also showed a monophasic displacement curve. A pK_i value and Hill slope could be calculated for CGS21680 (5.37 ± 0.07 [-1.01 ± 0.02]) but not the A_1-selective agonist CPA (N^6-cyclopentyladenosine, 74 ± 3 % control binding at 10^{-4} M).

Taken together, these preliminary data indicate that the novel antagonist radioligand [^{3}H]-ZM241385 binds with high affinity, good selectivity and low non-specific binding to A_{2A} adenosine receptors in the rat brain. The low affinity, monophasic nature of the agonist competition curve suggests that the radioligand may be binding preferentially to a low agonist affinity form of the receptor, as has also been suggested for other A_{2A} antagonist radioligands (Zocchi *et al.* 1996).

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28P INHIBITION BY PD165929, A BB_1 ANTAGONIST OF DESENSITISATION AT THE HUMAN BB_1 RECEPTOR

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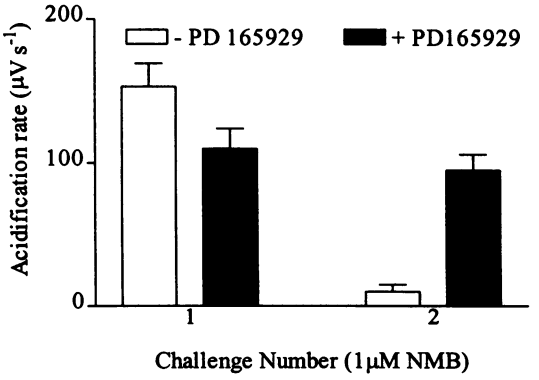
We have previously reported that whilst PD165929 (2-[3-(2,6-Diisopropyl-phenyl)-ureido]-3-(1H-indol-3-yl)-2-methyl-N-(1-pyridin-2-yl-cyclohexylmethyl)-propionamide) acts as a surmountable antagonist at the human BB_1 receptor (Eden *et al.*, 1996), it also enhances the maximal acidification response by an unknown mechanism (Smart *et al.*, 1997). Therefore, the aim of the present study was to characterise further the pharmacology of PD165929 using microphysiometry.

Activity at the human BB_1 receptor was assessed using CHO cells expressing the cloned human BB_1 receptor (CHO-BB_1) in a Cytosensor microphysiometer. The cells were seeded into Cytosensor cups (~0.6x10^6 cells per cup) and perfused at 120µl min^{-1} with bicarbonate-free Hams F-12 (pH 7.4), with the acidification rate being measured every 2min. Neuromedin B (NMB, 1pM-1µM) was serially added to the perfusate in the presence or absence of PD165929 (0.1-1µM) and/or signal transduction modifying agents. The peak responses were normalised to that evoked by 3µM UTP, which activates constitutive P_{2U} receptors. All data are presented as mean±s.e.mean unless otherwise stated.

NMB caused a concentration-dependent increase in the acidification rate of CHO-BB_1 cells, with a pEC_{50} of 9.33±0.66 (n=8). This response was antagonised by PD165929 (0.1-1µM) in a concentration-related, surmountable manner, with an apparent pK_B (derived from the concentration ratio between values for EC_{50} in the presence and absence of antagonist) of 7.79±0.05 (n=5). However, PD165929 also increased the maximum response by 28±8% (n=5) in a concentration-independent manner, but had no effect on the basal acidification rate. Moreover, not only did PD165929 (0.1-1µM) inhibit the acidification response to an initial 1µM NMB challenge, it also inhibited the profound desensitisation of the response to a subsequent NMB challenge (Fig. 1), although unlike the former, this latter effect was not concentration-related. Neither the protein kinase A (PKA) inhibitor, H-89 (1µM) nor the protein kinase C (PKC) inhibitor Ro31-8220 (1µM) had an effect on this anti-desensitisation action of PD165929 (n=4).

Fig. 1. Inhibition by PD165929 (1µM) of the NMB-induced desensitisation of the human BB_1 receptor



These data show that PD165929 enhances the maximal NMB-induced acidification response in a concentration-independent manner, but has no effect on the basal acidification rate, suggesting that PD165929 may not be an inverse agonist. Rather, the current data clearly show that PD165929 inhibits the NMB-induced desensitisation of the BB_1 receptor, via a PKC- and PKA-independent mechanism.

