99P QUANTITATIVE ENZYME RADIOAUTOGRAPHY REVEALS AN UP-REGULATION OF MAO-A AND MAO-B IN DISCRETE BRAIN REGIONS OF PATIENTS WITH HUNTINGTON'S CHOREA

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Huntington's disease is an autosomal dominant, inherited disorder that results in progressive degeneration of the basal ganglia and other forebrain structures and is associated with a clinical profile of movement, cognitive and psychiatric impairments for which there is at present no effective therapy (Huntington's Disease Collaborative Research Group, 1993). The disease is caused by expansion of CAG trinucleotide repeats within the coding region of a novel gene *huntingtin*. Neuropathological, neurochemical and behavioural features of the disease can be reproduced in animals by local injection of excitotoxic or metabolic toxins into the neostriatum (see Faull et al., 1995).

Monoamine oxidases (MAO. EC 1.4.3.4) are integral proteins of outer mitochondrial membranes. Isoenzymes (MAO-A and MAO-B) occur in various cells (both neuronal and non-neuronal in the CNS and peripheral organs) where they oxidatively deaminate biogenic and xenobiotic amines. In the CNS they play not only a physiological role in the metabolic inactivation of released monoamine transmitters (catecholamines, serotonin, histamine) and in the detoxification of xenobiotic amines but perhaps also a pathological role by indirectly generating cytotoxic free radicals during aging and in neurodegenerative diseases. Quantitative enzyme radioautography (using enzyme-selective reversible inhibitors of MAO-A and MAO-B, namely Ro 41-1049 and lazabemide, respectively) has been used to demonstrate, with high resolution, both age- and disease-related changes in enzyme activity in rat and human brain (see Richards et al., 1998). Thus, in Alzheimer brains increased binding of [^{3}H]lazabemide was shown to occur in plaque-associated astrocytes.

Samples of human brain (at the levels of caudate nucleus, globus pall-idus, substantia nigra and pons) from 5 non-diseased control individuals and 5 Huntington's disease cases were obtained at autopsy (5-11h post mortem) from The New Zealand Neurological Foundation Human Brain Bank (University of Auckland) and stored at -80^{0}C. Cryostat sections (16μm) of fresh-frozen tissue were prepared for *in vitro* binding with [^{3}H]Ro 41-1049 [TRK1062, Amersham] and [^{3}H]lazabemide [TRK1063, Amersham] (see Richards et al., 1998 for details); non-specific binding was determined in the presence of 1μM clorgyline or l-deprenyl, respectively. The binding was quantified radioautographically by exposing the radiolabelled sections, together with tritium microscales, to Hyperfilm Tritium (Amersham) for 4 weeks at 4^{0}C. The films were measured with a MCID M2 image analysis system (Imaging Res. Inc, St. Catherines, Ontario, Canada).

MAO-A was increased significantly (~50%; $p< 0.01$) in the putamen, central medial thalamic nucleus, substantia nigra pars compacta and in pons. Higher significant increases in MAO-B (75-200%) occurred in the putamen, ventral striatum, globus pallidus externus and internus, and insula cortex.

We conclude that MAO activities increase in regions of HC brains which are known to undergo neurodegeneration accompanied by glioses. Whether the increased enzyme activity is a cause or effect of the resulting loss of GABAergic neurons is yet to be clarified.

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100P STUDIES ON A NOVEL SELECTIVE β_3-ADRENOCEPTOR AGONIST IN HUMAN RIGHT ATRIAL APPENDAGE AND HUMAN WHITE ADIPOCYTES

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Most evidence for β_3-adrenoceptor-mediated responses in human tissue derives from studies that use CGP-12177 as the β_3-adrenoceptor agonist. However, recent work, especially in β_3-adrenoceptor knock-out mice, suggests that CGP-12177 can stimulate lipolysis via a putative β_4-adrenoceptor (Preitner *et al.*,1998). Moreover, CGP-12177 is far more potent as a β_1- or β_2-adrenoceptor antagonist than as a β_3- or "β_4"-adrenoceptor agonist. Here we describe a novel β_3-adrenoceptor agonist without the drawbacks of CGP-12177. Methods are described in detail in Sennitt *et al.*, (1998).

SB-251023 ((4-{1-[2-(S)-hydroxy-3-(4-hydroxyphenoxy)-propylamino]cyclopentylmethyl}phenoxymethyl)phenyl-phosphonic acid lithium salt) stimulated adenylyl cyclase activity ($pD_2 = 6.25 \pm 0.20$; intrinsic activity (IA) relative to isoprenaline = 0.66 ± 0.10, n=5) in Chinese hamster ovary cell membranes that expressed human cloned β_3-adrenoceptors (390 fmol/mg protein). It was more potent than, and of similar efficacy, to CGP-12177 in these experiments ($pD_2 = 5.61 \pm 0.11$; IA = 0.50 ± 0.07). SB-251023 had no significant agonist activity at human cloned β_1- or β_2-adrenoceptors and it had low affinities for β_1-adrenoceptors ($pK_i = 3.91 \pm 0.10$, n=6) and β_2-adrenoceptors ($pK_i = 4.37 \pm 0.05$, n=5) in binding studies using [^{125}I]-iodocyanopindolol.

SB-251023 stimulated human white adipocyte lipolysis, its IA (not maximal) at 10 μM being 0.45 ± 0.07 and its pD_2 relative to this response 6.19 ± 0.07, n=3. Maximum responses were achieved in the same experiments to isoprenaline ($pD_2 = 8.56 \pm 0.23$) and CGP-12177 ($pD_2 = 6.52 \pm 0.19$; IA = 0.38 ± 0.13). Nadolol (1μM) antagonised the response to isoprenaline ($pK_B = 7.94 \pm 0.11$), but not CGP-12177; it had little effect against SB-251023 (pK_B values <6, 6.3, 7.4).

SB-251023 did not stimulate atrial contractility nor (at 6μM) antagonise the "β_4"-adrenoceptor-mediated stimulant effect of CGP-12177 (1μM) in the presence of (-)-propranolol (200nM). In the presence of both nadolol (10μM) and SB-251023 (6μM) the effect of CGP-12177 was however blunted by (-)-bupranolol (1μM), consistent with the involvement of "β_4"-adrenoceptors. SB 251023 had a cardiodepressant effect ($pD_2 = 6.49 \pm 0.04$, n=7, 4 patients) that was not affected by (-)-propranolol (200 nM), nadolol (10 μM), or nadolol plus (-)-bupranolol (1μM), showing that it was not mediated by β_1- , β_2- , β_3- or putative β_4-adrenoceptors. In conclusion, SB-251023 is a selective human β_3-adrenoceptor agonist that lacks "β_4"-adrenoceptor agonist or antagonist activity, but has a non-β-adrenoceptor-mediated cardiodepressant effect. Its nadolol-insensitive lipolytic activity is consistent with data obtained using structurally and pharmacologically similar compounds in supporting a role for β_3-adrenoceptors in human white adipocyte lipolysis (Sennitt *et al.*, 1998).

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101P COMPARISON OF THE APPARENT AFFINITY OF HISTAMINE H_3-RECEPTOR LIGANDS IN GUINEA-PIG CORTEX AND ILEUM LONGITUDINAL MUSCLE MYENTERIC PLEXUS: FURTHER EVIDENCE FOR RECEPTOR HETEROGENEITY

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Recently, we developed a histamine H_3-receptor (H_3-R) radioligand binding assay, in guinea-pig cortex, using the antagonist [^{3}H]-clobenpropit ([^{3}H]-clo) (Harper *et al.*, 1997a). We found that the apparent affinity (pK_i') values of antagonists remained unaltered in the presence of high concentrations of metal ions, whilst the pK_i' estimates for agonists were reduced by an amount which appeared to correlate with the expression of agonist intrinsic activity (Harper *et al.*, 1997b). Furthermore, the pK_i' values, estimated in the presence of metal ions, were comparable to the agonist pK_A' values estimated in functional bioassays. In another study, we noted that histamine and it's homologues expressed different pK_i' values for H_3-Rs in guinea-pig cortex and ileum longitudinal muscle myenteric plexus (LMMP) (Harper *et al.*, 1997c). Although the between tissue differences could be interpreted as evidence for H_3-R subtypes, it was also possible to explain them by assuming that the agonists' induced the formation of, or bound to high affinity states of the receptor (Kent *et al.*, 1980). Here, in an attempt to discriminate between these explanations, we have developed a H_3-R radioligand binding assay in the LMMP using [^{3}H]-clo and a buffer containing a high concentration of metal ions. The aim was to determine whether the tissue specific differences in pK_i' values for ligands would remain when agonist high affinity binding was eliminated.

Cortex and LMMP membranes were prepared as described previously (Harper *et al.*, 1997d) and diluted in 20mM Hepes-NaOH buffer containing 300mM NaCl and 3mM metyrapone (buffer A). A 4mg ml^{-1} added tissue concentration was used for all cortex experiments and experimental procedures were as described previously (Harper *et al.*, 1997b). In buffer A there was a linear relationship between the specific binding of [^{3}H]-clo and added LMMP concentration up to 40mg ml^{-1}. At a 30mg.ml^{-1} added LMMP concentration 5.8±1.2% of the added [^{3}H]-clo was bound and specific binding was 47.7±2.0%. The specific binding to both membrane preparations was saturable and Hill plot slopes were not significantly different from unity (LMMP n_H=0.84±0.08; n=3±s.e.mean; cortex n_H=0.98±0.01; n=3±s.e.mean). There was no significant difference between the estimated apparent affinity (pK_D') of [^{3}H]-clo for H_3-Rs in the two tissues (LMMP=9.45±0.12; and cortex=9.44±0.17). The LMMP H_3-R B_{max} (0.78±0.19fmol mg^{-1} o.w.w. n=3±s.e.mean) was significantly lower than that estimated in the cortex (5.92±1.68fmol mg^{-1} o.w.w. n=3±s.e.mean) (ANOVA p<0.01). The estimated pK_i' values of 7 of the 11 ligands (*R*-α-MH, imetit, imbutamine, impentamine, imhexamine, thioperamide and GR175737) were significantly higher in the cortex than in the LMMP (Table 1). Principal components analysis (Meester *et al.*, 1998) indicated that the pK_i' values obtained in the LMMP were significantly different from those obtained in the cortex ($F_{(1,66)}$=29.0, p<0.01). This study adds support to the view that there are H_3-R sub-types which can be distinguished by higher (>C3) histamine homologues (Harper *et al.*, 1999).

Table 1 pK_i' values in LMMP and cortex (*p<0.05; **p<0.01; ***p<0.001)

Compound	pK_i' LMMP	n	ΔpK	pK_i' cortex	n
R-α-MH	6.63 ± 0.07	4	0.80**	7.43 ± 0.12	4
histamine	6.22 ± 0.16	5	-0.06	6.16 ± 0.05	4
imetit	7.53 ± 0.09	5	0.70**	8.24 ± 0.12	4
homohistamine	6.56 ± 0.10	5	-0.29	6.28 ± 0.19	4
imbutamine	7.62 ± 0.12	5	0.64*	8.26 ± 0.26	4
impentamine	7.61 ± 0.13	4	0.76**	8.37 ± 0.09	4
imhexamine	7.38 ± 0.08	4	1.04***	8.32 ± 0.03	4
thioperamide	8.39 ± 0.05	5	0.72***	9.11 ± 0.11	4
JB96132	8.73 ± 0.17	4	0.49	9.22 ± 0.17	3
GT2016	6.98 ± 0.15	4	0.08	7.06 ± 0.07	4
GR175737	7.67 ± 0.10	5	0.54*	8.21 ± 0.26	4

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102P CHARACTERISATION OF THE BINDING OF [^{3}H]-HISTAMINE TO HISTAMINE H_3-RECEPTOR SITES IN GUINEA-PIG CEREBRAL CORTEX MEMBRANES

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Previously, we found that the apparent affinity (pK_i') values estimated for *R*-α-methylhistamine (*R*-α-MH) in guinea-pig cerebral cortex and ileum longitudinal muscle myenteric plexus (LMMP) assays were indistinguishable when [^{3}H]-*R*-α-MH was used as radiolabel (Harper *et al.*, 1997a). In contrast, in the same study, histamine (HA) and several close structural homologues (homohistamine, imbutamine, impentamine) but not other chemical classes of H_3-receptor ligands, expressed lower pK_i' values (~2 log units) in the guinea-pig cerebral cortex than in the LMMP. To investigate this result, which is not compatible with the existence of a homogenous H_3-receptor population, we have now used [^{3}H]-HA as radiolabel in the same guinea-pig cortex assay.

Cortex membranes and the experimental procedures were as described before (Harper *et al.*, 1997b). A final assay concentration of 1nM [^{3}H]-HA was used for tissue concentration, kinetic and competition studies. 4mg of tissue was added to each 500µl final assay volume. The specific binding of [^{3}H]-HA was linearly related to added tissue concentration up to 15mg ml^{-1}. At a 10mg ml^{-1} tissue concentration, 1.6±0.1% of the [^{3}H]-HA was bound (specific binding=69.6±2.3%; n=4±s.e.mean). The specific binding of [^{3}H]-HA was saturable, Scatchard plots were linear and Hill plot slopes were not significantly different from unity (n_H=0.93±0.08; n=5). The pK_D' of [^{3}H]-HA was 9.05±0.07 (previously, [^{3}H]-*R*-α-MH=9.91±0.07) and the B_{max} was 2.80±0.40 fmol mg^{-1} o.w.w. (n=5). Specific binding reached equilibrium after 40min and remained constant for >4h. The dissociation $t_{½}$ was 8.45±0.40 min (n=4). The H_1- and H_2-receptor antagonists, pyrilamine and famotidine, had pK_i' values of 4.64±0.06 and 3.78±0.04, respectively, indicating that these receptor classes were not significantly labelled. In the competition studies (Table 1) only the n_H values for thioperamide (0.83±0.06) and JB96132 (0.70 ± 0.11) were significantly different from unity. When [^{3}H]-HA was used, the pK_i' values for compounds 2, 3, 4, 5 and 6 were significantly higher than those estimated using [^{3}H]-*R*-α-MH in the LMMP (Table 1). In contrast, when [^{3}H]-HA was used the pK_i' values for compounds 2, 4, 5, 6 and 8 were significantly lower than those estimated previously when [^{3}H]-*R*-α-MH was used as radiolabel in the cortex (Table 1).

These data enhance the view there are at least two H_3-receptor sites (West *et al.*, 1990; Leurs *et al.*, 1996). Moreover, it appears that these sites can be distinguished by some close structural analogues of histamine but not by some of those ligands often used as reference compounds for this receptor.

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Table 1 pK_i' values (***p<0.001; **p<0.01, *p<0.05 ANOVA) 11=JB96132 (*N*-[4-1*H*-imidazol-4-butyl]-*N*'-(4-chlorobenzyl)-sulphamide).

Compound	[^{3}H]-*R*-α-MH LMMP	ΔpK	[^{3}H]-HA cortex	ΔpK	[^{3}H]-*R*-α-MH cortex
1= *R*-α-MH	9.82 ± 0.09 (9)	+0.02	9.84 ± 0.04 (5)	+0.23	10.07 ± 0.16 (8)
2= histamine	7.98 ± 0.16 (7)***	+0.71	8.69 ± 0.11 (5)	+1.15	9.84 ± 0.14 (6)***
3= homohistamine	7.59 ± 0.07 (4)**	+0.65	8.16 ± 0.09 (5)	+0.31	8.47 ± 0.14 (6)
4= imbutamine	8.37 ± 0.09 (4)***	+1.23	9.60 ± 0.06 (5)	+0.78	10.38 ± 0.09 (6)***
5= impentamine	8.98 ± 0.14 (6)*	+0.41	9.39 ± 0.06 (5)	+0.92	10.31 ± 0.15 (6)***
6= imhexamine	7.67 ± 0.21 (6)***	+1.20	8.87 ± 0.05 (5)	+1.01	9.88 ± 0.09 (6)***
7= imetit	10.01 ± 0.12 (5)	-0.19	9.82 ± 0.04 (5)	+0.25	10.07 ± 0.13 (6)
8= thioperamide	8.64 ± 0.06 (16)	+0.01	8.65 ± 0.13 (5)	+0.43	9.08 ± 0.13 (17)**
9= clobenpropit	10.07 ± 0.13 (7)	+0.20	10.27 ± 0.17 (5)	+0.22	10.49 ± 0.16 (10)
10= iodophenpropit	9.69 ± 0.16 (5)	-0.21	9.48 ± 0.08 (5)	+0.48	9.96 ± 0.23 (6)
11= JB96132	8.56 ± 0.14 (3)	+0.27	8.83 ± 0.12 (3)	+0.33	9.16 ± 0.12 (6)

103P SELECTIVE INHIBITORY EFFECTS OF NIFLUMIC ACID ON 5-HT-INDUCED CONTRACTION OF THE RAT ISOLATED FUNDUS

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Recent evidence has indicated that depolarization due to activation of calcium-activated chloride (Cl_{Ca}) channels, leading to calcium entry via voltage-dependent calcium channels (VDCCs), may constitute an excitatory pathway utilized by neurotransmitters to contract smooth muscle (Large & Wang, 1996). Niflumic acid (NFA), a selective blocker of Cl_{Ca} channels, may be used to assess possible involvement of these channels in the contractile responses of agents in isolated smooth muscle (Criddle *et al.*, 1996, 1997). The present study has evaluated the effects of NFA and nifedipine on the contractions induced by acetylcholine (ACh), 5-hydroxytryptamine (5-HT) and KCl in the rat stomach fundus.

Longitudinal strips of stomach fundus from male Wistar rats (250-350g) were mounted for recording of isometric tension using conventional techniques. Tissues were maintained in aerated Tyrode's solution at 37°C at pH 7.4. The effects of NFA (1-30μM) and nifedipine (1μM) on contractions to 10μM ACh, 10μM 5-HT and 60mM KCl were assessed.

The contractions induced by 60 mM KCl (2.5 ± 0.3g, n=7) were not significantly altered by NFA (1-30μM); the response was 102.2 ± 5.9% & 94.7 ± 16.3% of the control in the presence of 10 & 30μM NFA, respectively (n=7). Nifedipine (1μM) completely abolished the response to 60 mM KCl, whilst reducing contractions elicited by 10μM ACh by 32.4 ± 11.8% (n=6). ACh-induced contractions were not inhibited by NFA (3-30μM), the response being 122.6 ± 5.4% of the control value in the presence of 30 μM NFA (n=8). In contrast, NFA induced a concentration-related inhibition of the contractions to 5-HT; the responses being 63.8 ± 10.0%, 43.1 ± 9.5%, 25.3 ± 7.2% & 15.5 ± 6.0% of the control value with 1, 3, 10 & 30μM NFA, respectively (n=8). Similarly, nifedipine (1μM) reduced the 5-HT contraction to 15.2 ± 4.9% of the control response (n=6). The effects of nifedipine and NFA were not additive, such that in the combined presence of 1μM nifedipine and 30 μM NFA the contraction induced by 5-HT was not significantly different from that with nifedipine alone (10.4 ± 4.7% of the control, n=8).

Our results suggest that NFA has selective inhibitory effects on the contraction induced by 5-HT in the rat fundus. Since this contraction was inhibited similarly by NFA and nifedipine, the activation of Cl_{Ca} leading to calcium entry via VDCCs may be an excitatory pathway used by 5-HT (but not ACh) to contract this smooth muscle preparation.

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104P CHARACTERISATION OF TACHYKININ NK_3 RECEPTORS IN THE RAT ISOLATED OESPHAGUS

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Rats express the tachykinin NK_3 receptor throughout their GI tract, mainly on nerve cell bodies. However, in the oesophagus, NK_3 receptors are located on smooth muscle cells, (Mann *et al*, 1997). A preliminary study suggested that the rat oesophagus contains functional NK_2 and NK_3 receptors (Stables *et al*, 1991). We have used the selective NK_3 receptor antagonists; SB 223412 (Sarau *et al.*, 1997) and SR142,801 (Emonds-Alt *et al.*, 1995) to further characterise the response to the potent and selective NK_3 agonist, senktide, in the rat isolated oesophagus.

Oesophagi were isolated from male rats (CD 200-250g), the skeletal muscle layer removed and each tissue halved transversely. Tissues were mounted for isometric tension recording in Krebs'-Henseleit solution (37°C, aerated with 95% O_2, 5% CO_2) and allowed to reach a resting tension of 200-300mg over a one hour equilibration period. Repeat contractions to 10μM carbachol were obtained until responses were consistent. Agonist cumulative concentration-effect curves (CEC) were constructed and expressed as percentages of the carbachol maximum response. Antagonist studies required construction of a preliminary CEC to senktide in all tissues. Following washout, tissues were incubated with either vehicle (0.1% DMSO), SR142,801 (2hr) or SB223412 (1hr) and a second senktide CEC constructed. Agonist activity (pEC_{50} ± SEM) is defined as the negative log of the concentration of agonist which produces 50% of the response to 10μM carbachol. Antagonist potency (pK_B ± SEM) was calculated according to the Gaddum equation: pK_B=log(CR-1)-log[B], or the method of Schild (pA_2±SEM; slope±SEM).

All neurokinin receptor agonists studied, except substance P (SP) evoked monophasic concentration-dependent contractions of the rat isolated oesophagus. Rank order of potency was: Senktide≥NKB≥ [MePhe^7]NKB> [β-Ala^8]NKA-4-10 (BANA)>>SP= 0, (see Table 1).

Table 1: Activity of Neurokinin Receptor Agonist (n = 9 - 15)

Agonist	pEC_{50}	E_{max} (% Carbachol Max)
Senktide	8.20 ± 0.06	87.42 ± 3.38
NKB	8.11 ± 0.07	82.85 ± 1.95
[MePhe^7]NKB	8.04 ± 0.06	66.65 ± 2.96
BANA	7.68 ± 0.10	90.17 ± 5.07

SR142,801 caused a rightward displacement of senktide-induced contractions at both 1 and 10μM, giving pK_B values of 7.01 ± 0.05 (n=10) and 6.62 ± 0.14 (n=4) respectively. Similarly, SB223412 gave pK_B values of 6.94 ± 0.17 (n=3) and 6.38 ± 0.13 (n=4) at 1 and 10μM respectively. Schild analysis using antagonist concentrations of 1, 3 and 10μM, demonstrated that SR142,801 had an apparent pA_2 of 7.71 with a slope of 0.58 (95% Confidence Limits 0.30- 0.86) and SB223412 had an apparent pA_2 of 7.99 with a slope of 0.47 (95% CL 0.07 - 0.87). Both slopes were significantly different to unity (F-test).

In agreement with immunoreactivity studies by Mann *et al.*, (1997) and preliminary functional studies of Stables *et al.*, (1991), our results with agonists indicate the presence of functional NK_3 and NK_2 receptors in the rat oesophagus. The effect of SR142,801 and SB223412 further define and support the concept that senktide induced contractions are mediated by NK_3 receptors. However, as noted for other tissues from the rat with NK_3 receptors the potency of these antagonists is significantly lower than that reported at NK_3 receptors in human and guinea pig, (e.g. Sarau *et al.*, 1997). The Schild slopes of less than unity might suggest that the antagonism by SR142,801 or SB223412 was not competitive or that senktide is interacting with more than one receptor population.

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105P EFFECT OF OVARIECTOMY ON MUSCARINIC RECEPTORS IN RAT ISOLATED UTERUS *IN VITRO*

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The nature of the muscarinic receptor subtype mediating contraction of rat or guinea-pig myometrium has not been definitively identified (Boxall *et al.*, 1998; Munns & Pennefather, 1998). In the present study, we examined the pharmacological characteristics of muscarinic receptors in rat isolated uterus. Uteri from sham operated (Sh.) and ovariectomized (Ov.) rats were investigated in parallel to examine the effect of ovarian steroids (4 month post-surgery).

Radioligand binding studies were conducted using membranes prepared from Sprague-Dawley rat uteri suspended in 50 mM Tris-HCl, 1mM EDTA buffer, pH 7.4, 25°C and 1.5 nM [^{3}H]-N-methyl scopolamine ([^{3}H]NMS; specific activity 81 Ci mmol^{-1}). Nonspecific binding was defined with 1μM atropine. Saturation experiments were conducted with eight concentrations of radioligand and most competition displacement curves were generated with six concentrations of antagonist. To define M_2 and M_3 receptor proportions, 24 concentrations of tripitramine were used. The curves were analyzed by non-linear iterative curve fitting and IC_{50} converted to K_i values using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). All affinity estimates were expressed as pK_i (-logK_i).

The methodology for contractile studies was similar to that described by Boxall *et al.* (1998). Cumulative concentration-response curves to carbachol (1μM - 1mM) were established in the absence and presence of antagonists (60 min equilibration) and antagonist affinities (pK_B) were determined using one concentration of each antagonist.