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29P EXTRACELLULAR MATRIX PROTEINS ASSOCIATED WITH HUMAN BRONCHIAL EPITHELIAL CELLS MEDIATE EOSINOPHIL ADHESION

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Cytokine-priming of eosinophils increases the responsiveness of these cells to chemoattractants in allergic inflammation. We have previously shown that tumour necrosis factor-α (TNFα) primes C5a-stimulated eosinophil adhesion to human bronchial epithelial cells (HBEC) and that a combination of monoclonal antibodies (mAbs) against eosinophil β_1- and β_2-integrins inhibits primed adhesion (Burke-Gaffney *et al.*, 1997; Burke-Gaffney & Hellewell, 1998a). Intercellular adhesion molecule-1, expressed on HBEC, is a ligand for eosinophil β_2-integrins (Burke-Gaffney *et al.*, 1997), but the ligands for β_1-integrins on HBEC are unknown. In the present study, we investigated whether extracellular matrix proteins, associated with HBEC monolayers, serve as ligands for eosinophil β_1-integrins.

HBEC (Clonetics, San Diego, USA) were maintained in basal epithelial growth medium supplemented with antibiotics. Monolayers of HBEC were grown to confluence for 6 days on 96-well plates. A specific enzyme-linked immunosorbant assay (Burke-Gaffney & Hellewell, 1998b) was used to measure expression of the extracellular matrix proteins, fibronectin and laminin, associated with HBEC monolayers and results were expressed as mean optical density (OD_{405}) ± s.e. mean of 3 experiments. Human eosinophils isolated from peripheral blood of three mildly atopic adult donors and labelled with a fluorescent dye (Calcein-AM, 10μM; Burke-Gaffney and Hellewell, 1998b), were incubated for 30 min with HBEC in the presence of TNFα (10ng ml^{-1}) or Krebs-Ringer-Phosphate-Dextrose buffer. C5a (10^{-7}M) or buffer were added to HBEC/ eosinophil co-culture for a further 30 min and adhesion measured at 60 min. Results were expressed as mean ± s.e mean of percent adherent cells over total cells (1×10^5) added per well, as determined by fluorescence.

OD_{405} values measured after incubation of HBEC monolayers with mAb against fibronectin (FN-15; 1μg ml^{-1}; 0.39±0.01) or laminin (LAM-89; 1μg ml^{-1}; 0.23±0.05) were significantly greater ($P<0.05$) than that measured in the presence of a control antibody, MOPC21 (1μg ml^{-1}; 0.12±0.01). C5a-stimulated eosinophil adhesion to HBEC was significantly reduced from 30.1±1.8 to 20.9±3.3% with FN-15 (10μg ml^{-1}; $P<0.05$), or from 32±1.8 to 19.5±1.5% with LAM-89 (10μg ml^{-1}; $P<0.001$). C5a-stimulated eosinophil adhesion was increased following priming with TNFα and FN-15 or LAM-89 significantly reduced ($P<0.001$) primed adhesion from 44.4±3.4 to 27.5±3.2% with FN-15, or from 53.2±2.1 to 34.7±2.1% with LAM-89. FN-15 or LAM-89 in combination with an anti-β_2-integrin mAb (6.5E; 10μg ml^{-1}) abolished C5a or TNFα-primed adhesion. These results show that fibronectin and laminin are associated with HBEC monolayers and that they mediate, in part, eosinophil adhesion to HBEC. We conclude that HBEC-associated extracellular matrix proteins, may play an important role in facilitating eosinophil adhesion to HBEC.

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30P INHIBITION OF FMLP-STIMULATED EOSINOPHIL IN VITRO CHEMOTAXIS BY N^{ω}-NITRO-L-ARGININE METHYL ESTER

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Recent evidence suggests that a nitric oxide (NO)-dependent pathway may have a regulating role in eosinophil emigration from the vascular into extracellular tissue where the cell plays a role in the inflamatory response.
Chronic treatment of rats with the NO-synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) inhibits stimulated eosinophil migration both *in vivo* and *ex-vivo* (Ferreira et.al.,1996) Immunohistochemical evidence further demonstrated the existence of a NOS functionally coupled to the cyclic-GMP transduction pathway in the rat eosinophil (Zanardo et. al. 1997). This work aims to demonstrate that such a NO-dependent migration pathway also exists in the human eosinophil.

60ml peripheral blood was taken from healthy volunteers, eosinophils were isolated from percoll-gradient separated granulocytes by magnetic cell sorting using a method described by Hansel et.al. (1991). Isolated eosinophils (>92% purity) were suspended in MEM (Eagle's minimum essential medium,pH 7.2) and incubated with L-NAME (0-3mM) for 30 minutes at 37°C, 5% CO_2. Migration of treated eosinophils through a (5μm pore) polycarbonate filter was stimulated by $1x10^{-7}$M FMLP (N-formyl-methionyl-leucyl-phenyl-alanine) and performed in a 48-well microchemotaxis chamber at 37°C, 5%, CO_2 for 60 minutes. Migrated cells adherent to the filter were stained with Diff-Quick (Baxter healthcare Corp.,USA). Rate of chemotaxis was measured by counting eosinophils that had completely migrated through the filter in five random high-power fields (1000x) per well.

Statistical comparisons were made using Student's t-test.

Inhibition of eosinophil NO production by L-NAME caused a dose dependent inhibition of FMLP-stimulated eosinophil chemotaxis *in vitro*. Inhibition reached 49.5% in eosinophils treated with 2mM L-NAME (n=3). See Figure 1. Results indicate that there is a NO-dependent pathway involved in the human eosinophil migration process.

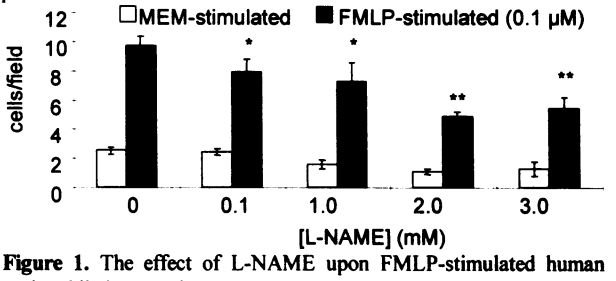

Figure 1. The effect of L-NAME upon FMLP-stimulated human eosinophil chemotaxis *in vitro*.
Mean ± S.E. shown (n=3), * $p<0.05$, ** $p<0.01$ compared to cells stimulated with MEM alone.

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31P INTERACTIONS BETWEEN LIPOTEICHOIC ACID AND LIPOPOLYSACCHARIDE ON HUMAN LUNG MICROVASCULAR ENDOTHELIAL CELL ADHESION MOLECULE EXPRESSION AND INTERLEUKIN-8 RELEASE

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Expression of cell adhesion molecules (CAM) and release of chemokines, such as interleukin-8 (IL-8), from endothelial cells facilitates the recruitment of leukocytes to sites of bacterial infection. Leukocyte recruitment acts to limit the spread of bacteria from the tissue into the blood stream. We have previously shown that lipopolysaccharide (LPS; a gram negative bacterial cell wall component), or lipoteichoic acid (LTA; a gram positive bacterial cell wall component) upregulates CAM expression and IL-8 production in human lung microvascular endothelial cells (HLMVEC; Blease *et al.* 1998a & b). In the present study we have investigated the effects of co-stimulating HLMVEC monolayers with LPS in the presence of LTA on expression of the CAMs E-selectin and intercellular adhesion molecule-1 (ICAM-1) and release of IL-8.

HLMVEC (Clonetics) were maintained in endothelial cell growth medium. Confluent monolayers of HLMVEC grown on 96-well plates were incubated for 2, 6 or 24h with medium, LPS (*Escherichia Coli* B55:05; 0.1μg ml^{-1}), LTA (*Staphylococcus Aureus*; 0.3 - 30μg ml^{-1}) or combinations of LPS and LTA. The conditions for HLMVEC stimulation did not cause cell damage, as assessed using ethidium homodimer-1 (Burke-Gaffney & Hellewell, 1997). A specific enzyme-linked immunosorbant assay (Blease *et al.* 1998a) was used to measure expression of ICAM-1 (24h) and E-selectin (6h) and results were expressed as mean optical density (OD_{405}) ± s.e. mean of 4 experiments. Functional consequences of CAM expression on HLMVEC (at 6h) were assessed by measuring adhesion to HLMVEC of human neutrophils, labelled with Calcein-AM (Blease *et al.*1998a) and results expressed as percent adherent neutrophils. A radioimmunoassay was used to measure concentration of IL-8 protein released into culture supernatants (24h; Au *et al.* 1994). IL-8 mRNA was assessed using reverse transcription polymerase chain reaction following extraction of total RNA from HLMVEC using TRIzol reagent (Pueyo *et al.* 1998). Results were expressed as a ratio of IL-8 mRNA to β-actin, the expression of which is not altered by LPS or LTA.

The effect of increasing concentrations of LTA on IL-8 release, ICAM-1 or E-selectin expression was bell-shaped. In the presence of LTA (30μg ml^{-1}), LPS-induced ICAM-1 expression was significantly reduced ($P<0.01$) from OD_{405} 0.77±0.09 to 0.21±0.03 and E-selectin expression from 0.46±0.01 to 0.07±0.01. Neutrophil adhesion to LPS-treated monolayers, was reduced in the presence of LTA (30μg ml^{-1}) from 32±0.6%, to 18±2.1%. LTA (3μg ml^{-1}) also significantly ($P<0.01$) reduced LPS-induced IL-8 protein release from 708±56 pM to 276±73pM and mRNA expression from 0.53±0.10 to 0.06±0.01 ($P<0.001$). These results show that LTA inhibited LPS-induced CAM expression and IL-8 induction. This suggests that cell wall products from gram positive and gram negative bacteria may interact to reduce leukocyte recruitment to sites of bacterial infection within tissue, leading to the spread of infection to the blood stream often culminating in life-threatening sepsis.