Radioligand binding studies revealed that the affinity (K_D) and total number of specific binding sites (B_{max}) for [^{3}H]NMS was 0.12±0.08 nM and 44.7±10.9 fmol/mg in Sh. rat uterus (n=3) and 0.16±0.04 nM and 67.4±12.4 fmol/mg in Ov. rat uterus (n=4). These parameters were not significantly different. Competition radioligand binding studies (n=4) with the selective M_2 antagonist, tripitramine, revealed high (representing 33±8 % and 38±2 %) and low (67±8 % and 62±2 %) affinity binding sites in both Sh. and Ov. rat uterus, respectively. These proportions, likely representing M_2 and M_3 receptors respectively, were not significantly different in the two tissues.

The apparent antagonist affinity estimates (pK_i) from competition radioligand binding studies are summarized in Table 1.

Carbachol induced concentration-dependent contractions in both Sh. and Ov. uterus (pEC_{50} = 5.69±0.18, max. tension=1.29±0.10 g and pEC_{50} = 5.15±0.07, max. tension=1.22±0.07 g, respectively) with no time-dependent change in sensitivity. The effects of carbachol were surmountably antagonized by several muscarinic receptor antagonists (Table 1).

Table 1. Functional (pK_B) and apparent radioligand binding (pK_i) affinity values for muscarinic antagonists in sham operated and ovariectomized rat uterus.

Antagonist	Sham operated		Ovariectomized	
	pK_B	pK_i	pK_B	pK_i
Pirenzepine	6.48±0.25	6.39±0.06	7.21±0.09	6.41±0.09
Methoctramine	6.79±0.11	6.88±0.14	7.49±0.18	7.39±0.16
Zamifenacin	9.19±0.16	8.22±0.04 [a]	9.18±0.24	7.88±0.02
AF DX 116	6.26±0.12	5.68±0.21 [a]	6.61±0.21	5.94±0.23
Tripitramine	7.23±0.12	7.26±0.15 [b]	7.54±0.10	7.80±0.48 [b]
p-F-HHSiD	8.50±0.08	7.25±0.07 [a]	9.06±0.13	7.23±0.07
Himbacine	7.21±0.18	7.53±0.23	7.41±0.31	7.49±0.15
MTx 3	<7.00		<7.00	
PD 102807	<7.00		<7.00	

Values shown are means ± s.e.mean, n≥3. [a] Hill slopes significantly different to unity. [b] Displacement curve was resolved with two components (pK_{iH} = 9.46±0.19 and 9.12±0.16, pK_{iL} = 6.77±0.11 and 6.76±0.13 in Sh. and Ov. uterus respectively).

This study suggests that the muscarinic receptor mediating contraction in the rat uterus operationally resembles the M_3 muscarinic receptor. Radioligand binding experiments indicate the presence of M_2 receptors, in addition to M_3 receptors. These results probably explain the discrepancies between functional and binding affinities. These data support previous findings by Munns and Pennefather (1998) but the proportions of the two populations of muscarinic receptors found differed from other tissues: i. e. a predominant population of M_3 receptors. This investigation further suggests that the pharmacological profile and proportions of the two populations of muscarinic receptors are unaffected by ovariectomy.

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106P AUTORADIOGRAPHIC STUDY OF [^{3}H]MIVAZEROL BINDING SITES IN THE RAT HEART FOLLOWING CHEMICAL SYMPATHECTOMY

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Mivazerol is an α_2-adrenoceptor agonist and an antihypertensive agent (Noyer *et al.*, 1994). Mivazerol effects have been shown to be antagonised by the α_2-adrenoceptor antagonist, rauwolscine (Kharkevitch *et al.*, 1991), indicating the main effects of mivazerol are via α_2-adrenoceptors. However, we have previously shown [^{3}H]mivazerol binding to the rat heart was unaffected by rauwolscine (Parker *et al.*, 1997), indicating the presence of another binding site(s) termed the non-adrenoceptor mivazerol binding sites (NAMBS). The aim of this study was therefore to chemically lesion noradrenergic terminals in rat and investigate the effect on [^{3}H]mivazerol binding to the rat heart.

Male Wistar rats (100 g; n=4 for each treatment group) were given either (100 mgkg^{-1}) i.p. injection of saline or 6-hydroxydopamine (6-OHDA) dissolved in sterile saline containing 0.1% ascorbic acid on day 1, with a further injection of 250 mgkg^{-1} on day 2 of treatment. On day 7 sodium pentobarbitone overdose (90 mgkg^{-1}) was administered, followed by cardiac perfusion. Hearts were removed and sectioned transversely at 12 micron thickness at the level of the atria (through the atrioventricular node) and ventricles. Autoradiography was performed on all hearts using [^{3}H]mivazerol alone (3 nM), or in the presence of unlabelled mivazerol; miconazole (cytochrome P_{450} inhibitor); propranolol (β-adrenoceptor antagonist); or αβ-methylene ATP (non-selective P_{2x}, P_{2y}-purinoceptor agonist; (all 10 μM)). Sections were apposed to tritium-sensitive hyperfilm (6 weeks).

Results failed to show any significant (Student's unpaired t-test) reduction in levels of [^{3}H]mivazerol bound to the cardiac tissue following chemical lesion. This is shown in table 1, where binding was comparable in both treatment groups. Furthermore, unlabelled mivazerol was the only compound to displace [^{3}H]mivazerol at both the level of the atria and ventricles.

	Unlesioned		**Lesioned**	
	Atria	*Ventricles*	*Atria*	*Ventricles*
Total	59.8±6.1	34.8±3.1	47.2±11.7	27.3±5.0
Mivazerol	nd	nd	nd	nd
Miconazole	42.0±8.3	27.4±5.7	23.1±5.5	18.6±5.6
Propranolol	35.9±6.3	25.6±6.8	24.0±6.1	15.7±3.7
Methylene ATP	37.0±2.3	23.9±3.2	31.4±9.2	17.5±5.3

Table 1 Quantitated data of [^{3}H]mivazerol bound to cardiac tissue in rat hearts either in the presence or absence of 6-OHDA lesion (mean and s.e.mean. in fmol mg^{-1} protein; nd = not detectable).

This finding agrees with our previous studies suggesting [^{3}H]mivazerol binds to a population of sites distinct from α_2-adrenoceptors within the rat heart, which are not affected by chemical sympathectomy.

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107P DIFFERING AFFINITIES OF Rec 15/2615, Rec 15/2627, Rec 15/2856, Rec 15/3043 AND SNAP 5089 AT α_{1A}-ADRENOCEPTORS IN RAT EPIDIDYMAL VAS DEFERENS AND PORTAL VEIN

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The affinities of a range of α_1-adrenoceptor subtype selective antagonists in the epididymal vas deferens, portal vein or human prostate correlated highly with binding affinities of the same compounds at cloned α_{1a}-adrenoceptors but one antagonist, RS 17053, had a much higher affinity in the vas than in the other two tissues (Burt *et al.*, 1995, Marshall *et al.*, 1995, 1996). The present aim was to see if other antagonists could differentiate between the α_{1A}-adrenoceptors in the vas and portal vein.

The epididymal vas deferens and portal vein (male Sprague Dawley rats, 350-450g) were set up in Krebs solution at 37°C and bubbled with 95/5% O_2/CO_2 (Marshall *et al.*, 1996). For the vas the Krebs also contained cocaine (10^{-5}M) and β-oestradiol (10^{-5}M) and for the portal vein the K^+ was raised to 50mM (by reducing Na^+) to suppress spontaneous phasic activity. Antagonists were equilibrated with tissues for 30 minutes. Rec 15/2615 and Rec 15/2627 (Testa *et al.*, 1997), Rec 15/2856 (N-[3-[4-(2-methoxyphenyl)-1-piperazinyl]propyl]-3-methyl-4-oxo-2-(4-phenoxyphenyl)-4H-1-benzopyran-8-carboxamide monomethanesulphonate), Rec 15/3043 (N-[3-[4-(2,5-dimethoxyphenyl)-1-piperazinyl]propyl]-3-methyl-4-oxo-2-phenyl-4H-1-benzopyran-8-carboxamide) and SNAP 5089 were synthesised and supplied by Recordati (Milan, Italy).

Most results in the vas, except for SNAP 5089, were consistent with competitive antagonism (Table 1) but this was not so in the portal vein (Schild plot slopes between 1.60 and 2.62). The Recordati compounds and SNAP 5089 differentiated between the α_{1A}-adrenoceptors in the vas and the portal vein, being about 10- to 30-fold more potent in the former tissue. This is further evidence that the α_{1A}-adrenoceptor in the portal vein differs from that in the epididymal vas deferens.

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Table 1. Affinities of antagonists (pA_2, slope±s.e.mean or $^*pK_B$, concentration, n≥4) in epididymal vas deferens and portal vein

Antagonist	Rat vas deferens	Rat portal vein
Prazosin	9.20 (1.02±0.03)[1]	9.20 (0.84±0.16)[2]
RS 17053	9.50 (1.07±0.08)[2]	7.10^* ($1x10^{-7}$M)[2]
Rec 15/2615	8.70 (0.90±0.14)	7.39^* ($1x10^{-7}$M)
Rec 15/2627	9.10 (0.98±0.29)	7.50^* ($3x10^{-7}$M)
Rec 15/2856	8.66 (1.10±0.33)	6.74^* ($3x10^{-7}$M)
Rec 15/3043	8.63 (0.92±0.17)	7.90^* ($3x10^{-8}$M)
SNAP 5089	9.15^* ($3x10^{-9}$M)	7.36^* ($1x10^{-7}$M)

[1]Value from Burt *et al.*, 1995; [2]Value from Marshall *et al.*, 1996

108P A POINT MUTATION IN THE THIRD INTRACELLULAR LOOP OF THE HUMAN D_{2short} DOPAMINE RECEPTOR INCREASES AGONIST BINDING AFFINITIES

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G-protein coupled receptors (GPCRs) exist in an equilibrium between an inactive (R) and a partially active (R*) conformational state, the R* state being able to couple to G-proteins to form the active R*G complex. Agonists display a preferentially higher affinity for the R* and R*G states, thus stabilising the active conformation of the receptor. Mutagenesis studies have indicated that substitution of amino acids in the C-terminal portion of the third intracellular loop of many GPCRs can increase constitutive (agonist-independent) activity. These constitutively activated receptors mimic the R* form of wild-type (WT) receptors. In the present study, ligand binding experiments were performed on human D_{2short} dopamine (D_{2s}) receptors in which a threonine residue in the third intracellular loop (residue 343) was mutated to an arginine.

Chinese hamster ovary (CHO) cell lines stably expressing the WT and mutated (T343R) receptors were used to determine the binding affinities of two dopamine agonists (dopamine and 10,11-dihydroxy-N-n-propylnorapomorphine hydrochloride (NPA)) by competition assays versus [^{3}H]spiperone (0.25nM). The effect of GTP on these binding parameters was also determined. Saturation studies using [^{3}H]spiperone (0.01-2nM) were undertaken to define receptor expression levels. Membrane preparations and binding assays were performed as described previously (Gardner *et al.*, 1996).

Saturation analysis of [^{3}H]spiperone binding to WT and T343R receptors indicated a single class of high affinity binding sites with K_D values (mean ± s.e.mean) of 0.07nM (pK_D 10.2 ± 0.1) and 0.06nM (pK_D 10.2 ± 0.2) respectively. Expression levels (B_{max}) for WT and T343R receptors were 1.2 ± 0.1 pmol/mg and 1.1 ± 0.3 pmol/mg (mean ± s.e.mean; 3 observations) respectively. The K_D and B_{max} values of the two receptors were not significantly different (Student's *t* test; $p > 0.05$).

Competition experiments for dopamine and NPA in the absence of GTP were best described by a two-site model (Table 1). The affinities of dopamine and NPA for the high (K_h) and low (K_l) affinity binding sites were increased approximately 10-fold for the T343R receptor as compared with the WT receptor. When experiments were performed in the presence of GTP (100μM) however, the data were best described by a one-site model. The K_{iGTP} values of dopamine and NPA for the mutated receptors were significantly lower when compared with the WT receptor (Table 1).

Table 1. Agonist binding affinities for WT and T343R receptors

Receptor:	Agonist	pK_h (K_h, nM)	pK_l (K_l, nM)	pK_{iGTP} (K_{iGTP}, nM)
WT:	Dopamine	6.81 ± 0.05 (155)	5.05 ± 0.07 (8912)	5.25 ± 0.02 (5623)
WT:	NPA	9.72 ± 0.08 (0.19)	7.81 ± 0.06 (15)	7.58 ± 0.05 (26)
T343R:	Dopamine	7.79 ± 0.07 (16) **	6.07 ± 0.19 (851) **	5.61 ± 0.04 (2454) *
T343R:	NPA	10.73 ± 0.09 (0.019) **	9.60 ± 0.06 (0.25) **	8.71 ± 0.16 (1.9) **

*, $p < 0.05$; **, $p < 0.005$ compared with corresponding WT affinity (Student's *t* test). Data are mean ± s.e.mean of 3-6 experiments. In the absence of GTP, % of receptors in the high-affinity state was 28-47%.

Substitution of a threonine residue (T343) with an arginine in the human D_{2s} receptor resulted in an increased affinity for two dopamine agonists (dopamine and NPA) in the absence and presence of GTP. This mutated receptor may therefore have an increased tendency to adopt the R* conformation, leading to the observed increase in agonist affinity.

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109P INVESTIGATING MECHANISMS OF AGONIST ACTION AT THE HUMAN D_{2SHORT} DOPAMINE RECEPTOR

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The aim of this study was to investigate how each of the stages in the Ternary Complex Model (DeLean *et al* .,1980. Wreggett & DeLean. 1984) contributes to the efficacy of agonist action. A CHO cell line stably expressing the human D_{2short} receptor was used in competition binding studies versus [^{3}H]spiperone, in the presence and absence of GTP, to define the higher and lower binding affinities (K_h & K_{iGTP}) of an homologous series of agonists. Functional properties of agonists were investigated using agonist stimulated [^{35}S]GTPγS binding and agonist inhibition of adenylyl cyclase deriving EC_{50} values and maximal agonist effects (Gardner *et al.*, 1997).

In both ligand binding (K_{iGTP}, K_h) and functional assays (EC_{50}), the same rank order of potency was observed (5-OH DPAT>7-OH DPAT> S-DPAT>6-OH DPAT≥8-OH DPAT). When comparing ligand binding affinity to functional response, greater maximal effects and amplifications (K_{iGTP}/EC_{50}) were generally seen in the adenylyl cyclase assays. compared with the [^{35}S]GTPγS binding assays and this is consistent with response amplification along the signal transduction pathway (Table 1).

Table 1.Agonist binding and functional parameters.

	K_{iGTP}/K_h	K_{iGTP}/EC_{50} [^{35}S]GTPγS binding	K_{iGTP}/EC_{50} adenylyl cyclase	% Maximal activity ([^{35}S]GTPγS binding)± s. e. mean	% Maximal activity (adenylyl cyclase) ± s. e. mean
5-OH DPAT	100	28.9	812.8	95.1 ± 4.6	98.3 ± 11.6
6-OH DPAT	60.3	38.9	66.1	76.3 ± 6.9	106.6 ± 20.5
7-OH DPAT	72.4	29.5	562.3	84.1 ± 5.8	100.5 ± 12.4
8-OH DPAT	26.9	9.1	72.4	44.6 ± 8.5	103.8 ± 14.2
S-DPAT	57.6	21.9	1412.5	98.3 ± 5.2	96.0 ± 6.1

The Ternary Complex Model predicts that the ratio of dissociation constants for the low and high affinity sites (K_{iGTP}/K_h) provides a measure of the ability of the agonist to promote coupling of receptor to G protein (DeLean *et al.*, 1980, Wreggett & DeLean, 1984) and thus could correlate with maximal agonist effect and the degree of system amplification. A prior investigation (Payne & Strange, 1988), using a series of compounds related to dopamine. concluded that agonist binding ratios (K_{iGTP}/K_h) cannot be used to predict agonist efficacy. A trend between the ratio of K_{iGTP}/K_h and agonist efficacy was observed in the present study using the structurally more rigid dipropylated aminotetralins (Table 1). However, in agreement with the findings of Gardner *et al.* (1997) . there was no clear correlation between these two parameters.

This lack of correlation implies that events following ternary complex formation may also be involved in the modulation of agonist efficacy.

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110P ALLOSTERIC REGULATION OF THE HUMAN D_{2SHORT} DOPAMINE RECEPTOR BY 5-(N-MEMTHYL-N-ISOBUTYL) AMILORIDE

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Allosteric ligands have been described for several G-protein coupled receptor systems (reviewed in Birdsall *et al.* 1995). Their ability to inhibit or enhance the binding of orthosteric ligands and the potential for receptor subtype selectivity make allosteric agents interesting alternatives to competitive ligands for therapeutic intervention in disease states.

The interaction of the allosteric ligand 5-(N-methyl-N-isobutyl)amiloride (MIA) (Hoare and Strange 1996) with [^{3}H]spiperone and [^{3}H]raclopride at the human $D_{2(short)}$ dopamine receptor expressed in recombinant Chinese hamster ovary (CHO) cells (Gardner *et al.* 1997) was investigated in both equilibrium and kinetic binding experiments.

The dissociation of radioligand from the receptor over 20 minutes was measured in the presence of a range of concentrations of an allosteric ligand. MIA accelerated the dissociation of both radioligands in a concentration dependent manner with a pEC_{50} of 4.49 ± 0.07 (mean ± s.e.m.) (33μM) (n=4) for [^{3}H]spiperone and 4.70 ± 0.12 (20μM) (n=4) for [^{3}H]raclopride. The difference in EC_{50} of 1.6 fold was not significant ($p>0.05$. Student's t-test).

Pseudo-competition equilibrium binding experiments were performed with MIA. as described (Hoare and Strange 1996). and pooled data from four independent experiments were fit to a four-parameter logistic equation. Hill coefficients of -1.44 ± 0.19 for [^{3}H]spiperone and -1.63 ± 0.17 for [^{3}H]raclopride were both found to be significantly greater than unity ($p<0.05$. F-test).

The dissociation constants of the two radioligands were determined under conditions that minimised radioligand depletion (assay volume 1ml for [^{3}H]raclopride and 10ml for [^{3}H]spiperone) and values of 9.40 ± 0.06 (pK_d ± s.e.m., n=3. K_d=0.4nM) and 10.83 ± 0.05 (pK_d ± s.e.m., n=4. K_d=0.015nM) were found. respectively.

These radioligand K_d values were applied in the method of Cheng and Prusoff (1973) to correct IC_{50}s from competition experiments and the resultant affinities of MIA were 6.01 ± 0.02 (pKi ± s.e.m., n=3, Ki=975nM) and 5.79 ± 0.03 (pKi ± s.e.m., n=3, K_i=1620nM) from competition with [^{3}H]spiperone and [^{3}H]raclopride, respectively. The difference in affinity was approximately 2 fold and was statistically significant ($p<0.05$. Student's t-test) The affinities of three orthosteric competing ligands were also compared and no significant difference was found (Table 1).

Table 1. *Dissociation constants (nM) of ligands determined in competition experiments with [^{3}H]spiperone and [^{3}H]raclopride.*

Competing Ligand	$K_{i(raclopride)}$	$K_{i(spiperone)}$
(+)-Butaclamol	0.575	0.346
Clozapine	33.1	43.5
Haloperidol	0.55	0.606
MIA	1620	975

Mean values were calculated using the normally distributed pK_i and in all cases the standard error of the mean was less than 4% of that value, n=3.

It is concluded that the allosteric interactions of MIA with the two radioligands. [^{3}H]raclopride and [^{3}H]spiperone. at the human $D_{2(short)}$ dopamine receptor are similar as determined through both kinetic and equilibrium radioligand binding experiments.

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111P INTERACTION BETWEEN L-DOPA AND 3-O-METHYL-L-DOPA AT THE LEVEL OF THE BLOOD-BARRIER DEPENDS ON CELLULAR LOCATION OF THE SUBSTRATES

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The present study was aimed to determine the kinetics of L-3,4-dihydroxyphenylalanine (L-DOPA) uptake in an immortalised cell line of rat capillary cerebral endothelial cells (clones RBE4 and RBE 4B) (Roux et al., 1994), to define the type of interaction with 3-O-methyl-L-DOPA (3-OMDOPA), sensitivity to 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BHC), N-(methylamino)-isobutyric acid (MeAIB) and sodium. RBE 4 (passages 25-29) and RBE 4B cells (passages 2-6) were grown in Minimum Essential Medium/Ham's F10 (1:1) supplemented with 300 ng ml^{-1} neomycine, 10% fetal bovine serum, 1 ng ml^{-1} basic fibroblast growth factor, 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B, 100 μg ml^{-1} streptomycin and 25 mM HEPES. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 μg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, cells were preincubated (30 min) with Hanks' medium with added pargyline (100 μM), tolcapone (1 μM) and benserazide (50 μM). L-DOPA and 3-OMDOPA were assayed by h.p.l.c. with electrochemical detection. Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Non-linear analysis of the saturation curves for L-DOPA and 3-OMDOPA revealed in RBE 4 cells K_m values (in μM) of 72 (53, 91) and 40 (25, 57) and in RBE 4B cells K_m values (in μM) of 60 (46, 74) and 44 (13, 75), respectively. IC_{50} values for 3-OMDOPA (RBE 4, 642 [542, 759] μM; RBE 4B, 482 [475, 489] μM) obtained in the presence of a nearly saturating (250 μM) concentration of L-DOPA were greater than the corresponding K_i values (RBE 4, 143 [121, 170] μM; RBE 4B, 93 [92, 95] μM) obtained in the presence of a nearly saturating (250 μM) concentration of 3-OMDOPA; this is compatible with a competitive type of interaction between L-DOPA and 3-OMDOPA. Uptake of both L-DOPA and 3-OMDOPA in RBE 4 and RBE 4B cells was sensitive to BHC with similar IC_{50} values. MeAIB (up to 2.5 mM) was found not interfere with the uptake of both L-DOPA and 3-OMDOPA. Uptake of (250 μM) L-DOPA and 3-OMDOPA in the absence of sodium in the incubation medium was similar to that observed in the presence of increasing concentrations of sodium (20 to 140 mM). Homogenates of both cell lines were endowed with considerable COMT activity (RBE, V_{max} = 3.7±0.1 nmol mg $protein^{-1}$ h^{-1}, K_m = 4.2 [3.6, 4.7]; RBE 4B, V_{max} = 4.6±0.1 nmol mg $protein^{-1}$ h^{-1}, K_m = 3.6 [2.8, 4.5]. Incubation of RBE 4 and RBE 4B cells with L-DOPA (25 μM) in the presence of a methyl donor (S-adenosyl-L-methionine) resulted in the formation of 3-OMDOPA; this was abolished by 1 μM tolcapone. The fractional outflow of intracellular L-DOPA through the luminal and abluminal cell side was not affected by the presence of intracellular 3-OMDOPA. The fractional outflow of exogenous 3-OMDOPA applied from the luminal cell border was similar to that observed from 3-OMDOPA with origin in L-DOPA. It is concluded that RBE 4 and RBE 4B cells are endowed with the L-type amino acid transporter through which L-DOPA and 3-OMDOPA can be taken up, and 3-OMDOPA behaves as a competitive inhibitor for the uptake of L-DOPA. This, however, only occurs for luminal cell inward movement but not for abluminal cell outward movement of the substrates.

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112P REPORTER ASSAYS FOR HUMAN CANNABINOID CB1 AND CB2 RECEPTORS FOR THE IDENTIFICATION OF NOVEL AGONISTS

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The aim of this work was to establish assays that may be used in the identification of receptor selective cannabinoid compounds. Reporter genes encode the synthesis of an easily measurable protein under the control of a genetic element that responds to changes in intracellular second messengers. 96 well plate assays have been developed which utilise the secreted placental alkaline phosphatase (sPAP) gene as a reporter for agonist activation of human cannabinoid CB1 and CB2 receptors. Both receptors signal via $G_{\alpha i}$ and $G_{\alpha o}$ G proteins and cause a decrease in cAMP. The sPAP reporter gene is positioned down stream of a cassette containing 6 tandem copies of the consensus cAMP response element linked to modulate expression of the reporter protein. Hence, sPAP secretion is used as an indicator of the levels of intracellular cAMP. A panel of cannabinoid agonists and antagonists have been used to evaluate the reporter system.

A cell line stably transfected with the sPAP reporter construct was used as a host cell line for the further transfection of cDNA encoding CB1 or CB2 receptor genes. Clones stably expressing the CB receptors were selected for further characterisation. Cells were grown to 80-90% confluency and quiesced for 24 hours in serum free media harvested, washed and plated into 96 well plates preloaded with test agonist. Following a 15 minute incubation at 37°C, forskolin was added to wells and left for a further 5 hours. A 30 minute incubation with antagonist could also be included prior to addition of agonist. sPAP accumulation was measured by production of coloured product from a dephosphorylated substrate (p-nitrophenol pyrophosphate) added to the plate. Plates were read at A_{405} for 5 minutes and a Vmax for sPAP activity was derived.