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32P THE MODULATION OF NF-κB AND SUBSEQUENT CYTOKINE PRODUCTION IN HUMAN PURIFIED MAST CELLS: A ROLE FOR PROTEIN KINASE A

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Human lung mast cells play a critical role in atopic allergic inflammation. In addition to generating mediators associated with the early phase allergic response, such as histamine, eicosanoids and proteases, they have been demonstrated to generate cytokines including IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, GM-CSF and TNFα. This study examines the activation and regulation of mast cell NF-κB and its role in TNFα, GM-CSF and IL-8 production.

Human lung mast cells were dispersed and enriched to >95% by affinity purification. Immunocytochemistry was used to assess the number of mast cells expressing activated NF-κB (mab 2C7, Pharmacia Upjohn) and the cytokines TNFα, GM-CSF and IL-8. The number of mast cells expressing activated NF-κB at rest was 3.4 ± 0.5% (mean ± SEM, n=5 for all experiments). Following activation with TNFα (5 ng/ml) and stem cell factor (SCF, 5 ng/ml) activation was upregulated to 77.4 ± 2.6% ($P<0.001$) within 15 min and remained elevated for 24 h. In these experiments, the number of mast cells expressing TNFα, GM-CSF and IL-8 rose from 3.6 ± 0.5%, 2.2 ± 0.4% and 2.8 ± 0.8% to 29.0 ± 1.8%, 31.2 ± 1.3% and 20.6 ± 2.0% respectively all $P<0.05$) following 12 hours of activation.

A role for cyclic AMP in the modulation of NF-κB activation and cytokines expression was suggested by the inhibitory effects of the β_2 agonist, salbutamol, and the type IV phosphodiesterase inhibitor, rolipram. The IC_{50} values of salbutamol and rolipram for inhibition of NF-κB activation were both 0.03 μM. For the inhibition of TNFα, GM-CSF and IL-8 expression, the IC50 values for salbutamol were 2.1, 41.6 and 26.7 μM respectively. The corresponding values for rolipram were 0.5, 11.2 13.6 μM. The variability of these values indicates that NF-κB is not the only transcription factor involved in the synthesis of these cytokines.

To explore the involvement of protein kinase A (PKA) in the modulation of NF-κB activation, mast cells were incubated with the PKA activator, Sp-5-5DCI CBIMPS (100 μM for 10 min), and the PKA inhibitor, R-P-8-Br-cAMP BOO1 (1 mM for 30 min) before activation with TNFα. From a baseline of 77.4 ± 2.6%, the PKA activator reduced the number of cells expressing activated NF-κB to 16.0 ± 2.2% ($P<0.001$). Further, the effects of salbutamol and rolipram were both significantly ($P<0.01$) enhanced by the PKA activator. With the PKA inhibitor, the number of cells expressing activated NF-κB was 79.3 ± 3.5% (n.s.). This drug significantly ($P<0.01$) reduced the inhibitory effect of rolipram. However, it had little effect on the action of salbutamol, suggesting mechanisms in addition to PKA stimulation for the inhibition by the β_2 agonist.

In conclusion, TNFα stimulates the activation of NF-κB and consequential cytokine production in human lung mast cells. Furthermore, the modulation of the activation of NF-κB by protein kinase A supports a role in acute allergic inflammation of agents which elevate intracellular cyclic AMP.

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33P EFFECT OF HEPARIN UPON ADHESION OF POLYMORPHONUCLEAR LEUCOCYTES TO HUMAN STIMULATED UMBILICAL VEIN ENDOTHELIAL CELLS AND ADHESION MOLECULE EXPRESSION *IN VITRO*

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Heparin, in addition to its role as an anticoagulant, is known to possess anti-inflammatory characteristics. We have demonstrated previously that heparin inhibits the adhesion of human polymorphonuclear leucocytes (PMNs) to stimulated human umbilical vein endothelial cells (HUVECs) *in vitro* when heparin is co-incubated with HUVECs and the relevant stimuli for six hours (Lever & Page 1997). In the present study, we have investigated the effect of unfractionated heparin upon expression of the endothelial adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and E-selectin. Additionally, we considered the effects of unfractionated heparin upon adhesion when applied to pre-stimulated HUVECs. The purpose of these studies was to determine whether the previously observed effect of heparin was due to inhibition of adhesion molecule expression.

HUVECs were grown to confluency in 96-well plates and some wells were stimulated for six hours with interleukin-1β (IL-1β; 10U ml^{-1}), lipopolysaccharide (LPS; 2.5μg ml^{-1}) or tumour necrosis factor-α (TNF-α;125U ml^{-1}) and were then washed to remove the stimuli. In adhesion experiments, venous blood was taken from healthy human volunteers (n = 6) and PMNs (>95% neutrophils) were separated by density-dependent centrifugation. Resultant cell pellets were radiolabelled with ^{51}Cr. PMNs were applied to HUVEC monolayers for 30 minutes at 37° C in the absence and presence of heparin (50U ml^{-1} - 1000U ml^{-1}), following which non-adherent cells were removed by washing and adherent cells were lysed and quantified by gamma-counting.

For enzyme linked immunosorbant (ELISA) assays, HUVECs stimulated in the absence and presence of heparin (50U ml^{-1} - 1000U ml^{-1}) were washed and incubated for one hour with mouse anti-human-ICAM-1 or -E-selectin IgG. Monolayers were washed again and were incubated for one hour with goat anti-mouse IgG-horseradish peroxidase conjugate. Following further washes, substrate was added and the expression of ICAM-1 or E-selectin was determined by colorimetric analysis at 450nm. Results are expressed as mean ± s.e. mean and were analysed using a modified t-test.

At all concentrations tested, against all three stimuli, heparin inhibited adhesion of PMNs to HUVECs ($P < 0.05$; maximum inhibition 74% ± 9% with heparin 1000U ml^{-1}; as measured against either IL-1β 10U ml^{-1} or LPS 2.5 μg ml^{-1}). Heparin had some inhibitory effect upon ICAM-1 expression ($P < 0.05$; maximum inhibition 33% ± 9% with heparin 1000U ml^{-1}; as measured against LPS 2.5μg ml^{-1}) but no significant effect upon E-selectin expression ($P > 0.05$; maximum inhibition 17% ± 5% with heparin 500 U ml^{-1}; as measured against LPS 2.5μg ml^{-1}).

These data suggest that whereas inhibition of endothelial adhesion molecule expression may contribute to the previously observed effects of heparin upon PMN adhesion, this is unlikely to be the predominant mechanism of action, as demonstrated by the ability of heparin to inhibit potently the adhesion of PMNs to HUVECs previously stimulated with cytokines.

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34P INTERLEUKIN-1β INHIBITS A TETRAETHYLAMMONIUM-INDUCED SYNAPTIC POTENTIATION IN THE RAT DENTATE GYRUS *IN VITRO*

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The pro-inflammatory cytokine interleukin-1β (IL-1β) has been shown to exert many actions in the CNS including an inhibition of NMDA-receptor dependent forms of tetanically induced long-term potentiation (LTP) in the CA1 and dentate gyrus of the rat hippocampus *in vitro* (Bellinger *et al.*, 1993; Cunningham *et al.*, 1996). IL-1β has also been shown to inhibit voltage dependent calcium channel (VDCC) currents and in our laboratory, NMDA-receptor mediated currents in the dentate gyrus (Coogan and O'Connor, 1997). In the present study we examined the effects of IL-1β on a form of NMDA-receptor-independent synaptic potentiation brought about by application of the K^+ channel blocker tetraethyl-ammonium (TEA; Aniksztejn and Ben-Ari, 1991).

All experiments were carried out in slices of the dentate gyrus (350μm) prepared from young adult Wistar rats (50-120g) following decapitation. Recordings of field excitatory postsynaptic potentials (EPSPs) were made from the medial perforant pathway using a monopolar glass electrode. EPSPs were evoked at a frequency of 0.05Hz at a stimulus strength adjusted to give a response of 30 to 50% maximal EPSP amplitude. All experiments were carried out in the presence of 100μM picrotoxin to eliminate $GABA_A$ receptor mediated responses. Data were analysed statistically with the paired Student's t-test and are expressed as mean±s.e.mean.

Bath application of TEA (25mM) for 10 min gave rise to a depression of the EPSP slope (65±7% of baseline; n=6; P<0.01, Students t-test). Upon washout of TEA a potentiation of the EPSP slope developed (TEA-LTP; 125±5% of baseline 1h following TEA washout; n=6; P<0.05). Application of 100μM D-AP5 (the NMDA receptor antagonist) or 20μM nifedipine (the L subtype VDCC blocker) had no significant effect on the TEA-LTP (120±5% and 127±4% respectively; n=5 for both). The TEA-LTP was found to be $NiCl_2$ (50μM; a T subtype VDCC blocker) and (s)-MCPG (100μM; non specific mGluR antagonist) sensitive (89±5% and 95±4%; n=5 for both respectively; P<0.01 compared to TEA-LTP).

Pre-incubation with IL-1β (1ng/ml) for 30 min prior to TEA application inhibited the potentiation (90±4%; n=6; P<0.01 compared to TEA alone). Application of IL-1β (1ng/ml) 30 min after the establishment of TEA-LTP did not have any affect on the maintenance of the potentiation (118±4% 1h post TEA application; n=5). Pre-incubation of slices with 50ng/ml of the interleukin-1 receptor antagonist reversed the IL-1β inhibition (123±4% 1h post TEA application; n=5).