CB agonists caused a concentration dependent inhibition of the forskolin response, which could be inhibited by pertussis toxin. Agonist inhibition of forskolin induced sPAP accumulation was calculated and plotted to derive pEC_{50} estimations. (Table 1).

Table 1 (n=minimum of 3)

		pEC50		
Cannabinoid	**CB1**	**sem**	**CB2**	**Sem**
Hu210	10.09	0.01	9.81	0.05
+WIN 55,212	7.55	0.09	9.90	0.36
MF Indole 9 (Gallant, 1996)	>5.0	-	9.14	0.49
Anandamide	>5.0	-	6.05	0.68
CP 55940	9.94	0.08	9.77	0.24
Methanandamide	6.63	0.33	7.04	0.04
Δ9 THC	6.98	0.12	Not	Tested
(L-768,242)	>5.0	-	7.90	0.43

Potency estimations from these sPAP assays agree with published values in other functional assays (Felder, 1995). The CB1 selective antagonist, SR141716A, was tested against Hu210 (n=2) to provide pKB values of 8.85 and >6.0 at CB1 and CB2 receptors respectively. SR144528, a selective CB2 antagonist (Rinaldi-Carmona, 1998), gave pKB values of >7.0 and 7.93 (n=2) at CB1 and CB2 receptors respectively.

These reporter assays provide a cheaper, non radioactive and expeditious alternative over traditional readouts for measurement of CB receptor activation. They may allow identification of novel cannabinoid compounds for use in further defining the role of CB receptors in disease states.

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113P [^{3}H]SUBSTANCE P BINDING IN STRIATAL SLICES: INTERACTION WITH SUBSTANCE P FRAGMENTS

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Several substance P (SP) fragments modulate dopamine release from striatal slices (Khan *et al.*, 1996), with a potency similar to SP. However, N-terminal SP fragments, such as SP(1-7), lack affinity for NK_1 receptors in membrane homogenates. To clarify this discrepancy, we studied the binding of [^{3}H]SP and SP fragments under the experimental conditions used in the functional assay. Striatal slices from male Wistar rats (250-300 g) were incubated with [^{3}H]SP at 34°C or 25°C in oxygenated Krebs-bicarbonate buffer, containing 0.05% BSA, thiorphan 10 µM, bacitracin 80 µg/ml, chymostatin 8 µg/ml, leupeptin 8 µg/ml. After 30 minutes incubation, the slices were washed by vacuum filtration with ice cold buffer containing 0.05% BSA (8 x 0.5 ml) and then homogenized in 1 ml water prior to radioactive counting. Non-specific binding was determined in the presence of 10 µM SP.

The specific binding after 10 min incubation of slices with 1 or 10 nM [^{3}H]SP at 34°C represented <5% of total binding whereas at 25°C, the specific binding increased to 30 - 40%. At 4 nM [^{3}H]SP specific binding reached a plateau after 30 min, when it represented 6 ±1.6 fmol/mg protein. Saturation data (Fig. 1) were fitted to a one site binding model using GraphPad Prism 1.03 (GraphPad Software Inc, San Diego CA) which gave estimates (± standard error) for Kd and Bmax of 3.9 ± 1.39 nM and 9.1 ± 3.5 fmol/mg protein, respectively. Increasing concentrations of SP (0.1 nM-10 µM) displaced 4 nM [^{3}H]SP with an EC_{50} of 10.7 nM. SP(1-7) or SP(5-11) (0.1 nM-10 µM) failed to displace [^{3}H]SP. The data show that it is possible to characterize binding of [^{3}H]SP in fresh brain slices under conditions used in functional assays.

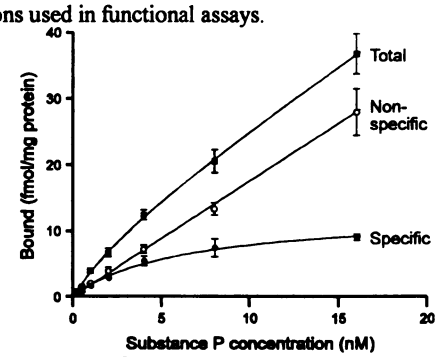

Figure 1. Binding of [^{3}H]SP (mean ± s.e.mean of 2-8 experiments in triplicate) to rat striatal slices at 25°C as a function of total concentration.

Using the displacement of heterologous binding, we showed a lack of affinity of SP fragments for the sites labelled by [^{3}H]SP. Therefore, the discrepancy between functional and binding data cannot be explained by the different types of preparations used. Alternatively, there may be interaction of SP fragments with an NK_1 receptor subpopulation different from that labelled by [^{3}H]SP. Homologous binding studies using labelled SP fragments should clarify this issue.

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114P EFFECTS OF CARIPORIDE (Hoe 642) ON INTRACELLULAR CALCIUM HOMEOSTASIS DURING ACIDOSIS IN CARDIOMYOCYTES

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Intracellular acidosis may occur in cardiac ischemia and has been discussed to be important in altering excitation-contraction coupling (Karmazyn, 1996). The aim of this study was to determine how intracellular acidosis may affect intracellular sodium and calcium handling. Cardiomyocytes were isolated from the hearts of adult male guinea pigs by standard techniques and superfused with modified Tyrode solution at room temperature, either HEPES-buffered containing 10 mM $NaHCO_3$ or HEPES-buffered without $NaHCO_3$, in order to examine a possible interaction with the sodium-bicarbonate symport. The whole cell voltage clamp technique was used utilizing 3MΩ pipettes filled with (mM): Cs-aspartate 120, CsCl 20, $MgCl_2$ 1, NaCl 5, Mg-ATP 2, HEPES 10 and either 100 µM Fura-2 or 100 µM SBFI. The pH of the pipette solution was either 7.2 (n=12) or 6.5 (n=12). Cells were kept at a holding potential of -80mV and after a pre-pulse to -40mV the membrane was continuously clamped to potentials from -30 to +80mV. Intracellular [Ca^{2+}] or [Na^+] were estimated using the Fura-2 or SBFI-technique (impermeable salt), respectively. The cardiac Na^+/H^+-exchanger was inhibited using the Na^+/H^+-exchange inhibitor cariporide (Hoe 642) (1µM) (n=12). In $NaHCO_3$ free experiments we found an increase in intracellular sodium reflected by a rise in the SBFI-ratio of 0.0459 ± 0.0147 /min upon intracellular acidification, in contrast to cells perfused at pH=7.2 (no significant increase in intracellular [Na^+]) ($p<0.05$). There was no difference in intracellular calcium handling between cells perfused with solutions of pH=7.2 or 6.5 (Fura-2-ratio: 0.8 ± 0.09 versus 0.82 ± 0.07). The L-type calcium current also remained unchanged (I_{max}: -0.83 ± 0.1 versus -0.8 ± 0.09 nA). Blockade of the Na^+/H^+-exchanger by Hoe 642 had no influence on cells perfused at pH=7.2 but inhibited the increase in intracellular [Na^+] at pH=6.5 (0.0019 ± 0.0002 /min in presence of Hoe 642 versus 0.046 ± 0.015 /min without Hoe 642) without affecting $[Ca^{2+}]_i$ or the L-type calcium current. In cells superfused with a Tyrode's solution containing $NaHCO_3$, the increase in intracellular sodium concentration was even more pronounced. Under these conditions cariporide also antagonized this increase in intracellular sodium but without reaching the control level.

We conclude that intracellular acidification causes an increase in $[Na^+]_i$ without changing intracellular [Ca^{2+}] or the L-type calcium current. This indicates that Na^+/Ca^{2+} exchange is significantly inhibited at pH=6.5. In addition, during intracellular acidosis the increase in sodium is enhanced probably by the $Na^+/HCO3^-$-symport. Cariporide antagonizes predominantly the rise in intracellular sodium during acidosis in this model.

Karmazyn M: Can J Cardiol 12: 1074-1082, 1996

115P EVIDENCE FOR CONSTITUTIVE AND INDUCIBLE CYCLOOXYGENASE EXPRESSION IN CULTURED GLIA

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Work using cultured cells has suggested that glia are likely to be a major source of prostanoids in the CNS (Murphy *et al.*, 1988). However, little is known about the precise biological responses mediated by these molecules or of the isoform of cyclooxygenase (COX) responsible for their generation. It is well established that COX can exist in one of two isoforms. COX-1 is constitutive and thought to be involved in normal physiological events whilst COX-2 is inducible and plays an important role in inflammation (Smith *et al.*, 1996). The advent of selective inhibitors for the two COX isoforms provides the opportunity to determine which of these is responsible for generating prostanoids in glia.

Glial cultures, which contained predominantly astrocytes, were prepared from newborn rat cerebral cortex (Dutton *et al.*, 1981) and maintained *in vitro* for at least 14 days. COX activity was determined by the ability of these cells to release thromboxane A_2 (TXA_2) in response to the calcium ionophore A23187. Cultures were incubated in Kreb's buffer for 30 min in the presence or absence of 3 μM A23187 and the released TXA_2 was determined as its stable breakdown product TXB_2 by radioimmunoassay with a detection limit of 0.01 ng/ml. In those experiments where COX inhibitors were used these were added 10 min prior to the addition of the ionophore. Under these conditions basal TXB_2 formation was 0.34 ± 0.05 ng/ml (mean ± s.e.m., n = 3) which increased to 2.83 ± 0.39 ng/ml (mean ± s.e.m., n = 3) in the presence of A23187. In some cases cultures were maintained in serum-free growth medium for 4 days prior to experimentation. This regime was employed because it has been shown that serum promotes the expression of COX-2 in a variety of cell types (Mitchell *et al.*, 1995). It was expected therefore that serum removal would result in a reduced level of COX-2 activity in these cells.

The inhibitors used were piroxicam and indomethacin which are COX-1 selective, nimesulide and NS398 which are selective for COX-2, and ibuprofen which does not distinguish between the two isoforms (Frölich, 1997). The effect of the inhibitors was examined in normal cells and in those which had been deprived of serum. Table 1 shows the IC_{50} values obtained for each inhibitor in the presence and absence of serum.

inhibitor	IC_{50} (μM)		ratio
	+ serum	- serum	+/-
indomethacin	0.06	0.045	1.33
piroxicam	0.1	0.035	2.86
ibuprofen	0.25	0.63	0.40
NS398	0.18	0.32	0.56
nimesulide	2.0	4.0	0.50

All of the inhibitors reduced the accumulation of TXB_2 in response to A23187, indicating the presence of both isoforms of COX in the cells. After removal of serum there was a decrease in the overall level of both basal and stimulated TXB_2 production (with serum; basal = 0.47 ± 0.03 ng/ml, A23187 = 2.07 ± 0.26 ng/ml, mean ± s.e.m., n = 9; after serum depletion; basal = 0.21 ± 0.04 ng/ml, A23187 = 0.87 ± 0.17 ng/ml, mean ± s.e.m., n = 9) and changes in the IC_{50} values for all drugs tested. The drugs known to be more selective for COX-1 became more potent whilst those which were less selective or COX-2 selective became less potent in the absence of serum. These findings suggest that these cells produce TXA_2 using a combination of the two isozymes, and that the relative contribution of COX-2 is large and is reduced upon removal of serum. This suggests that the expression of the inducible COX-2 by glial cells may be induced or up-regulated by the infiltration of serum (which could occur *in vivo* in physical CNS injury), supporting the notion that glial cells actively participate in an inflammatory response to CNS damage through prostanoid production.

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116P COMPARISON OF THE G-PROTEIN COUPLING OF RECOMBINANT α_{1B}– ADRENOCEPTORS AND ENDOGENOUS M_3-MUSCARINIC RECEPTORS CO-EXPRESSED IN THE SH-SY5Y NEUROBLASTOMA

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The M_3-muscarinic and α_{1B}-adrenoceptor are known to preferentially couple to $G_{q/11}$ to generate phosphoinositide-derived messengers including diacylglycerol and inositol (1,4,5) trisphosphate ($InsP_3$). However, there is little direct evidence on the relative efficiency of coupling of these receptors in a common cell environment. Here we have transfected cDNA for the human α_{1B}-adrenoceptor into a SH-SY5Y human neuroblastoma cell line that expresses an endogenous M_3-muscarinic receptor.

SH-SY5Y cells were transfected with the cDNA encoding the human α_{1B}-adrenoceptor. Western blot analysis, using SH-SY5Y membranes, employed specific antibodies raised against the C-terminal domain of the human α_{1B}-adrenoceptor and the third intracellular loop of the M_3-muscarinic receptor. Saturation binding assays were performed using intact cells incubated with concentrations of [^{3}H]prazosin and [^{3}H]N-methyl scopolamine ([^{3}H]NMS) at 37°C for 60 minutes. Non-specific binding was determined in the presence of phentolamine (10μM) and atropine (10μM) for [^{3}H]prazosin and [^{3}H]NMS binding respectively. Total inositol phosphates production ([^{3}H]$InsP_x$) was estimated using cells loaded (for 48hrs) with [^{3}H]-*myo*-inositol (2μCi/ml). Cells were then incubated for 15min in buffer containing lithium chloride (10mM) prior to incubation with agonists for 30min at 37°C. $InsP_3$ levels were determined using a radioreceptor assay (Challiss *et al.*, 1990) and immunoprecipitation of [^{35}S]-GTPγS bound G-proteins was performed as described previously to the Society (Akam *et al.*, 1998).

Western blot analysis identified α_{1B}-adrenoceptor and M_3-muscarinic receptor immunoreactivity in membranes of the α_{1B}-adrenoceptor-transfected SH-SY5Y cell line. Wild-type cells displayed [^{3}H]NMS binding with a Bmax and Kd of 326±42 fmols/mg protein and 241±19 nM respectively (n=4). Two clones (termed 'high' and 'low') were selected that exhibited Bmax and Kd for [^{3}H]NMS binding of 272±30.8 and 114±12 fmols/mg protein; 249±16 and 336±30 nM, for high and low respectively (n=4). [^{3}H]prazosin binding revealed different Bmax (high, 3956±301, low, 306±40 fmols/mg protein) but that the Kd values were similar (high, 185±33, low, 162±34 nM) (n=4). The EC_{50} value of noradrenaline (NA)-stimulated inositol phosphates production for the high clone was 107±4nM with a maximum response of 5.9±0.3 fold basal (n=3). The low clone failed to display increases in [^{3}H]$InsP_x$ in response to NA. Methacholine (MCh) was effective at stimulating inositol phosphates production with EC_{50} values of 9.0±1.0, 10.9±3.0, 7.8±0.5 μM and maximum responses of 17±1, 18±1, 4±1.1 fold basal for wild-type, high and low clones respectively (n=3). Peak $InsP_3$ responses (after ~10sec MCh stimulation) were of a comparable amplitude (~300 pmols/mg protein) for all clones (n=3). NA was also effective at increasing $InsP_3$ levels but only in the high clone (peak = 328±22 pmols/mg protein) (n=4). The receptor activation of G-protein was assessed by immunoprecipitation of specific G-protein α subunits labelled with [^{35}S]GTPγS. In wildtype cells and the low clones, NA was ineffective at stimulating $G_{q/11}$, however MCh produced activation with an EC_{50} of 39±4 (wildtype, n=3) and 42±10μM (low, n=3). In the high clone, NA was more potent than MCh at stimulating $G_{q/11}$ (EC_{50}=94±16 nM and 40±6μM, respectively, n=3). In high clones the maximum activation of $G_{q/11}$ by MCh and NA was 4210±518 and 6530±400 cpm, respectively, n=3.

Two clones of α_{1B}-adrenoceptor transfected SH-SY5Y cell lines have been described, termed high and low based upon their [^{3}H]prazosin binding profile. The high, but not the low, clone expresses an α_{1B}-adrenoceptor capable of mediating a stimulation of [^{3}H]$InsP_x$ production. Despite the low clones having a similar density of α_{1B}-adrenoceptors and M_3-muscarinic receptors, only stimulation of the endogenous M_3-muscarinic receptor produced a rise in [^{3}H]InsPx. The high clones express >10 fold the density of α_{1B}-adrenoceptors than M_3-muscarinic receptors but display similar maximum levels of [^{3}H]$InsP_x$ production in response to NA or MCh. The efficiency of G-protein coupling to the receptor may underlie these differences since immunoprecipitation of [^{35}S]-GTPγS-labelled $G_{\alpha q/11}$ -subunit revealed that $G_{q/11}$ activation by NA was present in the high and not the low clone. Furthermore the maximum levels of NA- and MCh-stimulated $G_{q/11}$ activation were similar in high clones despite the aforementioned differences in receptor density. Further investigation of the inefficiency of α_{1B}-adrenoceptor-$G_{q/11}$ coupling may determine if this is a feature of the expression of a recombinant receptor in a cell line or an intrinsic feature of the α_{1B}-adrenoceptor.

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117P METHACHOLINE-EVOKED Ca^{2+}-SIGNALLING IN CHO-m1 CELLS IS INHIBITED BY Li^+ IN A *MYO*-INOSITOL-REVERSIBLE MANNER

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Lithium is a widely used drug in the treatment of bipolar (manic) depression. One mechanism by which it may exert this therapeutic action is via uncompetitive inhibition of inositol monophosphatase. Thus, Li^+ can inhibit the recycling of inositol phosphates causing a depletion of cellular *myo*-inositol, limiting phosphoinositide resynthesis, and thereby leading to a reduction in the agonist-evoked $Ins(1,4,5)P_3$ signal (Jenkinson et al., 1994). Although the effects of Li^+ on agonist-stimulated changes in phosphoinositide cycle intermediates have been widely reported, its effect on subsequent Ca^{2+}-signalling events are less well documented. Here we report the effects of Li^+ on Ca^{2+}-signalling in Chinese hamster ovary cells (CHO-m1) stably expressing the human recombinant M_1-muscarinic receptor.

CHO-m1 cells grown on 22 mm glass coverslips were incubated with fura-2-AM (5 μM, 120 min) at room temperature. $[Ca^{2+}]_i$ was assessed in a small field of 5-8 cells using the Photon Technology International fluorescence system. In all experiments cells were constantly perfused (1 ml min^{-1}) with Krebs-Henseleit buffer (KHB). A stimulation protocol was adopted which involved repeated, brief (60 s) applications of methacholine (MCh) separated by wash/recovery periods of 300 s. $Ins(1,4,5)P_3$ mass was determined in parallel experiments using confluent cells grown on 8-well plates.

MCh (100 μM) stimulated a large peak Ca^{2+} response in CHO-m1 cells (approx. 20-fold-over-basal), with an EC_{50} value of 210 nM (log EC_{50} (M), -6.69±0.10). In control experiments, repeated stimulation of CHO-m1 cells with MCh every 6 min for 100 min in KHB alone resulted in highly reproducible Ca^{2+}-transients with only a small decline in the peak Ca^{2+} signal (to 88±7% of the initial peak; n=7). When similar experiments were performed in the presence of 5 mM LiCl, the Ca^{2+} response declined markedly with repeated MCh challenge (to 18±2% of the initial peak at 100 min; n=9). In contrast, the effect of Li^+ on agonist-stimulated Ca^{2+} signalling was largely reversed (initial peak reduced to 61±11%; n=3) in the presence of *myo*-inositol (10 mM). The inhibitory effect of Li^+ on agonist-stimulated Ca^{2+} signalling could be reversed following Li^+ washout, with the Ca^{2+} signal gradually returned towards initial values over a period of 60-120 min. Furthermore, recovery was facilitated by inclusion of *myo*-inositol (10 mM) in the perfusion KHB.

Using a similar experimental design, the effects of agonist ±Li^+ on $Ins(1,4,5)P_3$ levels (expressed as pmol.mg $protein^{-1}$) in CHO-m1 cells were assessed. Application of MCh (100 μM) to naive cells caused a rapid increase in $Ins(1,4,5)P_3$ (basal, 21±4; +MCh, 463±36; n=8). After sixteen 60 s exposures to MCh over a period of approx. 100 min the peak $Ins(1,4,5)P_3$ signal had decreased to 168±15 (n=8). However, in the presence of Li^+ the peak MCh-stimulated $Ins(1,4,5)P_3$ response was almost completely abrogated (basal, 52±4; MCh + Li^+, 65±11). Consistent with the $[Ca^{2+}]_i$ data, inclusion of *myo*-inositol substantially protected the $Ins(1,4,5)P_3$ response to MCh from the effect of Li^+ (130±21).

In this study we have shown that agonist-stimulated Ca^{2+} responses in CHO-m1 cells are time-dependently inhibited in the presence of Li^+, and this effect is paralleled by a diminution in the agonist-stimulated $Ins(1,4,5)P_3$ response. Additionally, the effects of Li^+ on both Ca^{2+} and $Ins(1,4,5)P_3$ responses are largely prevented by *myo*-inositol suggesting that cellular inositol depletion underlies both effects. These data also provide evidence that agonist-stimulated $Ins(1,4,5)P_3$ responses must be substantially blunted before disruption of Ca^{2+} signalling is observed.

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118P EFFECTS OF CALCIUM ANTAGONISTS ON *MYO*-INOSITOL HEXAKISPHOSPHATE-STIMULATED Ca^{2+} MOBILISATION IN RAT CEREBRAL CORTICAL SYNAPTOSOMES

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D-*myo*-inositol hexakisphosphate (IP_6) stimulates elevation of Ca^{2+} in synaptosomes derived from the cerebral cortex of rat brains (Arkle & Timony, 1994). This effect depends upon mobilisation of extrasynaptosomal Ca^{2+} but its mechanism is largely undetermined. In order to elucidate this mechanism, the effects of agents that modify Ca^{2+} mobilisation were tested on IP_6-stimulated changes in Ca^{2+} activity in synaptosomes preloaded with fura-2 and exposed to IP_6.

Synaptosomes prepared from adult male Wistar rat brain cerebral cortices (Gray & Whittaker, 1962) were loaded with fura-2 and incubated in a Krebs Henseleit buffer containing 1mM Ca^{2+} and 0.6mM Mg^{2+}. Intrasynaptosomal Ca^{2+} was determined in aliquots of synaptosomal suspensions at 37°C by fura-2 fluorescence. The fluorescent signal was calibrated using 5μM ionomycin and excess $MnCl_2$ to estimate maximal and minimal fluorescence respectively. Estimates of Ca^{2+} were corrected for extrasynaptosomal fura-2 fluorescence.

IP_6 (30 and 100μM) stimulated an increase in synaptosomal Ca^{2+} that could be inhibited by a variety of agents (table 1). The marked effect of EGTA and failure of ryanodine and the phospholipase C inhibitor, U73122, to alter the response to IP_6 indicates that the stimulated increase in Ca^{2+} is produced largely through extrasynaptosomal and not intrasynaptosomal Ca^{2+} mobilisation. A role for voltage-dependent Ca^{2+} channels (VDCCs) in this is supported by the actions of verapamil, ω-conotoxin GVIA (ωCT GVIA) and $CoCl_2$ although it is noted that these agents produced only partial inhibition of response.

Table 1. Effects of agents on stimulated increase in Ca^{2+} (nM) (mean ± s.e.m. (n)). * denotes $p<0.05$ compared to control (paired *t*-test).

treatment (preincubation time)	30μM IP_6	100μM IP_6
None	195 ± 40 (10)	1084 ± 186 (10)
5mM EGTA (30s)	21 ± 15 (10)*	143 ± 72 (4)*
30μM verapamil (5 min)	87 ± 15 (5)*	470 ± 62 (7)*
10μM $CoCl_2$ (30s)	50 ± 18 (6)*	354 ± 94 (6)*
100μM $CoCl_2$ (30s)	30 ± 16 (7)*	241 ± 123 (7)*
10μM $NiCl_2$ (30s)	113 ± 34 (5)	1164 ± 534 (5)
100μM $NiCl_2$ (30s)	107 ± 26 (5)	600 ± 120 (5)
1mM $NiCl_2$ (30s)	61 ± 29 (4)*	101 ± 49 (4)*
30μM U73122 (5 min)	117 ± 20 (6)	912 ± 180 (8)
30μM ryanodine (5 min)	104 ± 13 (7)	745 ± 102 (8)
200nM ω-conotoxin GVIA (5 min)	89 ± 17 (3)*	449 ± 62 (3)*

The inhibition of response by verapamil suggests a role of L-type VDCCs and that by low concentrations of $CoCl_2$ suggests a role of T-type VDCCs although this is not supported by the action of $NiCl_2$ which had no effect at micromolar concentrations. Similarly, the inhibition by ωCT GVIA indicates an involvement of N-type VDCCs. We conclude that IP_6 increases synaptosomal Ca^{2+} by a mechanism which is largely dependent upon extracellular Ca^{2+} mobilisation and that this is achieved through activation of a variety of different VDCCs that include types L, T and N.

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119P CHRONIC M2 STIMULATION INCREASES FORSKOLIN-INDUCED CYCLIC AMP FORMATION IN HUMAN CULTURED AIRWAY SMOOTH MUSCLE CELLS

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Previous studies have suggested M2 receptor dysfunction may play a role in airway inflammation (Fryer *et al.*, 1994). Human cultured airway smooth muscle (HASM) cells express a M2 receptor and provide a useful model system for studying regulation of M2 receptor expression and coupling. (Hall *et al.*, 1992; Widdop *et al.*, 1993). In this study we have examined the effects of chronic carbachol pretreatment upon forskolin-stimulated cyclic AMP formation in these cells.

Culture of HASM cells and determination of [^{3}H]-cyclic AMP formation in HASM cells prelabelled with [^{3}H]-adenine was performed as previously described (Widdop *et al.*, 1993). Comparisons were made using t test or Tukeys as appropriate.

Forskolin (10μM, 10min) alone induced a 11.7 ± 1.9 fold increase (cf unstimulated) in cyclic AMP formation (n=4, $p<0.05$) in HASM cells. Initial experiments confirmed that carbachol (1μM, 10min) is able to inhibit forskolin stimulated cyclic AMP formation (26 ± 14% inhibition, n=4). In contrast, 18h pretreatment with a range of concentrations of carbachol resulted in a concentration dependent augmentation of forskolin (10μM) stimulated cyclic AMP formation (EC_{50} 99.7 ± 8.7 nM n=7: maximal increase 2.4 ± 0.5 fold cf forskolin alone, $p<0.05$). However, this augmented response to forskolin could still be inhibited by acute exposure to carbachol indicating that the M2 receptor is not profoundly desensitised under these conditions.

Augmentation of forskolin (10μM) stimulated cyclic AMP formation was observed following a minimum carbachol (1μM) preincubation time of 20min, and increased with longer carbachol exposure. Augmentation of forskolin induced cyclic AMP formation by carbachol was seen at all concentrations of forskolin inducing significant cyclic AMP responses. The increase in forskolin stimulated cyclic AMP formation seen with 18h carbachol (1μM) pretreatment was completely abolished by prior incubation (30 min) with pertussis toxin (50 ng/ml, n=4, $p<0.05$), whilst prior incubation (30min) with the M2 selective antagonist methoctramine resulted in a dose dependent inhibition of the effects of 18h carbachol pretreatment upon forskolin stimulated cyclic AMP formation (Kd 7.6 ± 1.9 nM).

These results demonstrate that chronic M2 stimulation increases forskolin stimulated cyclic AMP formation in human cultured airway smooth muscle cells via a Gi dependent pathway, possibly by upregulating adenylyl cyclase activity.

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120P DIFFERENCES IN ANTAGONIST PROFILE DETERMINED BY DIRECT AND REPORTER GENE MEASUREMENT OF CYCLIC AMP AT THE HUMAN CRH_1 RECEPTOR

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The corticotropin releasing hormone type 1 receptor (CRH_1) stimulates cyclic AMP (cAMP) production. Both peptide (astressin, Perrin *et al.*, 1995) and non-peptide (CP154,526; Schultz *et al.*, 1996) antagonists for the receptor have been identified. The aim of these experiments was to compare the antagonist profiles of astressin and CP154,526 using both direct cAMP measurement and a cAMP-responsive firefly luciferase reporter gene readout (6 CRE-luc; George *et al.*, 1997).

CHO cells expressing the 6 CRE-luc reporter construct were stably transfected with the human CRH_1 receptor. Cells were grown to confluence in 96 well plates (37°C). For direct cAMP measurements, IBMX (1mM) was included in the medium (phenol red free DMEM) 40 min prior to drug exposure. Cells were then incubated with antagonist or vehicle (1% DMSO/medium) for 30 min followed by agonist (30 min). cAMP was extracted with ethanol and assayed (Biotrak cAMP assay; Amersham). In parallel, for luciferase assays, cells were plated out as before, antagonist was added for 30 min followed by agonist and incubated for 4h. Firefly luciferase production was measured (Luclite Kit; Packard). pEC_{50} values were determined by fitting data using a four-parameter logistic equation. pK_B (or pA_2^*) values were determined using a modified form of the Schild equation (Lew & Angus, 1995) where the Schild slope was constrained to 1.

Rat/human (r/h) CRH (CRH_1 agonist), calcitonin (acts at endogenous calcitonin receptors in CHO cells) and forskolin (directly activates adenylyl cyclase) stimulated concentration-dependent increases in cAMP and firefly luciferase levels (Table 1). When cAMP levels were determined directly, astressin (0.03-0.3μM) and CP154,526 (0.1-1μM) behaved as simple competitive antagonists and produced concentration-dependent rightward shifts to r/hCRH concentration-response curves (CRC) with no change in max. responses. Neither antagonist altered calcitonin or forskolin responses or basal cAMP levels. Astressin antagonised agonist responses in a similar way when responses were measured using a luciferase readout (Table 1). Using this readout, CP154,526 produced a concentration-dependent rightward shift to the r/hCRH CRC but not CRCs to forskolin or calcitonin. However, interestingly CP154,526 concentration-dependently decreased max. responses to r/hCRH and forskolin but not calcitonin. Similar results were obtained using sauvagine (CRH_1 agonist) or 4h antagonist or agonist incubation times (data not shown).

These data show that CP154,526 behaves as a competitive antagonist in the cAMP assay, but an additional probably non-receptor effect is revealed in the reporter gene assay. This is not observed with astressin.

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	r/hCRH				Calcitonin		Forskolin	
Antagonist/Readout	pEC_{50}	pK_B/pA_2^*	Schild Slope	Max.Response	pEC_{50}	Max.Response	pEC_{50}	Max. response
Astressin/cAMP	8.01±0.23	8.36±0.12	1.14±0.12	95.6±0.1	9.50±0.18	105.6±0.1	4.61±0.35	95.6 ± 0.3
Astressin/Reporter	8.53±0.26	8.32±0.12	1.13±0.08	97.9±0.2	9.39±0.18	98.7±0.1	4.84±0.15	103.6±0.3
CP154,526/cAMP	8.20±0.18	7.50±0.11	1.06±0.07	100.2±0.2	9.61±0.21	102.3±0.1	4.97±0.86	98.6±0.2
CP154,526/Reporter	8.52±0.20	7.32±0.07#	0.94±0.10	65.9±0.1*	9.26±0.28	102.4±0.2	4.57±0.09	60.3±0.2*

Table 1. Mean pEC_{50}, pK_B, Schild slope and maximum agonist response values determined at the CRH_1 receptor. Values are mean±s.e.m, n=3-6. *P<0.05 (ANOVA). % max. response is calculated using the ratio of the maximum cAMP response in the absence and presence of maximum antagonist concentration

121P NOVEL GLUTAMATE-MEDIATED INHIBITION OF FORSKOLIN-STIMULATED CYCLIC AMP ACCUMULATION IN RAT CULTURED CEREBELLAR ASTROCYTES

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Recent data suggest that glia, like their neuronal counterparts (Porter & McCarthy, 1997), express a variety of neurotransmitter receptors, including the G-protein-coupled metabotropic glutamate receptors (mGlu receptors). We report here, that L-glutamate inhibits forskolin-stimulated cyclic AMP accumulation in cultured rat cerebellar astrocytes, with a pharmacology that appears to be distinct from that exhibited by any other known mGlu receptor

Astrocytes were prepared from the cerebellum of Wistar rats (1-2 day-old, either sex) as described previously (Baba *et al.*, 1993). Astrocytic identity (> 95 % cells) was confirmed by immunoreactivity to glial fibrillary acidic protein (GFAP). Cyclic AMP accumulation was determined by assay with a bovine adrenal cortical cyclic AMP binding protein (Brown *et al.*, 1972; Toms *et al.*, 1997). Briefly, cells were preincubated with the selective cyclic AMP phosphodiesterase IV inhibitor Ro 20-1724 (100 μM, 10 min, 37°C) in buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, 1 mM $MgSO_4$, 1.3 mM $CaCl_2$, pH 7.4). Following buffer renewal, cells were incubated for 5 min with agonists and/or antagonists and the reaction started by the addition of 10 μM forskolin. After 10 min, the reaction was terminated with 5 % TCA and endogenous cyclic AMP quantified.

Forskolin (10 μM)-stimulated cyclic AMP accumulation increased linearly for 15 min (basal = 158.98 ± 3.98; forskolin = 3197.56 ± 681.36 pmol. mg protein^{-1}; n=3). Several excitatory amino acid receptor agonists were found significantly to inhibit forskolin-stimulated cyclic AMP accumulation (10 min): L-cysteine sulphinic acid (IC_{50}= 7.35 ± 2.25 μM; E_{max}= 34.09 ± 2.62 % control) > L-aspartate (IC_{50} = 8.33 ±1.50 μM; E_{max} = 45.56 ± 6.32 % control) > L-glutamate (IC_{50} = 19.05 ± 2.96 μM; E_{max} = 38.41 ± 4.17 % control) (P< 0.05, Mann-Whitney U test, n=3). However, the following selective mGlu receptor agonists were without effect: the group I mGlu receptor agonist (S)-3,5-dihydroxyphenylglycine (100 μM, n=3), the group 2 mGlu receptor agonist ((2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine) (DCG-IV; 10 μM; n=4) and the group 3 agonist L-2-amino-4-phosphonobutanoic acid (L-AP4, 1 mM, n = 4). Several mGlu receptor antagonists (1 mM; n=3) were found to be ineffective versus 30 μM glutamate: L-2-amino-3-phosphonopropionic acid (L-AP3), (S)-4-carboxyphenylglycine, (2S)-α-ethylglutamic acid, (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), (S)-α-methyl-4-carboxyphenylglycine (MCPG). Ionotropic glutamate receptor involvement was discounted, since the agonists: NMDA, kainate, quisqualate and (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ((S)-AMPA) (100 μM, n=3) all failed to influence forskolin-stimulated cyclic AMP accumulation

In order to eliminate the possibility that glutamate activates a cyclic AMP phosphodiesterase insensitive to Ro 20-1724, the broad spectrum phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was used. However, in the presence of 1 mM IBMX, inhibition of forskolin-stimulated cyclic AMP accumulation by glutamate (100 μM) was unaffected. Furthermore, the glutamate inhibition of forskolin-stimulated cyclic AMP accumulation was insensitive to overnight incubation with *Pertussis* toxin (100 ng ml^{-1}, n=3).

Therefore, we conclude that glutamate and the putative excitatory amino acid neurotransmitters, aspartate and L-cysteine sulphinic acid, potently inhibit forskolin-stimulated cyclic AMP levels in cultured cerebellar astrocytes. This inhibition does not share a pharmacology with known mGlu receptors and appears to be mediated via a pathway distinct from the classical "G_i" type inhibitory pathway.

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122P EFFECTS OF A RANGE OF β2 AGONISTS ON INTRACELLULAR CYCLIC AMP CONTANT AND CYCLIC AMP DRIVEN GENE EXPRESSION IN PRIMARY CULTURES OF HUMAN AIRWAY SMOOTH MUSCLE CELLS

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β2 adrenoceptor agonists produce airway smooth muscle relaxation predominantly through cyclic AMP-dependent mechanisms and are the mainstay bronchodilator drugs used in the treatment of asthma. However, an elevation of intracellular cyclic AMP levels can also promote increased transcription of target genes containing cyclic AMP response elements (CREs) via PKA-phosphorylated CRE binding protein (CREB). The CRE consensus sequence is an 8bp palindrome TGACGTCA.

We investigated the effects of a range of short and long acting β2 agonists on cyclic AMP levels and CRE-mediated gene expression in primary cultures of human airway smooth muscle (HASM) cells. Primary cultures of HASM cells were grown and cyclic AMP levels measured as previously described (Hall *et al.*, 1992). Ethical approval was obtained for these studies.

Table 1

β agonist	Cyclic AMP EC50 (μM) (n)	Maximal cyclic AMP response (% of maximal isoprenaline response) (n)
isoprenaline	0.077 ± 0.008 (3)	100 (3)
terbutaline	2.3 ± 0.5 (4)	37.8 ± 1.2 (4)
salbutamol	0.6 ± 0.11 (4)	19.1 ± 0.6 (4)
salmeterol	0.0012 ± 0.0002 (4)	17.5 ± 0.5 (4)

In order to determine the ability of agents to drive gene expression we transiently transfected the cells with a cyclic AMP responsive luciferase reporter construct containing multiple copies of the consensus CRE upstream of a minimal thymidine kinase promoter (p6CRE/luc).

The β2 agonists salmeterol, salbutamol and terbutaline all produced concentration related cyclic AMP responses (20 min): however, compared with isoprenaline they gave markedly smaller maximal responses (see table 1). Interestingly, all of the β2 agonists, despite producing much smaller cyclic AMP responses in these cells, produced equivalent or greater (salmeterol) maximal increases in CRE-driven luciferase expression (24h) compared to the full agonist isoprenaline (see table 2). Both salbutamol and salmeterol were more potent at increasing luciferase expression than in elevating cyclic AMP levels in these cells.

These data suggest that important quantitative differences exist in the ability of β2 agonists to increase cyclic AMP formation and elevate gene expression via a CRE-dependent mechanism.

Table 2 *p<0.05 cf isoprenaline

β agonist	p6CRE/luc apparent EC50 (μM) (n)	Maximal p6CRE/luc response (% of maximal isoprenaline response) (n)
isoprenaline	0.31 ± 0.012 (8)	100 (8)
terbutaline	0.68 ± 0.43 (3)	133.4 ± 30.5 (8)
salbutamol	0.025 ± 0.008 (4)	107.2 ± 19.6 (8)
salmeterol	0.000032 ± 0.00002 (3)	166.3 ± 19.1 (8)*

Hall *et al.* (1992). *Br. J. Pharmacol.*, 107, 422-428.

123P THE AMINO TERMINI OF RAMPs DETERMINE THE GLYCOSYLATION STATE OF CRLR AND WHETHER IT IS AN ADRENOMEDULLIN OR CGRP RECEPTOR

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Calcitonin gene related peptide (CGRP) and adrenomedullin (ADM) are related peptides with distinct pharmacologies. We have shown that a seven transmembrane receptor, the Calcitonin Receptor Like Receptor (CRLR), can function as either a CGRP receptor or an ADM receptor, depending upon the co-expression of a novel family of single, transmembrane proteins, which we have called Receptor Activity Modifying Proteins or RAMPs (McLatchie *et al.* 1998). RAMP proteins are required to transport CRLR to the plasma membrane. RAMP1 presents the receptor at the cell surface as a mature glycoprotein and a CGRP receptor whereas RAMP2 and RAMP3 transported receptors are core glycosylated and represent ADM receptors.

We have considered three explanations for RAMP activity.

1. RAMP1 might participate in the binding of CGRP and so define CRLR as a CGRP receptor.
2. RAMPs might alter the conformation of CRLR and so alter its binding characteristics.
3. The sugar residues present on the two forms of CRLR might determine the binding of CGRP and ADM.

Characterisation of the RAMP2/CRLR and RAMP3/CRLR receptors by either radioligand binding (100pM ^{125}I-rat ADM as radioligand), functional assay (cAMP measurement) or biochemical analysis (SDS-PAGE) revealed them to be indistinguishable even though RAMP2 and RAMP3 share only 30% identity. The means±s.e.m. are shown of quadruplicate determinations.

	^{125}I-rat Adrenomedullin binding. IC_{50} (nM)		
	Native	Recombinant	
PEPTIDE	NG108-15 cells	CRLR/RAMP2	CRLR/RAMP3
CGRP1	606 ± 196	546 ± 136	311 ± 105
CGRP2	68.3 ± 13.4	168.3 ± 36.9	91.7 ± 9.3
$CGRP_{8-37}$	73.4 ± 13.3	67.1 ± 7.6	56.3 ± 12.1
ADM_{1-52}	0.20 ± 0.06	0.8 ± 0.04	4.6 ± 1.05
ADM_{13-52}	12.0 ± 1.4	5.5 ± 2.4	40.7 ± 14.7
ADM_{22-52}	46.8 ± 8.4	20.5 ± 1.2	72.2 ± 10.6

CGRP2 is a more potent agonist at the ADM receptor than CGRP1 although the two peptides differ by only 3 amino acids out of 37 and have previously been regarded as functionally identical. The CGRPs were more efficacious at the ADM receptor than ADM peptides ($p<0.01$), n=3.

	Peptide ED_{50} (nM) vs intracellular cAMP			
Transfection (CRLR+)	CGRP 1	CGRP 2	ADM 1-52	ADM 13-52
RAMP1	6.6 ± 2.7	7.8 ± 3.6	53.5 ± 7.9	87.4 ± 8.8
RAMP2	194.5 ± 61.1	21.2 ± 2.3	4.7 ± 0.5	2.7 ± 0.1
RAMP3	230.7 ± 50.2	35.3 ± 13.0	4.1 ± 0.8	6.2 ± 0.6

The ED_{50} values (means± s.e.m.) for the intracellular cAMP responses to these peptides over the three experiments are given above.

Chimeric proteins were created in which the transmembrane and cytosolic portions of RAMP2 were associated with the amino terminus of RAMP1 (RAMP1/2) and visa versa (RAMP2/1).

HEK293T cells co-transfected with CRLR and RAMP2 or RAMP2/1 showed predominantly ^{125}I ADM and not ^{125}I CGRP sites. Co-transfection of CRLR with RAMP1 or RAMP1/2 produced cells that displayed specific high affinity ^{125}I CGRP sites. RAMP2/1 was as effective as RAMP2, RAMP1/2 as effective as RAMP1.

The CGRP receptors were mature glycoproteins whereas the ADM receptors were core glycosylated. Interestingly, and in conflict with our functional data from *Xenopus laevis* oocytes, the RAMP1/2 chimera in HEK293T cells also enabled CRLR to bind ^{125}I ADM. In this instance CRLR was expressed as a combination of mature and immature glycoproteins. This may relate to the fidelity with which oocytes and HEK293T cells process CRLR with the RAMP1/2 chimera.

These studies have demonstrated that the pharmacology of CRLR (either a CGRP or an ADM receptor) is defined by the amino terminal portions of RAMP1 and RAMP2 and is related to the glycosylation state of CRLR.

McLatchie L.M. *et al.* (1998) *Nature* **393**:333-339

124P THE HUMAN RECOMBINANT sst_5 RECEPTOR COUPLES TO MULTIPLE G PROTEINS IN CHO-K1 MEMBRANES: QUANTIFICATION BY [^{35}S]GTPγS BINDING AND Gα IMMUNOPRECIPITATION

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The human recombinant somatostatin sst_5 receptor expressed in CHO-K1 cells ($CHOsst_5$) mediates both inhibition and stimulation of adenylate cyclase (Williams *et al.*, 1996), activation of phosphoinositide metabolism (Wilkinson *et al.*, 1997) and increases in extracellular acidification rates (Thurlow *et al.*, 1996). These effects occur via interaction with pertussis toxin (PTx)-sensitive and –insensitive G proteins. Although somatostatin receptors are thought to predominantly interact with PTx-sensitive G proteins the present study has employed a [^{35}S]-GTPγS binding and Gα immunoprecipitation strategy (Burford *et al.*, 1998; Akam *et al.*, 1998) to analyse sst_5 receptor-G protein coupling in $CHOsst_5$ membranes.

$CHOsst_5$ membrane receptor expression (B_{max} 3.01 ± 0.3 pmol mg receptor protein^{-1}) was measured by competition of [^{125}I]-Tyr^{11}-somatostatin-14 binding with somatostatin-14 (n=3). [^{35}S]-GTPγS binding and Gα-specific immunoprecipitation assays were carried out as described previously (Burford *et al.*, 1998) with some minor modifications. Following a 2 min agonist pre-incubation period (150 µg 100 µl^{-1} membrane protein in 10 mM HEPES, 100 mM NaCl and 10 mM $MgCl_2$), membranes were exposed to 2 nM [^{35}S]-GTPγS for 2 min at 30 °C. Data are shown as mean ± s.e.m. from 4-5 separate membrane preparations performed in triplicate.

In preliminary time course experiments, basal and somatostatin-14 (1 µM)-stimulated [^{35}S]-GTPγS binding to $G_{\alpha i1-3}$ increased over the first 5 min of the reaction. Optimal agonist-induced binding to $G_{\alpha i1-3}$ was resolved after 2 min in the presence of 10 µM GDP (170.2 % over basal). For binding to $G_{\alpha 13}$, $G_{\alpha S}$, and $G_{\alpha q/11}$ GDP was omitted. Somatostatin-14 induced concentration-dependent increases in binding to $G_{\alpha i1-3}$ (see Table 1). Significantly, somatostatin-14 also increased binding to $G_{\alpha 13}$ with a similar pEC_{50} value to that for $G_{\alpha i1-3}$ (Table 1.)

Table 1 Specific G protein activation in $CHOsst_5$ membranes by somatostatin-14. Data are mean ± s.e.m. of 3-5 experiments performed in triplicate.

	Basal d.p.m	**pEC_{50}**	**maximum (as % above basal)**
$G_{\alpha i1-3}$	1610 ± 167	6.76 ± 0.15	212 ± 9
$G_{\alpha 13}$	1087 ± 221	6.63 ± 0.23	221 ± 28
$G_{\alpha s}$	975 ± 261	6.29 ± 0.36	141 ± 2
$G_{\alpha q/11}$	1289 ± 308	7.23 ± 0.36	81 ± 18

Consistent with the PTx-insensitive stimulatory effects of somatostatin-14 on adenylate cyclase activity in $CHOsst_5$ cells at high agonist concentrations (Williams *et al.*, 1996), somatostatin-14 also activated $G_{\alpha S}$ (Table 1). The maximum stimulation of binding to $G_{\alpha q/11}$ was least marked of all the Gα antibodies tested, a finding consistent with the largely PTx-sensitive nature of somatostatin-14-induced phosphoinositide hydrolysis in these cells (Wilkinson *et al.*, 1997). Western analysis confirmed the presence of $G_{\alpha i1-3}$, $G_{\alpha 13}$, $G_{\alpha S}$ and $G_{\alpha q/11}$ in $CHOsst_5$ membranes using the same Gα-specific antibodies used for immunoprecipitation.

In conclusion, the present study indicates that [^{35}S]-GTPγS binding and Gα-specific immunoprecipitation can be used to measure receptor-G protein coupling in $CHOsst_5$ cells. These data also demonstrate for the first time that the human recombinant sst_5 somatostatin receptor can couple to $G_{\alpha 13}$ on agonist activation.

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125P INTERNALISATION CHARACTERISTICS OF THE HUMAN RECOMBINANT SOMATOSTATIN 4 (hsst$_4$) RECEPTOR EXPRESSED IN CHO-K1 CELLS

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Opposing conclusions regarding the internalisation of somatostatin (SRIF) hsst$_4$ receptor have been described (Hukovic et al.,1996; Roth et al.,1997). We have now studied the internalisation of the human epitope (HA)-tagged sst$_4$ receptor expressed in CHO-K1 cells and the endocytosis and recycling of the radioligand, [^{125}I]-[Tyr^{11}]-SRIF. Membranes prepared (Smalley et al.,1998) from CHO-K1 cells expressing either epitope tagged or untagged hsst$_4$ receptors showed similar ligand binding characteristics (Table 1).

Internalisation of [^{125}I]-[Tyr^{11}]-SRIF was measured as described by [Koenig et al.,1997]. Approximately 10^5 cells seeded into 24-well plates overnight were incubated with 0.1nM [^{125}I]-[Tyr^{11}]-SRIF, for increasing periods of time at 37°C before being washed for 20 minutes with DMEM/MES, pH5.0, 15°C, to remove surface bound radioligand. Cells were then solubilised with 0.1% v/v Triton-X100 and subsequently counted for radioactivity. All values are mean ± se mean from at least 3 experiments performed in duplicate. In CHOsst$_4$ membranes, >90% of [^{125}I]-[Tyr^{11}]-SRIF dissociated at 15°C, pH5.0 in the presence of Na^+ (140mM) and GTP (100μM), thus validating the acid-wash procedure.

Internalisation (molecules/cell) of [^{125}I]-[Tyr^{11}]-SRIF was time dependent; reaching a steady state at 60 min (14131±1253) and was inhibited at 4°C (2271±757) and by hypertonic (0.5M) sucrose (1741±68). Internalisation of [^{125}I]-[Tyr^{11}]-SRIF was also inhibited by increasing concentrations of SRIF, L-362855 and NNC-296100 with pIC_{50} values which were approximately ten fold lower than equivalent values for inhibition [^{125}I]-[Tyr^{11}]-SRIF binding to membrane homogenates (Table 1).

After acid-washing, some plates were heated back up to 37°C in fresh DMEM containing excess cold-SRIF (1μM) and the media collected for counting. After 45 min, only 6.8±0.6% of internalised radioactivity remained in the cell. The $t_{1/2}$. for recycling was3.9±0.7min.