This study indicates that IL-1β can serve to inhibit a form of synaptic potentiation in the dentate gyrus which is NMDA-receptor independent but may be dependent on mGluR and T-subtype VDCC activation.

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35P *EX VIVO* ASSAY TO DETERMINE THE CYCLOOXYGENASE SELECTIVITY OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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The discovery in the early 1990s of an inducible isoform of cyclooxygenase, COX-2, has lead to the idea that inhibition of COX-2 underlies the therapeutic efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) whilst inhibition of the constitutive COX-1 underlies their side effects (Mitchell *et al.*, 1993). Compounds that preferentially inhibit COX-2 should, therefore, be potent anti-inflammatories with minimal side effects. A considerable number of *in vitro* test systems have been used to characterize the effects of NSAIDs on COX-1 and COX-2. However, these systems are often of little use in predicting the efficacy and selectivity of NSAIDs *in vivo*. Here we describe experiments to establish *ex vivo* the selectivity of NSAIDs given *in vivo*. Anaesthetized (Inactin, 120 mg kg^{-1}) male Wistar rats (220-250 g) received a bolus (i.v., t = 0) of one of the following compounds (dose mg kg^{-1}): aspirin (20), diclofenac (3), L 745,337 (30), sodium salicylate (20), sulindac (10). Blood samples were taken at t = -60, 5, 60, 120, 180, 240, 300 and 360 min and the plasma separated and snap frozen. Rats were killed by an overdose of anaesthetic after the final blood sample was removed. To assay NSAIDs activity in the plasma, 10 µl samples were added to 100 µl medium bathing either, a) IL-1ß-treated A549 cells (COX-2 system) or, b) human washed platelets (COX-1 system). After 30 min calcium ionophore A23187 (50 µM) was added and incubation continued for a further 15 min. Medium was then removed and TXB_2 and PGE_2 levels determined by RIA as a measure of, respectively, COX-1 and COX-2 activity. For control, drugs (10^{-4} to 10^{-11} M) were also added directly to the same assay systems.

All drugs, with the exception of sodium salicylate, inhibited COX-1 and COX-2 with varying potencies when added directly to the assay systems (Table 1). Plasma taken from aspirin-treated rats was without effect on either COX-1 or COX-2 as early as 5 min following administration *in vivo*. This is consistent with rapid *in vivo* metabolism of aspirin to salicylate. Conversely, plasma taken from sulindac-treated rats inhibited COX-1 and COX-2 effectively, with potencies according with *in vivo* metabolism to sulindac sulphide. The activity of diclofenac present in the plasma reduced throughout the *in vivo* experimental period, such that 50% activity was lost within 180-210 min. In addition, the *ex vivo* assay demonstrated that sulindac sulphide was present within the plasma at a COX-1 selective concentration, whereas the level of diclofenac was COX-1/2 non-selective. Plasma from rats treated with the COX-2-selective inhibitor, L745,337, also selectively inhibited COX-2 over COX-1. This is consistent with L745,337 being a COX-2-selective inhibitor *in vivo*.

In conclusion, this test system permits the study of a) drug inactivation (aspirin to salicylate), b) activation of pro-drugs (sulindac to sulindac sulphide), and c) drug selectivity. These attributes make this assay of much potential usefulness in predicting the *in vivo* efficacy and selectivity of both existing NSAIDs and novel COX-2-selective inhibitors. In particular, this assay could be used to analyse plasma samples taken from humans similarly treated with test drugs.

Table 1. Effects of NSAIDs either directly (drugs) or indirectly following administration *in vivo* (plasma) on the activities of COX-1 and COX-2. I = inactive, ND = not determined.

	Drugs IC_{50} (µM)		Plasma % inhibition			
NSAIDs (n ≥ 4)	COX-1	COX-2	COX-1 5'	COX-1 360'	COX-2 5'	COX-2 360'
Aspirin	1.88	12.34	I		I	
Diclofenac	0.0027	0.0016	95	5	90	30
L745,337	23.45	0.027	I		70	70
Sodium salicylate	I	I	I		I	
Sulindac	13.85	196	70	85	10	20
Sulindac sulphide	0.017	0.55	ND		ND	

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36P AGONISTS OF THE PROSTANOID EP_3-RECEPTOR REDUCE MYOCARDIAL INFARCT SIZE IN A RAT MODEL OF MYOCARDIAL ISCHAEMIA AND REPERFUSION

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We have recently reported that the prostanoid-derivative, ONO-AE-248, selectively binds to and activates murine $EP_{3\alpha}$-receptors and reduces the infarct size caused by myocardial ischaemia and reperfusion in the rabbit (Zacharowski *et al.*, 1998). This study was designed to elucidate whether the selective EP_3-receptor agonists, M&B 28767 and GR 63799X, reduce the infarct size caused by regional ischaemia and reperfusion of the rat heart *in vivo*.