Table 1: Potency (pIC_{50}) of SRIF ligands for inhibition of (a) internalisation of [^{125}I]-[Tyr^{11}]-SRIF in epitope tagged CHO sst$_4$ cells, (b) specific [^{125}I]-[Tyr^{11}]-SRIF binding in epitope tagged CHOsst$_4$ membranes and (c) specific [^{125}I]-[Tyr^{11}]-SRIF binding in CHO sst$_4$ membranes. Values are mean ± se mean from at least 3 experiments in duplicate.

Ligand*	pIC_{50} (a)	pIC_{50} (b)	pIC_{50} (c)
SRIF-14	7.74 ± 0.03	8.83 ± 0.04	8.82± 0.02
L-362855	6.27 ± 0.11	7.30 ± 0.04	7.40 ± 0.06
NNC-296100	6.50 ± 0.10	7.88 ± 0.08	8.00 ± 0.03

* See Smalley et al., (1998) for structure

Confocal microscopic analysis of permeabilised, fluorescein labelled CHOsst$_4$-tagged receptors revealed little if any internal immunostaining after treatment with SRIF, L-362855 or NNC-296100 (1μM, 60 mins). In parallel studies in epitope tagged CHOsst$_2$ cells, immunostained vesicle-like structures were clearly visible following SRIF treatment.

Our results demonstrate that in CHO–K1 cells expressing hsst$_4$ receptors, [^{125}I]-[Tyr^{11}]-SRIF is internalised via a clathrin mediated process with subsequent rapid recycling to the extracellular medium. In view of this rapid recycling, it is unlikely that receptor internalisation could be measured by changes in cell surface receptor expression (see Roth et al.,1997) or as we have demonstrated, by confocal microscopy.

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126P EXPRESSION AND PHARMACOLOGICAL PROPERTIES OF THE MOUSE β_{3A} AND β_{3B}-ADRENOCEPTOR IN CHO-K1 CELLS

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The gene encoding the β_3-adrenoceptor (AR) differs from the β_1- and β_2-AR genes in that it contains three exons and two introns (Van Spronsen *et al.*, 1993). Using reverse transcription/polymerase chain reaction (RT/PCR) with intron-spanning primers, we have recently identified a β_3-AR splice variant (β_{3B}-AR) in which the receptor coding region is altered. Relative levels of this mRNA are highest in mouse hypothalamus and cortex, and lower in ileum smooth muscle and brown adipose tissue (BAT). The mRNA is produced by alternate splicing at a novel acceptor site 100 bp upstream from the previously characterised start of exon 2 (Summers *et al*, 1998). The β_{3B}-AR encoded by the alternately spliced mRNA has a C-terminus that has 17 amino acids (SSLLREPRHLYTCLGYP) following the sequence encoded by the first exon compared to 13 (RFDGYEGARPFPT) in the known receptor (β_{3A}-AR).

The entire coding regions of mouse β_{3A}-AR and β_{3B}-AR cDNAs were subcloned into the mammalian expression vector pcDNA 3.1+ (Invitrogen). PCR fragments were amplified and plasmid DNA was transferred into CHO-K1 cells by electroporation, and stable transformants selected using G418 (neomycin). Clonal cell populations were analysed for expression of β_3-AR mRNA, and 2 lines (CHO 23A-6 and CHO 9A-7) with equivalent levels of mRNA and binding were chosen for functional studies. Responses of cells expressing β_{3A}-(CHO 23A-6) or β_{3B}-AR(CHO 9A-7) were examined in the cytosensor microphysiometer®. CHO cells were plated at 10^6 cells/capsule, superfused with modified RPMI 1640 and the acidification rate determined at 2 min intervals. Cumulative concentration-response curves were constructed to the β_3-AR-selective agonists CL 316243, BRL 37344, CGP 12177 and the β-AR agonist isoprenaline. Curves were fitted and pEC_{50} values calculated using Graphpad Prism (table 1).

	pEC_{50}	
	β_{3A}-AR	β_{3B}-AR
CL 316243	8.85 ± 0.08 (8) ***	8.27 ± 0.10 (8) ***
BRL 37344	8.67 ± 0.04 (7)	8.74 ± 0.06 (6)
CGP 12177	8.59 ± 0.03 (3) **	8.88 ± 0.04 (4) **
Isoprenaline	7.71 ± 0.06 (4)	7.71 ± 0.07 (4)

Values are means ± s.e.mean; **P<0.01 and ***P<0.005 by Student's unpaired t-test.

CL 316243 was more potent at β_{3A}-AR whereas CGP 12177 was more potent at β_{3B}-AR. There was no significant difference between pEC_{50} values for BRL 37344 or isoprenaline in cells expressing either receptor. In conclusion, although the β_{3A}- and β_{3B}-adrenoceptors differ only at the C-terminus there is evidence that the receptors can be discriminated by CL 316243 and CGP 12177.

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127P MOLECULAR CLONING AND CHARACTERISATION OF SPLICE VARIANTS OF THE MOUSE $P2X_4$ RECEPTOR

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The $P2X_4$ receptor has a widespread distribution in both rat and the human (Buell *et al.*, 1996; Garcia-Guzman *et al.*, 1997). The rat $P2X_4$ receptor was initially characterised as being antagonist insensitive since the P2 antagonists suramin and pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) were weak or inactive. However, these compounds were more potent antagonists of the human $P2X_4$ receptor (Garcia-Guzman *et al.*, 1997). In this study we show that the full length mouse orthologue of the $P2X_4$ receptor displays pharmacological differences from both rat and human $P2X_4$ receptors.

Using an RT-PCR approach and primers based upon the rat $P2X_4$ DNA sequences encoding N- and C- terminals of the protein we isolated full length cDNAs from mouse NTW8 microglial cell line (Chessell *et al.*, 1997). One clone encoded the full length mouse orthologue of the $P2X_4$ receptor. The predicted protein of 388 amino acids displayed 86 and 95 % identity to the human and rat $P2X_4$ receptors, respectively. In addition to this protein ($P2X_{4(a)}$), three other alternatively spliced variants were identified in which 27 amino acids ($p2x_{4(b)}$) from the extracellular loop, or 22 amino acids ($p2x_{4(c)}$) from the TM2 domain, or both the 27 and the 22 amino acid segments ($p2x_{4(d)}$) were spliced out.

Stable expression of each of four mouse $P2X_4$ receptors was established in HEK293 cells and whole cell patch clamp studies were performed as described previously (Chessell et al., 1997). Briefly, inward currents were recorded in cells patch clamped at –90mV and studies were performed at 22°C. Agonists were applied for 2sec at 5 min intervals by concentration-clamp. Antagonists were superfused for 5mins prior to recording agonist responses.

All results are the mean±s.e.mean of at least 4 experiments.

All four mouse $P2X_4$ splice variants were efficiently translated into proteins and glycosylated *in vitro*. HEK293 cells transfected with the $P2X_{4(a)}$ receptor responded to ATP (pEC_{50}=5.87±0.05; maximum current = 2692±385pA; n=12) whereas, in preliminary studies, HEK293 cells transfected with the $p2x_{4(b)}$, $p2x_{4(c)}$ or $p2x_{4(d)}$ splice variants did not respond. The agonist potencies at the mouse $P2X_{4(a)}$ receptor were similar to those reported at human and rat $P2X_4$ receptors. Thus, pEC_{50} values for 2-meS-ATP and ATPγS were 5.72±0.04 and 4.26±0.14, respectively.

A lysine residue present at position 78 of the human $P2X_4$ receptor, which is replaced by glutamine in the rat receptor, has been proposed to confer sensitivity to suramin (Garcia-Guzman *et al.*, 1997). The mouse $P2X_{4(a)}$ receptor, like the rat $P2X_4$ receptor contains glutamine at position 78 and was only weakly blocked by suramin (pIC_{50}=3.23±0.16 vs 10μM ATP). In contrast to the rat receptor, however, the mouse $P2X_{4a}$ receptor was blocked by PPADS (pIC_{50}=4.72±0.02 vs 10μM ATP) and also cibacron blue (pIC_{50}=4.06±0.09 vs 10μM ATP).

These results further highlight the species differences in $P2X_4$ receptors. The finding that the mouse and rat receptors differ from human receptors in their suramin sensitivity while the mouse and rat receptor differ in their sensitivity to PPADS may prove useful in identifying the molecular determinants of antagonist binding. The significance of the multiple splice variants of the mouse $P2X_4$ receptor remains to be determined, although our preliminary studies suggest the $p2x_{4(b)}$, $p2x_{4(c)}$ and $p2x_{4(d)}$ receptors are not functional.

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128P QUANTIFICATION OF THE mRNA LEVELS OF SOME POTASSIUM CHANNELS IN HUMAN UTERUS AND OTHER HUMAN TISSUES

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Published data suggest that several potassium channels may be required for normal myometrial function (Cheuk et al, 1993; Ludmir & Erulkar 1993) leading to the question are their number regulated at the level of mRNA during pregnancy? Consequently, the initial aim of this study was to develop a method for measuring mRNA levels of the α-subunits of the large conductance calcium activated potassium channel (BK_{Ca}), intermediate conductance calcium activated potassium channel (IK_{Ca}) and inwardly rectifying ATP-dependent K channel (Kir 6.2) in human uterus.

To establish the system, the mRNA levels of BK_{Ca}, IK_{Ca} and Kir 6.2 channels were examined in commercially supplied total RNA (Ambion & Clontech) obtained from human uterus, lung, heart, placenta, breast and foetal liver. A two step semi-quantitative RT-PCR procedure was used (Brady & Iscove 1993; Toellner et al., 1996). In the first step, all polyA mRNAs were converted to cDNA using $Not1_{40}$ and amplified to give *polyA cDNA* representing all genes expressed in the samples. In the second step, the levels of cDNA for BK_{Ca}, IK_{Ca}, Kir 6.2 channels and a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAP) were examined by adding *poly A cDNA* to PCR reactions containing oligonucleotide sequences specific for BK_{Ca}, IK_{Ca}, Kir 6.2 channels and GAP.

Initial results showed that BK_{Ca} channels were highly expressed in human uterus compared to other tissues (Table 1). IK_{Ca} and Kir 6.2 channels were also detected in human uterus but with a lower abundance compared to BK_{Ca} channels. However, these potassium channels were differentially expressed in the other human tissues.

Abundance of individual K channel cDNA/10^6 molecules total *polyA cDNA*			
Human tissue	BK_{Ca}	IK_{Ca}	Kir 6.2
Uterus	10-100	1-10 ≅ 3	~1.0
Lung	<0.01	1-10	~1.0
Heart	<0.01	0.01-0.1	~5.0
Placenta	<0.01	~100	<0.1
Breast	<0.01	10-100 ≅ 80	~7.0
Foetal liver	<0.01	10-100 ≅ 20	~0.1

Table 1: The approximate expression levels of mRNAs for BK_{Ca}, IK_{Ca} and Kir 6.2 channels in human tissues measured by densitometry.

The results indicate that each K channel shows a distinct tissue-specific pattern of expression. BK_{Ca}, IK_{Ca} and Kir 6.2 are expressed in human uterus which supports the idea that they play an important role in its function. The differential expression of mRNA for the three potassium channels between human uterus and other tissues might indicate that their roles vary between tissues.

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129P MAO_A INHIBITION IS NOT RESPONSIBLE FOR THE IMPROVED GLUCOSE TOLERANCE FOLLOWING THE IMIDAZOLINE S 22068

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There is evidence to suggest that imidazoline I_2 binding sites represent regions on the enzyme monoamine oxidase (MAO); they have similar molecular weight and amino acid sequences and expression of MAO in yeast is accompanied by expression of I_2 binding sites (Tesson *et al*, 1995). Furthermore several I_2 ligands including 2-BFI, inhibit MAO, (Ozaita *et al*, 1997). We have previously reported that the imidazoline compound S-22068 (1,4-di-isopropyl-2-(4,5-dihydro-1-H imidazol-2-yl) piperazine) improves glucose tolerance at 69 μmol/kg but without stimulating insulin secretion or producing hypoglycaemia in CBA mice (Shih *et al.*, 1997); in contrast, the putative I_3 imidazoline S-21663 (PMS 812) improves glucose tolerance and stimulates insulin output in streptozotocin-diabetic rats (Wang *et al*, 1996). In order to see if the effects of S 22068 on glucose homeostasis involve the inhibition of MAO_A, we have investigated its effect on MAO_A activity in mouse brain and liver tissue.

Adult male CBA mice (30-34 g) were fasted overnight. Brain and liver were rapidly removed and homogenised in 50 mM KH_2PO_4 buffer at 4°C, pH 7.2. A mitochondria-enriched preparation was obtained by differential centrifugation. MAO_A activity was determined using a modification of the method of Otsuka (1964). Briefly, 100-150 μg mitochondrial membranes were incubated with [^{14}C]-5-HT (500 μM brain, 250 μM liver) at 37°C for 45 or 60 minutes (brain and liver respectively) in triplicate; the reaction was terminated using ice cold 2 M citric acid. The reaction product, 5-HIAA, was extracted into 0.5 ml toluene/ethyl acetate (1:1 v/v) containing 0.6% (w/v) 2,5-diphenyloxazole.

Kinetic parameters were derived from plots of 1/v vs 1/[S], and inhibtion values (IC_{50}, 95% confidence intervals) from non-linear regression of competition assays (9 points in triplicate), using Graphpad Prism.

MAO_A in mouse brain and liver was found to have a K_m of 475 μM and 261 μM, and a V_{max} of 2.3 and 0.567 nmol min^{-1} mg^{-1} respectively (n=3). MAO_A was almost completely blocked by clorgyline, a selective MAO_A inhibitor; activity was reduced by 97% in brain and 96% in liver with IC_{50} values of 58.7 (31.3 - 110.3) nM and 130 (56.6 - 299) nM respectively. 2-BFI reduced MAO_A activity by 94% in brain and 96% in liver, with IC_{50} values of 49.1 (34.7 - 69.3) μM and 24.5 (14.1 - 40.0) μM. Idazoxan reduced MAO_A activity by 90% in brain and 89% in liver, with IC_{50} values of 486 (397 - 595) μM and 129 (89 - 186) μM respectively. S 22068 had little effect on MAO_A activity in brain or liver; at 2.2 mM S 22068 reduced activity by 14% in brain and 17% in liver ($p<0.05$, n=3), but produced no inhibition at 0.22 mM (n=3).

We conclude that S-22068 has no effect on MAO_A activity at concentrations at which it would improve glucose tolerance. This suggests that the mechanism by which S 22068 effects glucose homeostasis in mice does not involve MAO_A.

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130P PROTEASE-ACTIVATED RECEPTOR (PAR-2) MEDIATED MITOGENIC RESPONSE IN HUMAN LUNG FIBROBLASTS

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A family of the novel protease-activated G protein-coupled receptors (PAR) is emerging PAR1,PAR2,PAR3 *(Molino, Raghunath,1998)* and the recently identified PAR4 *(Xu, Andresen,1998)*. PAR1 and PAR3 are proteolytically cleaved by thrombin,PAR2 by trypsin and tryptase and PAR4 by either thrombin or trypsin. Activation of PAR1 with the peptide SFLLR has been shown to stimulate mitogenesis in hamster CCL-39 fibroblasts *(Morley et al,1996)*. Rodent PAR2 activating peptide (SLIGRL) mediates a mitogenic response in BaF3 cells expressing human PAR2; human PAR2 activating peptide (SLIGKV) mediates cell proliferation in cultured human aortic smooth muscle cells *(Bono,1997)*. Mast cell tryptase activation of PAR2 receptor may contribute to fibroblast mitogenesis and fibrosis in human lung. In this study proliferation of a primary, human lung fibroblast cell line (AP11/3) was measured in growth-arrested conditions to investigate the presence of a functional PAR2 receptor.

^{3}H-Thymidine incorporation was measured in cells growth arrested for 48 hours, followed by 24 hours in presence of ligand then 1 hour with 1μCi ^{3}H-thymidine per well. ^{3}H was measured in a NaOH fraction of the TCA treated cell. Stimulation of cells with purified human tryptase increased ^{3}H-thymidine incorporation (Table 1). The human peptide PAR2 SLIGKV increased ^{3}H-thymidine incorporation and was maximal at 500uM but the response was less than that of thrombin (1mU).The rodent PAR2 peptide, SLIGRL had no effect on ^{3}H-thymidine incorporation at 200uM. RT PCR indicated the presence of PAR2 mRNA in AP11/3 cells. The modest mitogenic effect of 500uM SLIGKV compared to tryptase and the lack of ^{3}H-thymidine incorporation with SLIGRL indicates that PAR2 is not abundantly expressed on the AP11/3. Evidence of the PAR2 receptor in fibroblasts from other species and other tissues is conflicting. In this study the PAR2 mRNA was measurable and tryptase induced a mitogenic response. The lack of activity with the peptides indicates that the mitogenic response may not be via PAR2 but by an other tryptase activated receptor. Further investigation is required to determine the type and function of PARs expressed on the AP11/3.

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TABLE 1. [3H] thymidine incorporation in AP11/3 cells (cpms/well)

Tryptase		SLIGKV (human)		SLIGRL (rodent)	
control	9737±2074	control	693±109	control	604±116
1mU	41376±3043*	0.001mM	560±66	0.002mM	529±39
5mU	48020±3673*	0.002mM	610±56	0.01mM	612±137
10mU	48462±3672*	0.01mM	1640±63*	0.02mM	754±99
20mU	51845±7798*	0.02mM	2431±168*	0.1mM	911±56
50mU	57800±2716*#	0.1mM	2826±664*	0.2mM	833±268
		0.5mM	3152±290*#		
thrombin 1mU	36016±3293*	thrombin 1mU	15022±2023*	thrombin 1mU	45564±5888*

* P < 0.05 compared to control # P < 0.05 compared to thrombin n =3

131P ISOLATION OF A PARTIAL cDNA CLONE OF THE PORCINE 5-HT_{3A} RECEPTOR SUBUNIT

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To date, a cDNA encoding a 5-HT_3 receptor subunit (5-HT_{3A}) has been isolated from mouse, rat, human and guinea pig , and exists as two splice variants (5-$HT_{3A(a)}$ and 5-$HT_{3A(b)}$) encoded by a single gene in all species, although the longer splice variant of the 5-HT_3 receptor has not been detected in the human (Maricq *et al.*, 1991, Hope *et al.*, 1993, Belelli *et al.*, 1995, Lankiewicz *et al.*, 1998). Here we report the cloning of a partial cDNA coding for a species orthologue of the 5-HT_{3A} receptor from pig nodose ganglion. The availability of the pig 5-HT_{3A} subunit will be invaluable to the heterologous expression of a non-5-HT_{3A} protein subunit which we have previously purified from pig cerebral cortex (Fletcher & Barnes 1997). The isolation of the pig 5-HT_{3A} receptor subunit may also help to explain the inter-species variation in the pharmacology of the 5-HT_3 receptor (Fletcher & Barnes 1996).

Total RNA was isolated from pig nodose ganglion tissue (RNA isolation kit, Stratagene) and reverse transcriptase (RT)-PCR performed using oligonucleotide primers (P1 ATCCTCGAGGTGGATGAGAAGAACCAA/GGT and P2 TTCATCGATGGCTGCAGTGGTTA/G/C/TCCCAT), designed to hybridise to previously identified 5-HT_{3A} sequences and which have already been used successfully in the cloning of a guinea pig 5-HT_{3A} subunit (Lankiewicz *et al.*, 1998). Oligo (dT) primed cDNA and 100 ng of primers P1 and P2 were incubated in buffer (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01 % Tween-20; Bioline) containing 2 mM $MgCl_2$, 0.2 mM of dNTPs (NBL) and 5U of *Taq* DNA polymerase (Stratagene). 30 cycles (95 °C, 1 min; 60 °C, 1 min; 72 °C, 4 min) were performed. RT-PCR resulted in the amplification of a 968 bp cDNA product, which is the size expected by analogy to other 5-HT_{3A} cDNAs. The cDNA was cloned into the vector pCR Script 2.1 (InVitrogen) and sequenced on both strands.

The partial cDNA coding for the porcine 5-HT_{3A} subunit shows between 81% and 87% nucleotide sequence identity with other species orthologues and codes for a 322 amino acid polypeptide, showing 84%, 84%, 90% and 87% sequence identity with the mouse, rat, human and guinea pig 5-HT_{3A} subunits respectively. Furthermore, this partial 5-HT_{3A} cDNA encodes the shorter splice variant in which 6 amino acids are absent in the large cytoplasmic loop. The extracellular amino terminal domain contains a characteristic cys-cys loop and three putative N-glycosylation sites (i.e. N-X-S/T) at positions N86, N152 and N168. When compared to the other species orthologues, the channel forming M2 domain associated with the pig 5-HT_{3A} subunit is completely conserved. Furthermore, a consensus sequence for phosphorylation by casein kinase (i.e. S-X-E), located between transmembrane domains 1 and 2 is found in all cloned 5-HT_{3A} subunits. An additional casein kinase II site (i.e. S/T-X-X-D/E) located in the large cytoplasmic domain of human and guinea pig 5-HT_{3A} subunits is also found in the pig 5-HT_{3A} subunit at position T357.

In conclusion, we have identified a further species orthologue of the 5-HT_{3A} subunit.

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132P CHARACTERISTICS OF L-DOPA AND L-5-HTP TRANSPORT IN RBE4 CELLS, AN IMMORTALISED CELL LINE OF RAT CAPILLARY CEREBRAL ENDOTHELIAL CELLS

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The present study aimed to determine the kinetics of L-3,4-dihydroxyphenylalanine (L-DOPA) uptake in an immortalised cell line of rat capillary cerebral endothelial cells (clones RBE 4 and RBE 4B) (Roux et al., 1994), to define the type of inhibition produced by L-5-hydroxytryptophan (L-5-HTP), 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BHC) and N-(methylamino)-isobutyric acid (MeAIB) and its sodium dependence. RBE 4 (passages 25-29) and RBE 4B cells (passages 2-6) were grown in Minimum Essential Medium/Ham's F10 (1:1) supplemented with 300 ng ml^{-1} neomycine, 10% fetal bovine serum, 1 ng ml^{-1} basic fibroblast growth factor, 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B, 100 µg ml^{-1} streptomycin and 25 mM HEPES. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 µg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, cells were preincubated (30 min) with Hanks' medium with added pargyline (100 µM), tolcapone (1 µM) and benserazide (50 µM). L-DOPA and L-5-HTP were assayed by h.p.l.c. with electrochemical detection. Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Non-linear analysis of the saturation curves for L-DOPA and L-5-HTP revealed in RBE 4 cells K_m values (in µM) of 72 (53, 91) and 102 (75, 129) and in RBE 4B cells K_m values (in µM) of 60 (46, 74) and 118 (84, 152), respectively. IC_{50} and corresponding K_i values (shown in table 1) for L-5-HTP and BHC were obtained in the presence of a non-saturating (25 µM) and a nearly saturating (250 µM) concentration of L-DOPA .

Inhibitor	Substrate	IC_{50} (µM)	K_i (µM)
	[L-DOPA]	RBE 4 cells	
L-5-HTP	25 µM	207 (166, 258)	153 (123, 191)
	250 µM	1026 (644, 1636)	229 (144, 366)
BHC	25 µM	157 (146, 169)	117 (108, 126)
	250 µM	1177 (944, 1469)	263 (211, 329)
		RBE 4B cells	
L-5-HTP	25 µM	208 (134, 324)	155 (99, 241)
	250 µM	831 (787, 878)	186 (176, 196)
BHC	25 µM	125 (101, 155)	88 (71, 110)
	250 µM	799 (678, 942)	155 (131, 182)

MeAIB (up to 2.5 mM) was found not to interfere with the uptake of L-DOPA. In RBE 4 cells V_{max} values (in nmol mg $protein^{-1}$ 6 min^{-1}) for L-DOPA uptake were identical in the absence (21.4±0.56) and the presence of 150 µM L-5-HTP (25.8 ± 0.35) or 150 µM BHC (19.3 ± 0.10), but K_m values (µM) were significantly greater ($P<0.05$) when L-DOPA uptake was studied in the presence of L-5-HTP (91 [68, 115] vs 272 [248, 296]) or BHC 208 [200, 216]). Similar findings were observed when RBE 4B cells were used. Uptake of (250 µM) L-DOPA in the absence of sodium in the incubation medium was similar to that observed in the presence of increasing concentrations of sodium (20 to 140 mM). It is concluded that RBE 4 and RBE 4B cells are endowed with the L-type amino acid transporter through which L-DOPA and L-5-HTP can be taken up; both L-5-HTP and BHC act as competitive inhibitors for L-DOPA uptake.

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133P BTS 67 582 MAY MEDIATE INSULIN SECRETION VIA THE PUTATIVE PANCREATIC ISLET IMIDAZOLINE RECEPTOR

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BTS 67 582, 1,1-dimethyl-2-[2-(4-morpholinophenyl)] guanidine monofumarate has been shown to reduce blood glucose in both normal and streptozotocin-induced diabetic rats but does not interact at the sulphonylurea binding site (Jones *et al.*, 1997). BTS 67 582 promotes insulin secretion in perifused pancreatic islets, reverses the effects of diazoxide and can directly modulate activity at the ATP-dependent K^+ channel (Dickinson *et al.*, 1997; Jones, *et al.*, 1996). There have been several studies that indicate there is a novel imidazoline receptor that mediates insulin release in pancreatic islets (Chan, 1993). Therefore, we have evaluated if the guanidine containing BTS 67 582 can bind to the I_2-imidazoline receptor.

Rabbit kidney cortex microsomes were prepared from male New Zealand white rabbits (1.5-2 kg) according to published methods (Coupry *et al.*, 1987). [^{3}H]-Idazoxan binding was performed in a total assay volume of 500 µl in buffer (50 mM Tris.Cl, 2 mM EGTA, 1 mM $MgCl_2$ pH 7.4). Saturation studies were carried out using 11 concentrations of [^{3}H]-idazoxan (0.05 nM to 50 nM), with non specific binding defined by 10 µM idazoxan. Tubes were incubated at room temperature for 30 minutes and the reaction terminated by filtration. Drug displacements were determined at a single concentration of radioligand (2 nM). Binding parameters were calculated using EBDA/LIGAND (McPherson, 1983).

Three experiments on separate membrane preparations yielded mean Kd values (± SEM) of 1.1 ± 0.02 and 88 ± 19 nM and mean Bmax values of 0.56 ± 0.05 and 3.1 ± 1.3 pmoles/mg protein for the high and low affinity sites respectively. There was no effect of (-)-epinephrine or rauwolscine (up to 100 µM) indicating a lack of interfering α_2-adrenoceptor binding. IC50 values and associated Hill (H) slopes (mean ± SEM, 3 experiments) for idazoxan (3.6 ± 1.2 nM, H = 0.58 ± 0.04), phentolamine (5.1 ± 0.4 µM, H = 0.70 ± 0.03), clonidine (2.6 ± 1.6 µM, H = 0.46 ± 0.16), guanabenz (2.5 ± 1.4 nM, H = 0.39 ± 0.04), amiloride (0.13 ± 0.04 µM, H = 0.62 ± 0.09) and cimetidine (>0.1 mM) were obtained. The micromolar affinity for clonidine, high affinity for guanabenz and amiloride, lack of affinity of cimetidine and low Hill slopes are all consistent with binding to the I_2-imidazoline receptor (Regunathan & Reis, 1996). Displacement curves for BTS 67 582 (9 concentrations from 10 nM to 1 mM) were performed using 9 isotope concentrations from 0.1 nM to 50 nM. There was a linear relationship between the IC50 value and isotope concentration indicating that the Cheng-Prusoff relationship was valid. The extrapolated Ki value was 4.7 ± 0.6 µM (± SEM, n=3-6).

In conclusion, BTS 67 582, can bind to the I_2-imidazoline receptor with a potency consistant with its effect on insulin secretion. Thus, BTS 67 582 may bind to the putative pancreatic islet imidazoline receptor and regulate insulin release by an interaction at the ATP-dependent K^+ channel.

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134P THE IMIDAZOLINE$_2$ (I_2) SITE-SELECTIVE LIGAND BU224 ELEVATES PLASMA CORTICOSTERONE IN RATS

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Central catecholamines play an important role in maintaining basal homeostasis and in mediating the response to stress. Increased noradrenaline in the hypothalamic paraventricular nucleus (PVN) results in activation of the hypothalamo-pituitary-adrenal (HPA) axis (Plotsky, 1987). Acute administration of imidazoline$_2$ (I_2) site selective ligands results in an increase in extracellular catecholamines in the rat brain (Nutt *et al.*, 1997). We have now investigated the effect of the I_2 site selective ligand, 2-(4,5-dihydroimidaz-2-yl)-quinoline (BU224) on plasma corticosterone in rats, an index of HPA axis activation.

Male Sprague-Dawley rats (200-240g) were cannulated through the right jugular vein under sodium pentobarbital anaesthesia (48 mg kg^{-1}). Following surgery, animals were housed singly. The experiment began between 09.00h and 10.00h, 3 days after surgery. At time 0 min a pre-injection blood sample (0.4 ml) was taken from each animal. Rats were then given an i.p injection of either saline vehicle (control) or BU224 (2.5 or 10 mg kg^{-1}). Further blood samples (0.4 ml) were taken 30 min and 60 min after injection.

Total plasma corticosterone was measured directly by radioimmunoassay (Harbuz *et al.*, 1994). Data was analysed using Fisher's PLSD test following one-way analysis of variance.

Both doses of BU224 resulted in a significant elevation in plasma corticosterone levels relative to controls at 30 min and 60 min post injection (Figure 1). Furthermore, the elevation in corticosterone appeared dose-dependent with the elevation induced by 10 mg kg^{-1} BU224 being significantly greater than that induced by 2.5 mg kg^{-1}.

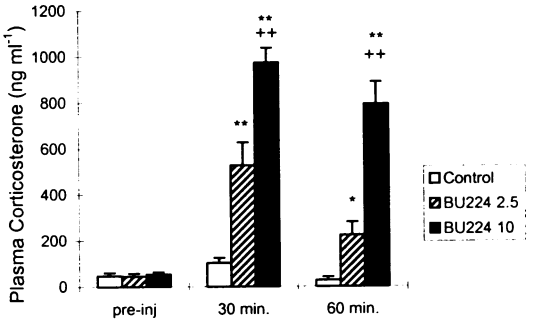

Figure 1. Effect of BU224 on plasma corticosterone. Results are expressed as mean ± S.E.M. (n = 5-8). **p < 0.01, *p < 0.05 compared to appropriate time-point controls; ++p < 0.01 compared to 2.5 mg kg^{-1} BU224.

In conclusion, peripheral administration of BU224 increases plasma corticosterone. The exact role of the HPA axis in this effect is curently under investigation.

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135P CHANGES IN UNCOUPLING PROTEIN mRNAs IN DIETARY-OBESE RATS AND THEIR RELATIONSHIP TO THERMOGENESIS

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Mitochondrial uncoupling proteins (UCPs') are a family of structurally related proteins that exhibit tissue specific distributions and have been suggested to be involved in thermogenesis through uncoupling of oxidative phosphorylation (Gura, 1998). Factors controlling UCP synthesis during altered energy balance are currently under investigation since they may be useful in combating obesity through disposing of excess energy stores via increased thermogenesis. We have investigated the changes in mRNA coding for UCPs' in dietary-obese rats to understand physiological factors that regulate thermogenesis and metabolism. Eighteen male Wistar rats (150g) were fed a highly palatable diet, rich in fat, for 8 weeks and became significantly heavier than control rats fed standard pellet chow (Widdowson et al., 1997). Rats were killed by CO_2 inhalation and gastrocnemius muscle, epididymal fat and brown adipose tissue (BAT) snap frozen in liquid N_2 and stored at –40°C. UCPs' were measured by Northern blotting using a digoxigenin-labelled oligonucleotide probe for UCP1, or digoxigenin-labelled cDNA probes for UCP2 and UCP3. Dietary-obese rats could be divided into two groups depending on the degree of weight gain, as compared to controls (420 ± 9g; n=9), low-fat gainers (460 ± 6g; n=8) and high-fat gainers (526 ± 8g; n=10). Low-fat gainers exhibited significantly increased epididymal fat pad masses (+82%, P<0.01; ANOVA and Bonferroni modified t-test) plasma leptin (+26%) and insulin (+43%) concentrations, as compared to controls. High-fat gainers demonstrated greatly increased fat pad masses (epididymal pad +145%; p<0.01) plasma leptin (+40%) and insulin (+55%) concentrations as compared to controls. Both low (37.6 ± 0.1°C; P<0.01) and high-fat (37.5 ± 0.1°C; P<0.01) gainers had raised rectal temperatures, as compared to controls (36.8 ± 0.1°C). Although BAT exhibited marked hypertrophy in both low- and high-fat gainers, UCP1, UCP2 and UCP3 mRNA concentrations were not significantly different in tissue samples of equal weight. There was also no difference in UCP2 mRNA concentrations in white adipose tissue between low-fat gainers, high-fat gainers and controls. However, both UCP2 and UCP3 mRNA concentrations were significantly increased (P<0.05) in gastrocnemius muscle of low fat gainers (UCP2 = 182% of control values; UCP3 = 223% of controls values) and high fat gainers (UCP2 = 164% of control values; UCP3 = 197% of control values). Gastrocnemius muscle weights of low-fat or high-fat gainers were, however, not significantly different from controls. In conclusion, we have demonstrated tissue specific increased UCP mRNA concentrations in muscle but not in BAT or white fat. The lack of correlation of UCP mRNA expression with the raised body temperature of dietary-obesity suggests that UCPs' may be involved in metabolic regulation other than uncoupling oxidative phosphorylation, possibly being influenced by the elevated muscle triglyceride and free fatty acid concentrations observed in these dietary-obese animals.

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136P MECHANISTIC PHARMACOKINETIC-PHARMACODYNAMIC MODELLING OF ANTI-LIPOLYTIC EFFECTS OF ADENOSINE A_1 RECEPTOR LIGANDS: PREDICTION OF TISSUE-DEPENDENT EFFICACY *IN VIVO*

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It has been suggested that agonists for adenosine A_1 receptors on adipocytes may be used as anti-lipolytic drugs but the pronounced cardiodepressant effects have been a major impediment for the clinical development (see Van Schaick *et al.*, 1998). A strategy to overcome this problem is based on the idea that low-efficacy agonists may display greater organ selectivity compared to high-efficacy ligands and compounds have been designed that behave as partial agonists for the adenosine A_1 receptor-mediated effect on heart rate in rats (see Van der Graaf et al., 1997). Very recently, we have shown that the 8-methyl-, 8-ethyl- and 8-butylamino analogues (hereafter referred to as 8MCPA, 8ECPA and 8BCPA, respectively) of N^6-cyclopentyladenosine (CPA), despite their limited cardiovascular action, behave as full agonists for the anti-lipolytic effects in rats (Van Schaick et al, 1998), suggesting that reducing efficacy is indeed a feasible strategy to enhance organ selectivity of adenosine A_1 receptor agonists. The aim of the present study was to obtain further "proof of concept" for this approach by analysing further the anti-lipolytic effects of the 8-alkylamino- and three other CPA analogues (2', 3' and 5'-deoxy-CPA; hereafter referred to as 2dCPA, 3dCPA and 5dCPA, respectively) and to develop a mechanistic model to quantify and predict the tissue-selective action of adenosine A_1 receptor agonists *in vivo*.

Details of the methods of the pharmacokinetic-pharmacodynamic experiments have been published previously (Van Schaick *et al.*, 1998). Briefly, two days before experimentation, the right femoral artery and right jugular vein of male Wistar rats (200-250 g) were cannulated for the collection of serial blood samples and administration of drugs, respectively. Animals were fasted for 24 h before experimentation, with free access to water. Freely-moving rats received an intravenous infusion of vehicle (765 µl 20% DMSO in 0.9% saline) or compound over 15 min (5.2, 0.25, 0.16, 4.0, 12.0 and 20.0 mg/kg for 2dCPA, 3dCPA, 5dCPA, 8MCPA, 8EBPC and 8BCPA, respectively). Arterial blood samples (~15) were taken at regular time intervals for the determination of concentration of drugs using HPLC analysis. For the determination of concentrations of non-esterified fatty acids (NEFAs), 24 blood samples of 50 µl each were taken over a period of 4 h and NEFAs plasma concentration was determined as described by Van Schaick *et al.* (1998).

All CPA analogues investigated produced a significant decrease in the NEFAs plasma concentration after intravenous infusion (Table 1). The pharmacokinetic behaviour of each ligand was described by a standard two-compartment model. The pharmacokinetic parameter estimates were then used to fit simultaneously the individual (n = 6-8) time-NEFAs concentration profiles for each agonist to the physiological indirect response model presented before (Van Schaick *et al.*, 1998) in combination with the Hill equation to obtain estimates of the NEFAs elimination rate constant (k_e) and upper asymptote (fractional inhibition), midpoint location and midpoint slope parameter (α, pEC_{50} and n_H, respectively) of the concentration-effect relationship (Table 1). Subsequently, the data were analysed with the operational model of agonism (see Van der Graaf *et al.*, 1997). Estimates of affinity (pK_A, Table 1) were not significantly different from the values obtained from our previous analysis of the effects on heart rate (Van der Graaf *et al.*, 1997) and it was estimated that the *in-vivo* density and/or coupling of adenosine A_1 receptors mediating anti-lipolytic effects is 36.5 ± 4.8 times higher compared to the receptors mediating bradycardia. The model predicts that it is possible to design ligands which produce significant inhibition of lipolysis and are completely devoid of cardiovascular effects *in vivo*.

TABLE 1 Pharmacodynamic parameter estimates (mean ± s.e.mean)

	k_e (min^{-1})	α	pEC_{50}	n_H	pK_A
2dCPA	0.07 ± 0.01	0.62 ± 0.02	6.81 ± 0.08	1.49 ± 0.18	5.55 ± 0.08
3dCPA	0.06 ± 0.01	0.61 ± 0.09	7.67 ± 0.18	1.21 ± 0.17	5.87 ± 0.17
5dCPA	0.08 ± 0.01	0.80 ± 0.06	9.18 ± 0.25	0.64 ± 0.15	6.70 ± 0.23
8MCPA	0.05 ± 0.01	0.68 ± 0.12	7.13 ± 0.47	1.01 ± 0.23	5.55 ± 0.19
8ECPA	0.07 ± 0.02	0.71 ± 0.05	6.80 ± 0.21	0.73 ± 0.29	5.34 ± 0.12
8BCPA	0.06 ± 0.01	0.66 ± 0.05	6.00 ± 0.09	1.85 ± 0.21	5.32 ± 0.09

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137P EVIDENCE FROM *IN VIVO* AND *IN VITRO* STUDIES THAT THE INHIBITORY ACTIONS OF LIPOCORTIN 1 ON ANTERIOR PITUITARY FUNCTION REQUIRE AMINO ACID RESIDUES 13-26

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Studies from our laboratory suggest that lipocortin 1 (LC1), a 37kDa protein, is an important mediator of glucocorticoid action in the rat neuroendocrine system, acting at the levels of both the hypothalamus and the anterior pituitary gland (Buckingham & Flower, 1997). In the present study we have used *in vitro* and *in vivo* models to identify the active sequences within the LC1 molecule.

The *in vitro* studies examined the effects of a number of peptides derived from the N-terminus of LC1 (N-acetyl $LC1_{7-12}$, N-acetyl $LC1_{2-12}$, N-acetyl $LC1_{2-26}$ and $LC1_{1-188}$), as well as the full length LC1 protein, on the resting and forskolin-stimulated release of ACTH and prolactin (PRL) from rat anterior pituitary tissue. The tissue was collected *post mortem* from adult male rats and incubated according to a well established protocol (Taylor *et al*, 1993). Hormones released into the medium were determined by radioimmunoassay and the results analysed by ANOVA and Duncan's multiple range test (n=6). A sub-maximal concentration of forskolin (Fk, 100µM, 1h contact) induced significant ($p<0.01$) increases in ACTH and PRL release; these were reduced significantly ($p<0.01$) by dexamethasone (Dex., 0.1µM, 3h contact) which, alone, was without effect. None of the peptides tested significantly influenced basal peptide release. N-acetyl $LC1_{7-12}$ and N-acetyl $LC1_{2-12}$ (0.2-200µg/ml) also failed to influence the Fk-stimulated release of ACTH and PRL. By contrast, N-acetyl $LC1_{2-26}$ (Ac2-26) partially inhibited the Fk-induced release ($p<0.01$) of ACTH (Basal 86±8 *vs.* Fk alone 242±30 *vs.* Fk + Dex. 105±13 *vs.* Fk + 2µg/ml Ac2-26 158±31pg/ml) and PRL (Basal 1±0.5 *vs.* Fk alone 20±1 *vs.* Fk + Dex. 2±1 *vs.* Fk + 2µg/ml Ac2-26 10±1ng/ml). $LC1_{1-188}$ and the full length LC1 were more potent and, at concentrations of 10ng/ml, both produced complete inhibition (100%, $p<0.01$) of Fk-stimulated anterior pituitary hormone release as did dexamethasone.

For *in vivo* studies, adult male CFY rats (~200g), handled daily for 10 days, were treated either with a purified anti-LC1 polyclonal antibody (LC1 pAb) raised against Ac2-26 or a corresponding volume (1ml/kg, s.c.) of similarly purified non-immune sheep serum (NSS). The following morning, rats from each group (n=6) were given either Dex. (10µg/kg, i.p.) or its saline vehicle (1ml/kg, i.p.) and 75min later IL-1ß (0.5µg/kg, s.c.) or its saline vehicle (1ml/kg, s.c.). Blood was collected 1h later and assayed for ACTH; data were analysed by Scheffe's test. In NSS-treated rats, IL-1ß produced significant ($p<0.01$) rises in plasma ACTH concentration which were abolished by Dex. (Basal 17±2 *vs.* IL-1ß 168±29 *vs.* Dex 16±4 *vs* IL-1ß + Dex 6±1pg/ml, $p<0.01$). IL-1ß also stimulated ACTH release in LC1 pAb-treated rats ; however, the ability of Dex to block the pituitary response to the cytokine in NSS-treated rats was partially quenched when rats were treated with the antibody (Basal 20±4 *vs.* IL-1ß 216±23 *vs.* Dex 14±3 *vs* IL-1ß + Dex 106±22 pg/ml, $p<0.01$).

These results suggest that sequences contained within the N-terminal of LC1 contribute to the inhibitory effects of LC1 on pituitary hormone release and that residues 13-26 may be particularly important in this regard.

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138P EXPRESSION AND LOCALISATION OF LIPOCORTIN 1 IN THE RAT ADRENAL GLAND

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Increasing evidence suggests that local factors in the hypothalamus, anterior pituitary and adrenal gland play a role in "fine-tuning" the functional activity of the hypothalamo-pituitary- adrenal (HPA) axis. Studies from our laboratory have shown that lipocortin 1 (LC1), a 37kDa glucocorticoid-inducible protein, is important in this regard serving as a paracrine/autocrine agent in the hypothalamus and pituitary gland (Buckingham & Flower, 1997). As a prelude to examining the potential role of LC1 at the adrenal level, we have used a combination of immunohistochemical and flow cytometric techniques together with western blot analysis to explore its expression and distribution in the rat adrenal gland.

Adrenal tissue collected *post mortem* from adult male CFY rats (200-220g) was processed for (a) SDS-PAGE western blot analysis using a standard protocol (Philip *et al.*, 1998) and a polyclonal antiserum (pAb) raised in-house against $LC1_{1-346}$, (b) immunohistochemistry using pAbs against $LC1_{15-35}$ (raised in-house, diluted 1:50,000) or tyrosine hydroxylase (anti-TH pAb, Chemicon, Temecula, CA, USA, diluted 1:50,000) with appropriate non-immune serum controls and (c) fluorescence activated cell (FAC) analysis. Cells for FAC analysis were dispersed enzymatically (collagenase, 0.2%; DNase, 0.002%), fixed (paraformaldehyde, 1%) and permeabilised (saponin, 0.02%); intracellular LC1 and TH were detected with a mouse anti-LC1 monoclonal antibody (mAb, coded 1B, 100µg/ml, Biogen Inc., Cambridge, MA, USA) and anti-TH pAb (1:500) respectively and labelled with fluorescent secondary antibodies conjugated to FITC or RPE. Anti-tubulin mAb (100µg/ml) and pAb (1:500, both from Sigma Chemical Company Ltd) and anti spectrin mAb (100µg/ml, Sigma) were used as positive and negative controls respectively. Morphological examination at the EM level confirmed that the structural integrity of the permeabilised cells prepared for FAC analysis was well preserved.

LC1 was readily detectable in the adrenal gland by SDS-PAGE western blot analysis with the major band corresponding to 37kDa native "species" of the protein. The immuno-histochemical studies demonstrated the well documented (Stephens *et al.* 1981) presence of TH in the adrenal medulla and outer capsule of the gland (n = 4 rats, 10 sections per rat). They also showed discrete staining of LC1 in the adrenal medulla and, to a lesser extent, in the capsule; no LC1-positive cells were observed in the cortex (n = 4 rats, 10 sections per rat). Data from preliminary double labelling studies suggested that there is some degree of co-localisation of LC1 and TH in the medulla. In accord with these data, FAC analysis identified populations of adrenal cells which show positive staining for TH or LC1 protein; these comprise 30.9±4.9% and 23.1±1.6% respectively of the total adrenal cell population with 7.0±0.9% showing co-localisation of the proteins (n = 3 experiments). As a function of fluorescence, the LC1 containing cells were estimated to have approximately 30,000 molecule per cell (n=3 experiments).

These results show that LC1 is expressed in the rat adrenal gland and pave the way for analysis of the functional role of the protein.

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139P ADRENOMEDULLIN (AM), A VASODILATOR PEPTIDE SECRETED BY HUMAN PULMONARY ARTERY-DERIVED CELLS, INCREASES INTRACELLULAR CAMP AND INHIBITS DNA SYNTHESIS

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Adrenomedullin (AM) is a 52 amino acid peptide secreted by vascular endothelial and smooth muscle cells in the rat. In rat vascular smooth muscle cells AM increases cAMP and inhibits cell proliferation (Ishizaka et al., 1994). Specific receptors for AM are present in rat tissues and most abundant in the lung, (Owji et al., 1995). AM is a powerful vasodilator in the lung and plasma levels are increased in pulmonary hypertension. We therefore investigated the effects of AM and its receptors on cAMP and DNA synthesis in cells derived from human pulmonary arteries.

Nine pulmonary artery samples were obtained at operation (informed consent given) and cells grown out from explants cultured in medium 199 with 20% FBS. AM-immunoreactivity and cAMP (in the presence of isobutyl methylxanthine, IBMX) were assayed by radioimmunoassay. DNA synthesis was measured by ^{3}H-thymidine uptake and AM receptor binding using ^{125}I-AM. Inhibition of DNA synthesis is expressed as a % of PDGF stimulation. These techniques were as previously detailed (Withers et al., 1996). Statistical significance was analysed using one-way ANOVA and post hoc Tukey's test.

All of the cells stained positive for smooth muscle alpha-actin. Six of the cell lines produced AM-IR (188 ± 80 fmol/10^5 cells/24h, n=6). In these cells AM had no effect on intracellular cAMP (basal 23.4 ± 5.3 vs 100 nM AM 24.5 ± 3.9 pmol/10^5 cells/15 min, n=6, NS).

Three cell lines released no AM (below detection limit 2 fmol/tube). However, these cells showed the presence of specific ^{125}I-AM binding (IC_{50} AM = 0.76 ± 0.16 nM, n=3) and AM stimulated cAMP (basal 24.8 ± 3.9, 10 nM AM 2243 ± 67 pmol/10^5 cells/15 min, n=3 P<0.0001, EC_{50} = 1.14 ± 0.39 nM, n=3). PDGF (5 ng/ml) stimulated ^{3}H-thymidine uptake (basal 19 ± 3%, PDGF 100 ± 3%, n=3) in the presence of IBMX (50 μM). This stimulation was inhibited by AM (0.1 nM 86 ± 10%, 10 nM 40 ± 1% of PDGF, n=3 P<0.001).

We have derived cell lines from human pulmonary arteries some secreting AM and the others expressing AM receptors. In the cells expressing receptors AM dose dependently increased cAMP and inhibited PDGF stimulated DNA synthesis. Thus AM may be involved in paracrine regulation of pulmonary vascular tone and in vascular remodelling.

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140P ACTIONS OF OESTROGENS ON PRIMATE ISOLATED AORTIC RINGS

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Oestrogens appear to offer cardiovascular protection in premenopausal women and those receiving hormone replacement therapy (Henderson *et al.*, 1991). The reduced morbidity and mortality observed may be related to the dilator actions of oestrogens reported in arterial preparations from various species which may involve inhibition of calcium influx (Shan *et al.*, 1994) or protein kinase C inhibition (Magness *et al.*, 1989). However, there are few reports of oestrogen action on primate vessels. In these experiments we compared actions of 17α and β oestradiol (αEST, βEST), 17α ethinyl oestradiol (ET-EST) and diethylstilboestrol (DEST) with the calcium channel blocker, nifedipine (NIF), on the aorta of the marmoset (*Callithrix jacchus*).

Endothelium-intact rings of aorta 3-5mm in length prepared from marmosets of either sex (310-380g) were suspended in Krebs' solution containing indomethacin (10μM) (37°C; 95% O_2, 5% CO_2) under 2g resting tension. Following 60 min equilibration rings were contracted with 80mM KCl three times to check response reproducibility.