Fifty-two male Wistar rats (240-350 g) were anaesthetised with thiopentone sodium (120 mg·kg^{-1} i.p.). All animals were tracheotomised and ventilated with room air (tidal volume: 8-10 ml·kg^{-1}; respiration rate: 70 strokes per min). Subdermal platinum electrodes were placed to allow the determination of a lead II electrocardiogram (ECG). The carotid artery was cannulated to measure mean arterial blood pressure (MAP) and the jugular vein was cannulated for the administration of drugs. The chest was opened by a left sided thoracotomy, the pericardium incised and an atraumatic needle was placed around the left anterior descending coronary artery (LAD). The animals were allowed to recover for 30 min and subsequently the LAD was occluded for 25 min and then reperfused (for 2 h). At the end of the experiment, the LAD was re-occluded, and 1 ml of Evans Blue dye (2% w/v) was injected into the jugular vein to determine the perfused and the non-perfused (area at risk, AR) myocardium. Infarct size (IS) was determined by incubation of the slices of the heart with nitro-blue tetrazolium (NBT, 0.5 mg·ml^{-1}). The following groups were studied: Infusion of (1) saline (vehicle, n=8), (2) M&B 28767 (0.3 µg·kg^{-1}·min^{-1}, n=7), (3) GR 63799X (3 µg·kg^{-1}·min^{-1}, n=7), (4) 5-hydroxydecanoate (5-HD, 5 mg·kg^{-1}, n=6), (5) 5-HD + M&B 28767 (n=6), (6) 5-HD + GR 63799X (n=6), (7) M&B 28767 sham (n=3), (8) GR 63799X sham (n=3), (9) 5-HD sham (n=3) and (10) control sham (n=3). All infusions started 10 min prior LAD-occlusion and were maintained throughout the experiment. All data are expressed as mean±s.e.mean. Statistical differences between groups were analysed by ANOVA followed by a Bonferoni's test.

Treatment of rats with M&B 28767 (GR 63799X) resulted in a significant reduction in IS from 60±3% (control) to 39±6% (38±4%; $p<0.05$) of the AR, respectively. The reductions in IS caused by the EP_3-receptor agonists were not due to a reduction in blood pressure or pressure-rate index. Pretreatment of rats with 5-HD, an inhibitor of mitochondrial ATP-sensitive potassium channels (Garlid *et al.*, 1997), attenuated the cardioprotective effects of M&B 28767 (to 60±4% of the AR, $p>0.05$ vs control) or GR 63799X (to 52±5% of the AR, $p>0.05$ vs control). However, 5-HD did not affect the IS in control (saline) rats (63±3% , $p>0.05$). The AR was not different between the animal groups studied (41±4% to 51±3%).

In sham-operated animals, the drugs used did not affect any of the parameters measured.

Thus, this study demonstrates that two, chemically distinct agonists of the prostanoid EP_3-receptor reduce the IS caused by regional myocardial ischaemia and reperfusion in the rat. We propose that the cardioprotective effects of these agents are due to the openning of mitochondrial ATP-sensitive potassium channels, and that the cardioprotective effects of E-type prostaglandins are, in part, due to activation of EP_3-receptors.

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37P THE STABLE NITROXIDE RADICAL TEMPOL REDUCES INFARCT SIZE CAUSED BY REGIONAL ISCHAEMIA AND REPERFUSION IN THE ISOLATED, PERFUSED HEART OF THE RAT

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Several studies correlate reperfusion injury with the generation of reactive oxygen species (ROS), including superoxide anions (O_2^-) and hydroxyl radicals (OH^-) (Bolli *et al.*, 1988). Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), is a stable piperidine nitroxide radical, which reacts with several ROS including O_2^- (Laight *et al.*, 1997). Here we investigate the effect of Tempol on (i) the infarct size caused by regional ischaemia and reperfusion in the isolated, buffer-perfused heart of the rat, and (ii) hydrogen peroxide (H_2O_2)-mediated injury of rat ventricular myoblasts (H9c2 cells).

Male Wistar rats (250-350 g) were anaesthetised with thiopentone sodium (120 $mg \cdot kg^{-1}$, i.p.) and heparinised (1400 $U \cdot kg^{-1}$, i.p.). The heart was rapidly excised and perfused with modified Krebs' solution in Langendorff mode (37°C, 80 mm Hg) and gassed with 95%O_2/5% CO_2. A suture was positioned around the left anterior descending coronary artery (LAD). After 15 min equilibration, the LAD was occluded for 35 min, followed by 120 min reperfusion. Vehicle (Krebs' solution, n=7) or Tempol (3 mM bolus followed by 1 mM infusion, n=6) was administered throughout reperfusion. At the end of the experiment, area at risk (AAR) and infarct size were determined using Evans Blue dye (0.5% w/v) and nitro-blue tetrazolium (0.5$mg \cdot ml^{-1}$, 37°C for 20 min), respectively.
H9c2 cells were cultured in 96-well plates containing DMEM (200 µl) supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum (FCS) until they reached confluence. Cells were preincubated (10 min) in media (1% FCS) alone or media containing Tempol (0.01-30 mM). Cells were then exposed to 1 mM H_2O_2 for 4 h and cell injury was assessed by measuring the reduction of MTT (3-(4,5-dimethyliazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan. Data are expressed as mean ± s.e.mean (*p< 0.05 compared to control, ANOVA followed by Dunnett's test).