Cumulative concentration-response curves were constructed to KCl (10-300mM) or phenylephrine (PHE, 0.1-40μM) and repeated following 40 mins incubation with βEST, αEST, ET-EST or DEST (10, 20μM) or NIF (0.1, 1μM). E_{max} was 1.9 ± 0.1g and 1.5 ± 0.1g for KCl and PHE respectively; n = 5-6. No vehicle effects were observed.

The oestrogens caused little change in responses to PHE: only DEST (20μM) significantly reduced E_{max} to 80 ± 8% of control (P<0.05, Student's t-test). In contrast, βEST (10μM) greatly reduced PHE-induced responses of rat aorta, shifting concentration-response curves rightward and reducing E_{max} to 52 ± 6% (P<0.01). KCl-induced responses of marmoset aorta were more affected by oestrogens: βEST (20μM) reduced E_{max} to 80 ± 5% (P<0.01). ET-EST and DEST (20μM) shifted the KCl concentration-response curve rightward, reducing E_{max} to 69 ± 4% and 52 ± 6% respectively (P<0.01). Marmoset aorta was relatively insensitive to nifedipine. NIF (0.1μM) reduced maximal responses to KCl by only 36 ± 3%, compared to 100% relaxation of contraction in rat aorta (Babaei *et al.*, 1995).

We conclude that β oestradiol inhibits contraction less in marmoset aorta than in human arteries (Mugge *et al.*, 1993 & Belfort *et al.*, 1996) and many mammalian vessels, e.g. rat aorta (Babaei *et al.*, 1995) where significant relaxation of agonist-induced contraction by oestradiol has been observed. Thus marmoset aorta is unlikely to provide a useful model of oestrogen action on the human vascular system.

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141P EFFECTS OF 17β-OESTRADIOL AND ENVIRONMENTAL OESTROGENS ON DEVELOPING RAT HYPOTHALAMIC DOPAMINERGIC NEURONES IN CULTURE

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At critical periods in early life sex steroid hormones programme sex differences in brain function. Studies in the rat have shown that a rise in testicular testosterone production during the perinatal period irreversibly masculinizes the hypothalamic circuitry controlling the neuroendocrine system principally via an estrogen receptor (ER) -mediated mechanism after its conversion to 17ß-estradiol (E_2) by central aromatase enzymes. There is thus concern that the environmental estrogens, which belong to diverse chemical classes, could interfere with these processes (Gorski, 1986). Further studies are hampered by the relative lack of information on the cellular targets for these organizational effects of estrogen. Our own studies have shown that E_2 can influence the development of dopaminergic (DA) neurones in primary rat hypothalamic cell cultures, as determined by [^{3}H]DA uptake (Murray & Gillies 1993). Therefore, this study aimed to investigate whether the environmental estrogens octylphenol, bisphenol A, and genistein might act similarly.

Cell suspensions were prepared from rat hypothalami taken at 18 days' gestation and cultured in 15mm wells ($5x10^5$ cells in 200µl) in a chemically defined, serum-free medium as described previously (Murray & Gillies 1993). From the time of plating the medium contained either no estrogen (control) or estrogenic compounds at a range of concetrations (10^{-14}-10^{-8}M for E_2; 10^{-11}-10^{-5}M for the environmental estrogens). At days 4, 9, 13 and 16 *in vitro* (DIV) cells were incubated with [^{3}H]DA (0.33µM for 1h) and specific uptake (>70% in the presence of 10^{-4}M nomifensine) was determined using scintillation counting as described in detail elsewhere (Murray & Gillies, 1993). Results were analysed by ANOVA followed by Student Newman-Keuls multiple range test (n=4). In control cultures uptake increased significantly with time (eg. 169.9±10.5, 403.5±23.3, 543.4±11.5, 412.6±27.1 pmoles per well at DIV 4, 9, 13 and 16 repectively; $p< 0.01$ for DIV 4 vs 9 and 9 vs 13). For the first few days in culture estrogenic compounds had no effect, but by DIV 9 all compounds increased uptake with significant effects observed at 10^{-14} for E_2, 10^{-11} for OP, 10^{-11} for BPA and 10^{-9}M for genistein (154.7±10.5, 141.8±8.9, 167.8±3.1, and 160.5±7.7% of control, respectively, $p<0.01$). These increments were near maximal for each compound tested. Similar results were obtained at later stages in culture. In the presence of the antiestrogen, ZM 182,780 (10^{-5}M, which alone had no effect on uptake), responses to E_2 (10^{-12}M) and OP (10^{-9}M) were completely blocked.

In summary, these results show that developing hypothalamic DA neurones are sensitive to very low levels of both the natural estrogen and the environmental estrogens via mechanisms which appear to involve the intracellular ER. They thus provide direct evidence that chemicals with potential endocrine disrupting ability could target the brain at a time when it is exquisitely sensitive to endocrine signals with the potential that neuroendocrine control could be affected in later life. Further studies using lower concentrations would be needed to investigate the reason for the absence of a dose-response realtionship for some of the compounds tested.

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142P THE ORL$_1$-RECEPTOR ANTAGONIST [Phe$^1\Psi$(CH$_2$-NH)Gly^2]NOCICEPTIN(1-13)NH$_2$ CAUSES CONTRACTION IN RAT AND MOUSE COLON

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Characterization of the pharmacological actions of nociceptin/orphaninFQ, the endogenous ligand for the ORL$_1$-receptor (Meunier et al., 1995; Reinscheid et al., 1995), has been hampered by the lack of an antagonist. Recently, [Phe$^1\Psi$(CH$_2$-NH)Gly^2]nociceptin(1-13)NH$_2$ was shown to antagonise the action of nociceptin in isolated tissues (Guerrini et al., 1998). We have used [Phe$^1\Psi$(CH$_2$-NH)Gly^2]nociceptin(1-13)NH$_2$ to characterise further the contractile activity of nociceptin in the rat and mouse isolated colon preparations (Corbett et al., 1998).

Sections of distal colon from male Wistar rats (~250g) and proximal colon from DBA/2 mice (25g) were set up in 3ml silanised organ baths containing Krebs solution at 37°C. Values for EC_{50} are presented as the mean (with range).

In rat colon, the µ-opioid agonist [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and the δ–opioid agonist, [Met^5]enkephalin were contractile with EC_{50} values of 6.4nM (2.2-9.8, n=5) and 0.33nM (0.17-0.83, n=13), respectively. The κ–opioid agonist CI-977 was inactive. Nociceptin and [Tyr^{14}]nociceptin produced concentration-dependent contractions with EC_{50} values of 0.25nM (0.07-0.45, n=6) and 0.63nM (0.12-1.13, n=5), respectively. Similar results were obtained with the mouse colon. The effects of the opioid peptides but not of nociceptin were blocked by naloxone (1µM). In both species, however, the putative ORL$_1$-receptor antagonist [Phe$^1\Psi$(CH$_2$-NH)Gly^2] nociceptin(1-13)NH$_2$ also caused concentration-dependent contractions; the EC_{50} values were 3.1nM (1.1-5.6, n=6) in the rat and 6.0nM (5.3-8.0, n=4) in the mouse.

Nociceptin-induced contraction of colonic smooth muscle is not mediated by opioid receptors, since it is not blocked by naloxone, but is presumably caused by an action at ORL$_1$-receptors. However, it was not possible to confirm this using the ORL$_1$-receptor antagonist [Phe$^1\Psi$(CH$_2$-NH)Gly^2]nociceptin(1-13)NH$_2$ as it also caused contraction. This analogue has also been found to have agonist activity at ORL$_1$-receptors in CHO cells (Butour et al., 1998) and in *in vivo* antinociceptive tests (Grisel et al., 1998). These data taken together imply that nociceptin and related peptides may interact with receptors other than the ORL$_1$-receptor.

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143P EFFECTS OF METHYL-EUGENOL ON THE CONTRACTILE ACTIVITY OF THE GUINEA-PIG ISOLATED ILEUM

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Methyl-eugenol (ME) is a common constituent of the essential oil of many Brazilian aromatic plants including *Croton nepetaefolius*. This plant has a rich essential oil content and is used in the folk medicine of the Northeast of Brazil as a stomachic, carminative, and intestinal antispasmodic. We have previously shown that the essential oil of *Croton nepetaefolius* exerts myorelaxant and antispasmodic effects on isolated intestinal smooth muscle, an action shared by several components including ME (Magalhães *et al.*, 1998). The present study has addressed the possible mechanism of action of ME.

Segments of ileum from male guinea-pigs (250-400g) were dissected and mounted for the recording of isotonic tension using a kymograph. The tissue was bathed in aerated standard Tyrode's solution maintained at 37°C, pH 7.4. The effects of ME on basal tone and the contractions induced by 60 mM KCl, acetylcholine (ACh) and histamine were examined. In addition, effects on resting membrane potential were determined using impalement with glass microelectrodes containing 3M KCl. Statistical differences were assessed using a non-paired Student's t-test.

ME (5 –780 μM) induced a concentration-dependent, reversible relaxation of basal tonus which reached a maximum of 31.7 ± 8.8 % of a 60mM KCl contraction (EC_{50} of 52.2 ± 18.3 μM, n=5). The response to 330 μM ME was not altered by tetrodotoxin (0.5 μM, n=6), hexamethonium (0.5 mM, n=4), indomethacin (1 μM, n=7), or L-NAME (100 μM, n=5). ME also completely relaxed the ileum pre-contracted with 60 mM K^+ (EC_{50} of 126.6 ± 32.2 μM, n=11). ME (330 μM) induced a slight hyperpolarisation of the resting membrane potential (E_m) from -57.3 ± 0.66 to -62.8 ± 0.60 mV (n=28-32 cells, $p<0.05$) but did not alter E_m in depolarised (60mM KCl) tissues (-23.6 ± 0.53 to -24.2 ± 0.6mV, n=24-25 cells). ME inhibited the contractions induced by sub-maximal concentrations of ACh (IC_{50} = 82.2 ± 19.4 μM, n=7), histamine (IC_{50} = 124.4 ± 12.2 μM, n=9), and 60 mM K^+ (IC_{50} = 65.0 ± 13.3 μM, n=6). ME (330 μM) further reduced the component of ACh-induced contraction that was insensitive to 1 μM nifedipine (55.5 ± 19.8% of control ACh contraction) to 0.91 ± 0.5% of the control value (n=8). Also ME (330μM) inhibited the contraction induced by ACh in Ca^{++}-free solution from 24.8 ± 4.0% to 12.5±3.8% of the control contraction in normal (Ca^{++}-containing) Tyrode's solution (n=7).

In conclusion these results suggest that ME induces relaxation of guinea-pig ileum by a direct action on smooth muscle via a mechanism largely independent of alterations of E_m and extracellular Ca^{++} influx.

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144P REGULATION OF β_3-ADRENOCEPTORS BY THYROID HORMONE IN RAT ILEAL SMOOTH MUSCLE

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Most studies of β_3-adrenoceptor (AR) regulation have been performed in adipose tissue *in vivo* but several recent studies have shown that regulation is tissue specific. Glucocorticoids markedly down-regulate β_3-AR expression in adipose tissue but do not affect that in gut (Evans *et al.*, 1998). Thyroid hormone regulation of β-ARs is both tissue and receptor subtype specific and differentially affects the expression of β_3-AR in brown and white adipose tissue (Rubio *et al.*, 1995). β_3-ARs are the predominant β-AR subtype mediating relaxation in rat ileum (Roberts *et al.*, in press) but the effect of thyroid hormone on gut β_3-ARs has not been addressed. This study examines the effect of thyroid hormone on β_3-AR-mediated relaxation responses and β-AR mRNA levels in ileal smooth muscle tissue cultures.

Longitudinal smooth muscle strips were dissected from segments of ileum from male Sprague-Dawley rats (250-320g) and either used immediately or incubated in Dulbecco's Modified Eagle's Medium with 5% foetal bovine serum at 37°C in 5% CO_2 in the absence or presence of 3,3',5-triiodo-L-thyronine (T_3, 100nM). After 24 and 48h the muscle strips were transferred to organ baths or frozen at -70°C for RNA extraction. Reverse transcription and polymerase chain reaction were used to measure β-AR mRNA's. In organ bath studies, muscle strips were precontracted with carbachol (3μM) and relaxed by (-)-isoprenaline (non-selective β-AR agonist) or CL 316243 (β_3-AR agonist). The pEC_{50} values for (-)-isoprenaline showed no change with time (7.31 ± 0.08 at 0h, 7.23 ± 0.12 at 24 h and 7.53 ± 0.10 at 48h, n=4-8). The potency of (-)-isoprenaline was unchanged by T_3 treatment (pEC_{50} values, 7.27 ± 0.11 at 24h and 7.34 ± 0.27 at 48h, n=3-5). The pEC_{50} values for CL 316243 were also unchanged with time (7.85 ± 0.11 at 0h, 8.07 ± 0.08 at 24h and 7.81 ± 0.17 at 48h, n=3-4) or with T_3 treatment (7.82 ± 0.13 at 24 h and 7.79 ± 0.29 at 48h, n=3). However, β_3-AR mRNA levels (expressed as % of 0h controls) declined to 50 ± 7% at 24h and 25 ± 6% at 48h in the absence of T_3. In the presence of T_3, β_3-AR mRNA levels were maintained at control levels (103 ± 11% at 24h and 84 ± 28% at 48 h) throughout the incubation period (n=6-7). In contrast, β_2-AR mRNA levels increased to more than 200% at both 24 and 48h in both the absence and presence of T_3 (n=5-7). β_1-AR mRNA levels were below levels of quantitation in all samples. This study indicates that β_2- and β_3-AR mRNA in rat ileal smooth muscle are differentially regulated by thyroid hormone but at times up to 48h the changes in β_3-AR mRNA are not directly reflected in reduced potency of β_3-AR agonists.

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145P GM-CSF IS PRODUCED IN HIGH CONCENTRATIONS BY INFLAMED COLONIC MUCOSA FROM PATIENTS WITH ULCERATIVE COLITIS AND IS DOWN-REGULATED BY CO-INCUBATION WITH DEXAMETHASONE

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Inflammatory bowel disease (IBD; ulcerative colitis, UC, or Crohn's disease, CD), is associated with increased production of prostanoids, due to up-regulation of cyclo-oxygenase-2 (COX-2), and pro-inflammatory cytokines (Beck and Wallace, 1997). Interestingly, nonsteroid anti-inflammatory drugs (NSAIDs), including the COX-2 selective inhibitor L-745,337, greatly increase the release of granulocyte macrophage-colony stimulating factor (GM-CSF) by human vascular cells (Mitchell et al., 1998). Since GM-CSF increases the production and life span of leukocytes associated with IBD we have investigated the possibility that a number of anti-inflammatory drugs may regulate GM-CSF production by colonic biopsies from patients with active IBD.

Colonic mucosal biopsies obtained at elective colonoscopy, were blotted, weighed and cultured. Presence (UC or CD) or absence (control) of IBD was confirmed histologically. Each patient provided three samples, one of which was used as control (vehicle, 0.1% DMSO) and two of which were used for paired drug treatment, either: (A) L-745337 (10 μM), or indomethacin (10 μM); or (B) 5-ASA (1 mM), or dexamethasone (1 μM) (n=8-12 for each). Biopsies were then placed into culture medium (DMEM plus antibiotics, 5 % CO_2, 37 °C) and incubated for 24 h. Conditioned medium was then removed and the content of GM-CSF measured by ELISA (PharmiGen) and standardised to the biopsy protein content.

UC and CD were associated with significant increases in the production of GM-CSF ($p<0.05$, ANOVA). Productions of GM-CSF were unaffected by L-745,337 or indomethacin at concentrations that block COX-2 activity. By contrast GM-CSF production by UC tissue was modestly reduced by 5-ASA and greatly reduced by dexamethasone ($p<0.05$, ANOVA) (Fig. 1).

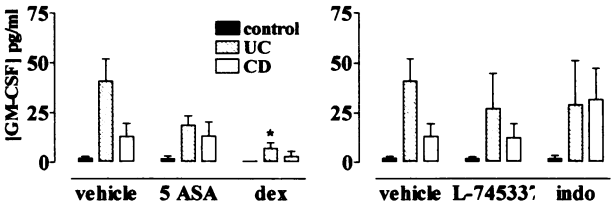

Fig. 1. Production of GM-CSF from colonic mucosa over 24 h

Here we show that IBD is associated with an enhanced ability of colonic tissue to form GM-CSF. In contrast to other human tissues, inhibition of COX-2 did not increase GM-CSF release. Interestingly, GM-CSF production was also greatly decreased (>80%) in samples taken from 2 patients with UC who were also receiving oral corticosteroids (data not shown). Thus, the beneficial effects of corticosteroids in IBD may be associated with reductions in GM-CSF production which is independent of prostanoid production.

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146P INDUCTION OF B_1 BRADYKININ RECEPTORS WITH LIPOPOLYSACCHARIDE TREATMENT IN B_2 BRADYKININ RECEPTOR KNOCKOUT MICE

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Bradykinin is produced in response to tissue injury and produces inflammation and pain. Its acute effects are mostly mediated through constitutively expressed B_2 receptors however, chronic inflammation results in induction of B_1 receptors. A problem with investigating the role of B_1 receptors in chronic inflammation is that data may be confounded by the presence of B_2 receptors. Production of a transgenic mouse in which the B_2 receptor gene has been disrupted (Borkowski et al., 1995) may provide a solution. In this study we examined the cardiovascular effects of bradykinin and the bradykinin B_2 receptor antagonist Hoe 140 in wild type and in B_2 receptor knockout mice. We also used lipopolysaccharide (LPS) to induce B_1 receptors in B_2 receptor knockout mice and investigated the cardiovascular effects of the B_1 receptor agonist $desArg^9$ bradykinin and B_1 receptor antagonist Leu^8,$desArg^9$ bradykinin.

Wild type (C57, Bk2r+/+) or B_2 knockout (hybrid C57 x J129 backcrossed with C57 for 2 generations, Bk2r-/-) mice (30 - 40 g) were anaesthetised with pentobarbitone sodium (60 mg kg^{-1}, i.p.). A tail vein and carotid artery were cannulated for administration of all test compounds and measurement of blood pressure and heart rate, respectively. Anaesthesia was maintained by pentobarbitone sodium (18 mg kg^{-1} hr^{-1}) via a jugular vein and body temperature maintained at 37 ± 1 °C.

In wild type mice, bradykinin (0.3 - 10 μg kg^{-1}) produced dose-related, transient, decreases in blood pressure (max ΔBP -42 ± 4%) which were significantly inhibited (max ΔBP -8 ± 2%) after Hoe 140 (3 μg kg^{-1}, n = 5) but were unaffected (max ΔBP -38 ± 3%) by vehicle (n = 4). In B_2 knockout mice, bradykinin (0.3 - 10 μg kg^{-1}, n = 6) or $desArg^9$ bradykinin (10 – 300 μg kg^{-1}, n = 3) had no significant effects on blood pressure. Three hours after LPS treatment (*E. coli* serotype 0111:B4, 1 mg kg^{-1}, i.v.), $desArg^9$ bradykinin (100 μg kg^{-1}) produced decreases in mean blood pressure which were significantly inhibited by Leu^8,$desArg^9$ bradykinin at 100 μg kg^{-1} (max ΔBP -17 ± 1% reduced to –7 ± 1%, n = 5) and 300 μg kg^{-1} (max ΔBP -21 ± 2% reduced to –4 ± 2%, n = 7), whereas its vehicle had no effect (max ΔBP –19 ± 3% to –18 ± 3%).

Since bradykinin is the preferred ligand at B_2 receptors and the selective B_2 receptor antagonist Hoe 140 blocked the hypotensive response to bradykinin in wild type mice, these results suggest that bradykinin produced hypotension in these mice via activation of B_2 receptors. We were unable to demonstrate a hypotensive response to similar doses of bradykinin in B_2 receptor knockout mice, confirming the disruption of functional B_2 receptors in these animals. The B_1 receptor agonist, $desArg^9$ bradykinin had no effects on blood pressure in B_2 receptor knockout mice indicating that B_1 receptors are not present in these mice under baseline conditions. However, after LPS, $desArg^9$ bradykinin produced a consistent hypotensive response which was blocked by the B_1 receptor antagonist Leu^8,$desArg^9$ bradykinin, indicating that the LPS had stimulated the induction of B_1 receptors.

This study demonstrates LPS-mediated induction of B_1 receptors in B_2 receptor knockout mice. As research into the treatment of chronic inflammation continues, this transgenic animal may provide a valuable system for investigating the role of B_1 receptors in chronic inflammation in the absence of B_2 receptors.

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147P LACK OF EVIDENCE FOR INTERACTIONS BETWEEN THE TACHYKININ NK_1 RECEPTOR AND IL-1β-MEDIATED EVENTS IN THE RAT CUTANEOUS MICROVASCULATURE

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The cytokine interleukin-1β (IL-1β) is a potent inflammatory mediator which can act via a variety of mechanisms. It stimulates neutrophil accumulation and evidence suggests that a neurogenic neurokinin-1 (NK_1) receptor component is involved in IL-1β neutrophil accumulation in the murine air pouch model (Perretti *et al.*, 1993; Ahluwalia *et al.*, 1998). In this study we have investigated whether there are links between IL-1 and the tachykinin neurogenic NK_1 receptor-mediated inflammatory component in rat skin.
Male Wistar rats (200-300g) were anaesthetised with thiopentone sodium (100 mg/kg i.p.) and body temperature was maintained at 36-38°C. Rats were prepared for either measurement of neutrophil accumulation in response to intradermally-injected agents in rat abdominal skin (Waller *et al.*, 1997) or plasma extravasation after saphenous nerve stimulation by measurement in the rat paw of the extravascular accumulation of i.v. ^{125}I-albumin (Towler *et al.*, 1998).
IL-1β (0.03-3 pmol) induced dose-dependent neutrophil accumulation, measured by assay of myeloperoxidase in skin sites. The NK_1 receptor antagonist SR140333 (240 nmol/kg i.v. + 240nmol/kg s.c.) was used at a high dose, that blocks NK_1-receptor-mediated plasma leakage for 5h (Amann *et al.*, 1995). This antagonist had no effect on neutrophil accumulation induced by IL-1β (see Table 1).
In separate experiments neurogenic plasma exudation was determined where the sensory saphenous nerve is stimulated (10V, 1ms, 2Hz for 5 min) in the anaesthetised rat and plasma accumulation measured for 30 min afterwards. IL-1β pretreatment (3 pmol/site, at t = -4 h into paw skin) had no effect on neurogenic plasma exudation. Results: IL-1β pretreated 21.1±2.2; saline-pretreated 20.6±4.6 (μl/100 mg tissue, mean ± s.e.mean, n=4). Our results suggest that IL-1β induces neutrophil accumulation by an NK_1 receptor-independent mechanism in rat skin. In addition, our results to date suggest that neurogenic oedema formation is not modulated by IL-1β pretreatment.

We cannot explain the reason for the differences between these results obtained in rat skin and those obtained in the mouse air pouch model with respect to neutrophil accumulation. It is possible that the results are due to the different species used. Alternatively, the NK_1-dependent mechanism in the murine air pouch may depend on vasoactive activity or a priming step.

Table 1. Effect of SR140333 pretreatment on neutrophil accumulation induced by IL-1β i.d. Results are expressed as mean ± S.E.M of 10^6 cells/site. n=4.

IL-1β (pmol/site)	vehicle	SR140333
Saline	0.62 ± 0.08	0.7 ± 0.1
0.03	2.29 ± 0.5	2.6 ± 0.15
0.1	3.77 ± 0.5	4.07 ± 0.3
0.3	6.52 ± 1.6	8.98 ± 1.0
3.0	10.55 ± 0.66	11.11 ± 0.95

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148P ANALYSIS OF NEUROKININ-1 (NK_1) RECEPTOR-MEDIATED OEDEMA FORMATION: USE OF NK_1 KNOCKOUT MICE

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The neurokinin-1 (NK_1) receptor has been demonstrated to have an important role in neurogenic inflammation. Here we have investigated the mechanism of NK_1 receptor involvement in oedema formation in mouse dorsal skin of SV 129 + C57BL/6 wildtype and NK_1 receptor knockout mice (Bozic et al, 1996).

Mice (30-35g) were anaesthetized with urethane (70μg/10g bodyweight). Dorsal skin was shaved and 50kBq [^{125}I]-albumin was injected i.v. Agents (50μl) were injected intradermally (i.d.) into randomly selected sites on the dorsal skin and 30 min was allowed for oedema formation, which was determined by [^{125}I]-albumin accumulation into skin sites. All results were expressed as mean±s.e.mean of n animals. Statistical analysis was by ANOVA followed by Bonferroni's or student t test.