AAR was not significantly different between the groups studied (control; 51±2%, Tempol; 57±3%). Reperfusion of hearts with buffer containing Tempol reduced infarct size from 54±4% (n=7) to 33±2%* (n=6) (see Figure 1). H_2O_2 caused an impairment in mitochondrial respiration which was attenuated by Tempol in a concentration-dependent manner (e.g. control; 100%, 1 mM H_2O_2; 10±6%, 1mM Tempol; 72±11%*).

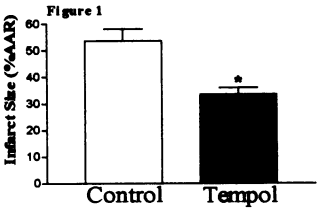

Figure 1 Effect of Tempol on infarct size caused by regional ischaemia (35 min) and reperfusion (2h) in the isolated perfused heart of the rat. Control (vehicle, open column, n=7) Tempol (3 mM bolus + 1 mM infusion, solid column, n=6) *p<0.05

Thus, Tempol, when administered during reperfusion, reduces infarct size caused by regional ischaemia and reperfusion of the isolated buffer-perfused heart of the rat. Tempol also attenuates the reduction in mitochondrial respiration caused by H_2O_2.

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38P AUTORADIOGRAPHIC DISTRIBUTION OF THE α_{1D} ADRENOCEPTOR IN THE RAT CNS

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Although the α_{1D} adrenoceptor (α_{1D}-AR) has been cloned and its mRNA localisation described (Day *et al.*, 1997), the receptor protein itself has not yet been identified in the CNS (Michel *et al.*, 1994).

This study describes the use of a novel α_{1D}-selective piperazine antagonist, SNAP 8493 (8-{2-[4-(2,4,5-trifluorophenyl) piperazin-1-yl]ethyl}-8-azaspiro[4.5]decane-7,9-dione)(Konkel *et al.*, 1998), in the *in vitro* autoradiographic localisation of α_{1D}-AR binding sites in the rat CNS. Brains and spinal cords were removed from 3 adult male Sprague-Dawley rats (250-300g), frozen on dry ice and sectioned coronally at 20 µm. Total α1-AR binding was determined in the presence of 0.3nM ^{3}[H]prazosin in 50 mM TRIS/5 mM EDTA/150 mM NaCl buffer (pH7.4) according to the method of Walden *et al.*, (1997). NS binding was determined in the presence of 10 µM phentolamine and the α_{1D}-AR binding image was determined by subtracting the image obtained in the presence of SNAP 8493 (20nM; K_D at α_{1D}-AR= 1.26 nM) from the total binding. Slide mounted sections were then apposed to a tritium sensitive film for 8 weeks and images analysed by computer assisted densitometry. For each rat five measurements were taken/region and levels of optical density were converted into fmol/g of α_{1D}-AR binding using Amersham tritiated microscales (Table 1).
The highest levels of α_{1D}-AR binding sites were found in the dorsal raphe, lateral amygdala, olfactory bulb, in several thalamic regions, the centromedial and lateral dorsal nuclei as well as in the lateral geniculate nucleus and also in layers IV-V of the somatosensory cortex. Regions with an intermediate number of binding sites include the inferior olives, locus coeruleus, hippocampus and remaining thalamic nuclei. Low α_{1D}-AR binding was found in the nucleus accumbens, caudate putamen and at all levels in the spinal cord, no binding was detected in the corpus callosum.

This is the first report of α_{1D}-AR localisation in the CNS and the distribution pattern strongly indicates a role for this receptor in the modulation and integration of somatosensory information.

Table 1:Regional distribution of $\alpha_{1D\text{-}AR}$ binding sites in rat brain (Mean Bmax, fmol/mg protein ± S.E.M. N=3)

dorsal raphe	112 ± 11.6
lateral amydgala	111 ± 12.2
olfactory bulb (EPL)	90.3 ± 8.8
centromedial thalamus	71.5 ± 13.4
locus ceruleus	51.7 ± 17.5
paravent. thalamic nuc.	43.4 ± 12.4
inferior olives	27.7 ± 6.9
caudate putamen	9.1 ± 2.5
nucleus accumbens	9.2 ± 3.2

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