Substance P (SP, 30-300pmol), the NK_1 receptor specific agonist, septide (3-30pmol) and the mast cell degranulating agent, compound 48/80 (C48/80, 100-500ng), all produced dose-related oedema formation in wildtype mice (+/+). Septide was 3-10 times more potent than SP in inducing oedema formation. In NK_1 knockout mice (-/-, see Table 1), SP (300pmol) and septide (30pmol) did not produce significant oedema formation compared to vehicle (Tyrodes). C48/80 (500ng) however, induced oedema formation in knockout mice that was significantly larger ($P<0.05$) than that of wildtype mice. Mepyramine, a histamine H_1 receptor selective antagonist ($3mgkg^{-1}$, -15min i.p.) significantly $P<0.05$) reduced septide (30pmol) induced oedema formation in wildtype mice (1.4±0.3μl) compared to vehicle (saline) pretreatment (2.3±0.2μl). Similar results (not shown) were obtained with the NK_1 agonist GR 73632.

Table 1 Comparison of plasma extravasation induced in wildtype (+/+) and knockout (-/-) mice (μl/site) by SP, septide and C48/80 in dorsal skin, n=number of animals. #$P<0.05$, compared to C48/80 in wildtype mice; **$P<0.01$; ***$P<0.001$ compared to Tyrodes in wildtype mice.

	Tyrodes	SP (300pmol)	Septide (30pmol)	C48/80 (500ng)
+/+ n=10	0.7±0.1	2.2±0.5**	2.3±0.3***	1.7±0.3**
-/- n=6	0.4±0.2	0.8±0.2	0.6±0.1	3.0±0.1#

In conclusion results confirm that the NK_1 receptor is involved in mediating tachykinin induced oedema formation. Septide is more potent than SP in inducing formation in the mouse, as previously shown for the rat (Ahluwalia et al., 1995). Furthermore, results suggest an NK_1 receptor-mast cell involvement in that H_1 receptor antagonist inhibited oedema formation induced by NK_1 agonists in the wildtype mouse. Thus it is suggested that NK_1 receptors are present on mast cells in addition to endothelial cells in these mice.

TC is the recipient of a BHF PhD studentship.

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149P DIFFERENTIAL EFFECTS OF UNFRACTIONATED HEPARIN AND POLY-L-GLUTAMIC ACID ON RABBIT CUTANEOUS RESPONSES

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Heparin has been shown to inhibit eosinophil accumulation in guinea-pig skin (Teixeira & Hellewell, 1993) and polycation-induced responses in rabbit skin (Jones *et al*, 1998a). However, when administered intradermally both unfractionated heparin (UH) and the non-sulphated polyanion poly-L-glutamic acid (PGA), inhibited poly-L-lysine (PLL)-induced responses in rabbit skin (Jones *et al*, 1998b). Here, we have investigated the effect of intravenous injection of UH, dextran sulphate (DS) or PGA on plasma exudation (PE) and polymorphonuclear leukocyte (PMN) accumulation in rabbit skin in response to formyl-met-leu-phe (fMLP, 10^{-11}mol site^{-1}), leukotriene B_4 (LTB_4, 10^{-10}mol site^{-1}) (both mixed with PGE_2, 100ng site^{-1}) and PLL (100µg site^{-1}).

Rabbit (NZW) PMNs were separated and labelled with ^{111}Indium (In), washed and injected (10^7 kg^{-1}) into pentobarbitone anaesthetised rabbits (2.5 - 3.0 kg). To measure PE, ^{125}I albumin (0.1 MBq kg^{-1}) was injected intravenously 1 h after the labelled cells. Intravenous injections of UH, DS, PGA or saline (control) were made 10 min prior to 0.1 ml intradermal injections in the shaved dorsal skin of these rabbits. Responses were measured over 45 min. ^{111}In-PMN accumulation in excised skin sites was expressed as cells site^{-1} and PE in the same sites as µl plasma. Mean ± s.e.m. values (n = 4 - 6) were calculated and data analysed using ANOVA. Differences between means were assessed using Student's t-test with Bonferroni correction.

I.v. injection of UH (10 - 5000 i.u. kg^{-1}; ~0.06 - 27.8 mg kg^{-1} had no significant inhibitory effect on any PE or PMN accumulation response. At 10,000 i.u. kg^{-1}; ~55.6 mg kg^{-1}, UH inhibited significantly PE induced by fMLP (from 79.05 ± 12.42 to 43.29 ± 7.28) and LTB_4 (from 68.13 ± 11.17 to 43.73 ± 13.11), and also fMLP- (from 35495 ± 5557 to 14629 ±1663) and LTB_4- (from 31466 ± 2444 to 14156 ± 1536) induced PMN accumulation ($P<0.05$). However, UH had no significant effect on PLL-induced responses. DS at a dose of 25 mg kg^{-1} inhibited significantly PE and PMN accumulation induced by fMLP (from 66.10 ± 7.7 to 39.25 ± 15.15 and 44758 ± 10492 to 16269 ± 5037) and LTB_4 (from 66.82 ± 7.33 to 29.16 ± 10.06 and 44835 ± 11104 to 14059 ± 3898), ($P<0.05$), but had no effect on PLL-induced responses. In addition PGA, also administered at 25 mg kg^{-1} inhibited significantly PMN accumulation induced by fMLP (from 61244 ± 16150 to 22769 ± 4610), LTB_4 (from 56174 ± 13954 to 23514 ± 4675) and PLL (from 10749 ± 2733 to 3284 ± 1064). However, PGA had no effect on the PE response to any of the stimuli.

These results show that, when administered *via* the i.v. route UH and DS inhibit PE and PMN accumulation responses to PMN-dependent mediators, but have no significant effect on responses to the polycation PLL. In contrast, PGA inhibits PMN accumulation induced by all 3 stimuli without significantly affecting PE. Therefore, it appears that PGA may be acting *via* a different mechanism to UH and DS. This may be related to the different anionic properties of these molecules.

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150P PARALLEL REGULATION OF L-ARGININE TRANSPORT AND NO SYNTHESIS, AND mRNA EXPRESSION FOR DIFFERENT CATIONIC AMINO ACID TRANSPORTERS AND INDUCIBLE NO SYNTHASE IN RAT ALVEOLAR MACROPHAGES

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NO synthesis by iNOS in alveolar macrophages (AMΦ) largely depends on the supply of the substrate L-arginine (L-Arg) (Hammermann *et al.*, 1998) which is taken up by the cells via specific cationic amino acid transport systems (Racké *et al.*, 1998). In the present study mechanisms involved in the control of L-Arg transport activity in rat AMΦ were compared with those controlling the expression of iNOS.

Rat AMΦ ($0.5*10^6$ cells per well) were cultured for 20 h in DME/HAM-F12 medium containing 5 % FCS in the absence or presence of 1 µg ml^{-1} lipopolysaccharides (LPS) and/or different test drugs. Thereafter, nitrite accumulated in the culture medium was determined and L-Arg uptake was studied by measuring the cellular radioactivity after 2 min of incubation with ^{3}H-L-Arg (37 kBq, 0.1 µM). In parallel experiments total RNA was isolated after the culture period and the expression of mRNA for iNOS and different cationic amino acid transporters was determined by RT-PCR. Data are given as means (± s.e.mean) of ≥ 6 experiments. Significance of differences was evaluated by ANOVA followed by the Dunnett's modified *t* test.

Control AMΦ accumulated 5.2±0.5 nmol nitrite/10^6 cells/20 h and ^{3}H-L-Arg uptake in control AMΦ amounted to 83 372± 4 356 dpm/mg protein. After exposure to LPS (up to 1 µg/ ml), L-Arg uptake and nitrite accumulation were enhanced by 173± 14 and 825±228 %, respectively (each $P < 0.01$ vs controls). Two different NFκB inhibitors (pyrrolidine dithiocarbamate, PDTC, 60 µM and N^{α}-p-tosyl-L-lysine chloromethyl ketone, TLCK, 100 µM) caused a reduction of L-Arg uptake by 30±8 and 63±5 %, and of nitrite accumulation by 69±5 and 54±8 %, respectively (each $P < 0.05$ vs controls), and prevented the respective stimulatory effects of LPS. The MAP kinase kinase inhibitor PD 098 059 (50 µM) caused a reduction in L-Arg uptake and nitrite accumulation by 39±2 and 41±8%, respectively (each $P < 0.01$ vs controls) and caused a shift to the right of the respective concentration response curves of LPS. Likewise dexamethasone (10 µM) caused a reduction of L-Arg uptake and nitrite accumulation by 41±6 and 65±7 %, respectively (each $P < 0.05$ vs controls)) and largely attenuated the respective stimulatory effects of LPS. Semi-quantitative RT-PCR demonstrated additionally that the expression of mRNA for iNOS and specific cationic amino acid transporters (CAT-1 and CAT-2B) changed in parallel to the functional observations.

In conclusion, the same intracellular signals responsible for the induction of iNOS in rat AMΦ appear also to upregulate the activity of L-Arg transport. The expression of specific cationic amino acid transporters appears to be linked to that of iNOS.

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151P DETECTION OF NITRATED PROTEINS IN PEROXYNITRITE-TREATED RAT SKIN AND EFFECTS OF NITRATED PLASMA PROTEINS ON PLASMA EXTRAVASATION

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Production of nitric oxide and superoxide at inflammatory sites can lead to the generation of peroxynitrite ($ONOO^-$). We have previously shown that $ONOO^-$ increases microvascular permeability in rat skin (Ridger *et al.*, 1997). The nitration of proteins by $ONOO^-$ may have pro-inflammatory consequences. We have investigated the formation and turnover of nitrated protein induced by $ONOO^-$ in rat skin.

Male Wistar rats (220-300 g) were anaesthetised with sodium pentobarbitone (50 mg kg^{-1}, i.p.). The dorsal skin was shaved and agents (0.1 ml $site^{-1}$) injected i.d. Agents were made up in either Tyrode's solution, rat plasma or nitrated plasma (produced by addition of 20 µl of 200 mM $ONOO^-$ to 1 ml plasma). 1-96 h after injection, animals were killed, the dorsal skin removed, sites punched out, frozen and homogenised. An ELISA assay (Khan *et al.*, 1998) was used to quantify protein bound 3-nitrotyrosine (3NT) with values expressed as nitrated BSA equivalents mg ml^{-1} 100 µg $protein^{-1}$). Data (expressed as mean±s.e.mean) was analysed by ANOVA followed by Tukey-Kramer's multiple comparison's test.

$ONOO^-$ (50, 100, 200 nmol $site^{-1}$) induced a dose dependent increase in protein nitration after 4 h (885±311, 1612±227, 2621±317 nitrated BSA equivalents mg ml^{-1} 100 µg $protein^{-1}$ respectively, n=4). Levels of nitrated protein were significantly increased after i.d. $ONOO^-$ (100 nmol, $P<0.05$; 200 nmol, $P<0.01$) compared to pH adjusted vehicle control.

Nitrated protein levels detected 1-24 h after $ONOO^-$ administration were greater ($P<0.001$) than levels found for Tyrode's solution and for decomposed $ONOO^-$ sites (figure 1). The half-life of protein bound 3NT induced by $ONOO^-$ (200 nmol $site^{-1}$) was 5.78 ± 1.43 h (n=4).

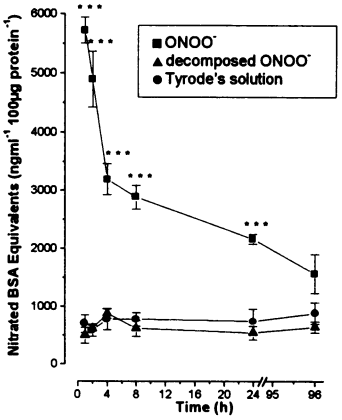

Figure 1 The turnover of protein bound 3NT induced by i.d. $ONOO^-$ (200 nmol $site^{-1}$) over 1-96 h. ***$P<0.001$, n=4.

Plasma and nitrated plasma injected i.d. had no significant effect on microvascular permeability, measured by the extravascular accumulation of ^{125}I-albumin (as Ridger *et al.*, 1997). Results as follows: Tyrode's 8±3, plasma 6±3, nitrated plasma 8±3 (expressed as µl plasma extravasation $site^{-1}$), n=3.

To our knowledge these results are the first to show the turnover of nitrated proteins within a tissue. The data also indicates that nitration of plasma proteins by $ONOO^-$ does not increase plasma exudation.

SG holds an ARC Ph.D. studentship.

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152P NO SYNTHASE INHIBITION BY KETOPROFEN ENANTIOMERS

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Electrical stimulation of the trigeminal ganglion or systemic substance P administration cause neurogenic inflammation (NI) in the rat meninges, which can be assessed as extravasation of [^{125}I]-labelled bovine serum albumin into the rat dura mater. (-)- and (±)-ketoprofen inhibit (NI) while (+)-ketoprofen and racemic acetaminophen have no or little effect, and these data are not explained by the known effects of these drugs on cyclooxygenase (Limmroth *et al.* 1999). Moreover, the NO synthase inhibitor N^G-nitro-L-arginine (L-NA) also inhibits of NI (Limmroth *et al.* 1999). Therefore, we have investigated whether ketoprofen isomers may cause inhibition of NO synthase or its induction.

NO synthase activity was assessed as conversion of [^{3}H]-arginine to [^{3}H]-citrulline as previously described (Ross & Iadecola, 1996). Neuronal NO synthase activity was determined in homogenates of rat cerebellum in the presence of 2 mM Ca^{2+}. Inducible NO synthase was determined in the absence of extracellular Ca^{2+} in homogenates of J774 cells, which had been pretreated with 100 U ml^{-1} interferon-γ and 1 µg ml^{-1} lipopolysaccharide for 16-18 h. To test the ability to inhibit NO synthase induction, pretreatments of J774 cells were also performed in the presence of the indicated test compounds. Non-specific [^{3}H]-citrulline formation was assessed as formation in the presence of 1 mM L-NA, and was subtracted from all values. Data are shown as the mean ± s.e.mean of n experiments. Statistical significance of differences was assessed by one-way analysis of variance.

In cerebellar homogenates (-)-, (+)- and (±)-ketoprofen concentrations up to 0.1 mM did not inhibit NO synthase activity. At higher concentrations inhibition became detectable which was 56 ± 5%, 45 ± 1% and 28 ± 1% for 1 mM of (-)-, (+)- and (±)-ketoprofen, respectively ($n = 4$; $P < 0.01$). In contrast, acetaminophen did not cause any inhibition up to 1 mM ($n = 5$).

A similar picture was obtained in J774 cells: At 1 mM acetaminophen lacked inhibitory effects (1 ± 4%), while (-)-, (+)- and (±)-ketoprofen caused 57 ± 7%, 65 ± 1% and 49 ± 3% inhibition, respectively ($n = 4$; $P < 0.01$).

(-)-, (+)- and (±)-ketoprofen also inhibited the induction of NO synthase activity in J774 cells. Again this was seen only in concentrations of more than 0.1 mM. At 1 mM the inhibition of induction was 60 ± 9%, 72 ± 6% and 62 ± 7% for (-)-, (+)- and (±)-ketoprofen, respectively, while acetaminophen did not cause inhibition ($n = 4$-7; $P < 0.01$).

We conclude that (-)-, (+)- and (±)-keteprofen inhibit neuronal and inducible NO synthase with similar potency, while acetaminophen does not. Similar data were also found for inhibition of NO synthase induction. However, the concentrations required for such inhibition are quite high. These data do not support the hypothesis that inhibition of NO synthase underlies the inhibition of NI by ketoprofen isomers. Based on these an other data (Limmroth *et al.* 1999), we propose that a yet to be discovered mechanism underlies inhibition of NI by ketoprofen.

Limmroth, V. *et al.* (1999) This meeting

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153P INHIBITION OF NEUROGENIC INFLAMMATION BY KETOPROFEN DOES NOT INVOLVE CYCLOOXYGENASE INHIBITION

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Neurogenic inflammation in the meninges has been proposed to reflect important aspects of headache pathophysiology. It develops following release of vasoactive neuropeptides from perivascular trigeminal nerve fibers, characterized by plasma protein extravasation (PE), platelet aggregation and mast cell degranulation It can be inhibited by non-steroidal antiinflammatory drugs such as indomethacin (Buzzi *et al.* 1989), indicating that prostaglandins may be involved. To further investigate this hypothesis, we have studied the effects of (-)-, (+)- and (±)-ketoprofen and racemic acetaminophen and the NO synthase inhibitors N^G-nitro-L-arginine (L-NA) and 7-nitro-indazole (7NI).

PE was studied in phenobarbitone-anaesthetized rats (Limmroth *et al.* 1996). It was induced by unilateral trigeminal stimulation (0.6 mA, 5 ms, 5 min) or substance P administration (1 nmol kg^{-1} i.v.) and determined as accumulation of [^{125}I]-labelled bovine serum albumin in the dura mater (c.p.m. mg^{-1} wet weight). Data are shown as the mean ± s.e.mean (n = 5-6) of the ratio between the stimulated and unstimulated side (electrical stimulation) or as % of vehicle-treated rats (substance P administration). A $P < 0.05$ was considered significant in a one-way analysis of variance.

Electrical stimulation caused a PE ratio of 1.69 ± 0.06. (-)- and (±)-ketoprofen inhibited PE ($P < 0.05$) with full suppression occuring at 3 and 10 mg kg^{-1} (ED_{50} 0.28 and 0.90 mg kg^{1}) respectively, while (+)-ketoprofen was without effect up to 10 mg kg^{-1}. Acetaminophen caused partial inhibition at the highest concentration of 30 mg kg^{-1} (PE ratio 1.45 ± 0.05). Systemic substance P administration caused a PE of 173 ± 9% of vehicle-treated rats. (±)- and (-)-ketoprofen reduced substance P-induced PE to 125 ± 10% and 122 ± 9% ($P < 0.05$), respectively, while (+)-ketoprofen had no effect (177 ± 3%; all tested at 10 mg kg^{-1}).

The NO synthase inhibitor L-NA (1 mg kg^{-1}) inhibited elect rical stimulation-induced PE completely, whereas 7NI, which is selective for the neuronal NO synthase, caused only partial inhibition (PE ratio 1.42 ± 0.0.2 and 1.39 ± 0.03 at 10 and 50 mg kg^{-1}, respectively; $P < 0.05$) .

We conclude that inhibition of electrical stimulation- and substance P-induced PE does not involve suppression of prostaglandin synthesis because (-)- and (±)-ketoprofen inhibited it more potently than (+)-ketoprofen, although ketoprofen is believed to inhibit cyclooxygenase through preferentially its (+)-enantiomer; moreover, acetaminophen also caused only weak inhibition of PE. The fact that ketoprofen inhibited substance P-induced inflammation indicates that the inhibition occurs at the level of the meningeal vasculature. Based on the experiments with L-NA and 7NI, we also conclude that a NO synthase distinct from the neuronal form may be involved in neurogenic inflammation. However, *in vitro* studies (Schubert *et al.* 1999) make it unlikely that NO synthase inhibition underlies the effects of ketoprofen.

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154P DIRECT INTRACELLULAR MEASUREMENT OF PROSTAGLANDIN (PG) E_2 SYNTHESIS IN MOUSE CULTURED FIBROBLAST CELLS: EFFECT OF CYCLOOXYGENASE (COX) INHIBITORS

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Cyclooxygenase converts arachidonic acid to prostaglandins, prostacyclin and thromboxane A_2. COX exists in at least two different isoforms. COX-1 is constitutively expressed, whereas COX-2 is induced by pro-inflammatory stimuli (Mitchell *et al*, 1994). PGE_2 is a major metabolite of COX activation, is a potent mediator of diverse physiological responses and contributes to the pathogenesis of inflammatory, autoimmune and neoplastic diseases (Zeng *et al*, 1995). In order to compare the action of COX inhibitors on PGE_2 synthesis and release, we investigated the response of Swiss 3T3 cells to the calcium ionophore A23187, a known mediator of AA metabolism (Ogata *et al*, 1995), and nonsteroidal anti-inflammatory drugs (NSAIDs).

Mouse 3T3 cells were cultured on 96-well culture plates in Dulbecco's Eagle's medium supplemented with 10% foetal calf serum and 4mM L-glutamine. The effect of NSAIDs (acetylsalicylic acid, ASA and indomethacin, IM) on intracellular PGE_2 generation and secretion was measured with competitive enzymeimmunoassay (Amersham Pharmacia Biotech). Cells were exposed to NSAIDs (1-1000μM) for 24 hours before stimulation with A23187 (1-100μM) for 5 minutes (37°C, 95% humidity, 5% CO_2) or for up to 2 hours in the presence of both A23187 and NSAID. For intracellular measurements, the culture supernatant was gently aspirated, cells thoroughly washed with PBS and lysed with 0.25% (w/v) dodecyltrimethylammonium bromide.

The cell lysis method described here had no effect on antibody: antigen binding. A23187 increased levels of intracellular PGE_2 in a dose-dependent manner. Typically, basal levels of intracellular PGE_2 were 51±2 pg/10^6 cells in the absence of A23187. Intracellular PGE_2 levels significantly increased ($p<0.001$) to 3288±127 pg/10^6 cells in the presence of 100μM A23187. When stimulated in the presence of NSAID, there was a significant reduction ($p<0.01$) in levels of intracellular PGE_2 compared with the control (50μM A23187, 4μM ASA, -71±4 pg/10^6 cells, 74% inhibition; 50μM A23187, 4μM IM, -16±3 pg/10^6 cells, 73% inhibition). Under the same experimental conditions, secretion of PGE_2 was not significantly reduced in the presence of NSAID (ASA -1.5± 0.2pg/10^6 cells,11% inhibition; IM -24±2 pg/10^6 cells, 26% inhibition). Statistical analyses were carried out with Student's t-test. Data are expressed as mean ± S.D. (n=5). Furthermore, in the presence of A23187 and IM, the kinetics of intracellular PGE_2 accumulation and secretion were significantly different (Figure 1).

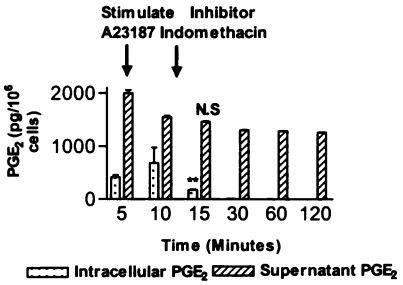

Figure 1. Effect of IM on the kinetics of PGE_2 synthesis and secretion. Cells were stimulated with 50μM A23187 for 5 minutes before addition of 4μM IM. PGE_2 levels were determined using EIA. Measurements were made at intervals over a 2 hour time period. The data are expressed as means ± S.D. (n=5). **p<0.01 versus maximum response, N.S= not significant.

Swiss 3T3 cells contain very low levels of PGE_2 under control conditions, but when stimulated with a calcium ionophore, COX is upregulated and cells contain significant amounts of an AA-metabolite. Intracellular PGE_2 generation was comprehensively inhibited in the presence of low concentrations of NSAID. However, this response was not observed when PGE_2 measurements were carried out on cell culture supernatants. We suggest these findings may aid the understanding of the mechanism of action of COX inhibitors and lead to the discovery new and more specific therapies.

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155P EFFECTS OF COX-1 AND COX-2-SELECTIVE NSAIDs ON PROSTAGLANDIN E_2 AND GM-CSF PRODUCTION BY HUMAN SYNOVIOCYTES

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Non-steroidal anti-inflammatory drugs (NSAIDs) are a diverse group which act through inhibition of the enzyme cyclo-oxygenase (COX). Beneficial effects of these drugs are attributed to inhibition of inducible COX-2 while inhibition of constitutive COX-1 may underlie their side effects, including gastric ulceration (Vane *et al*, 1998). Prostaglandin (PG)E_2, a product of COX activity has been shown to inhibit the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Agro *et al*, 1996). GM-CSF which is elevated in rheumatoid arthritis (RA) (al-Janadi *et al*, 1996) stimulates production, activation, and survival of neutrophils. Neutrophils are strongly associated with joint damage in RA. Here we have examined the effect of a range of NSAIDs on the production of PGE_2 by cytokine-treated synoviocytes, and looked for influences on the production of GMCSF.

Synovium was obtained from patients undergoing routine surgery. Explants of synoviocytes were cultured in DMEM supplemented with 2mM glutamine and 20% foetal calf serum (37°C, 5%CO_2). Explanted synoviocytes, identified by morphology were cultured to confluency. Serum deprived cells were then cultured for 24h with a combination of interleukin-1 (IL-1β), tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6) (all 10ng/ml) in the presence of a range of NSAIDs. PGE_2 and GMCSF production were determined by radioimmunoassay and specific sandwich ELISA respectively.

All NSAIDs tested and the selective COX-2 inhibitor DFP (Merck-Frosst, Canada, Gottesdiener *et al* 1998) reduced PGE_2 and increased GMCSF in concentration-dependent manners.

Drug (n=6-9)	PGE_2 IC_{50} nM (95% CI)	GMCSF EC_{50} nM (95% CI)
Indomethacin	3.8 (1.9 – 6.4)	8.3 (5.4 – 23405)
Diclofenac	0.3 (0.2 – 0.7)	490 (145 – 28230)
Meloxicam	15 (0.7 – 21)	3.9 (0.04 – 182)
DFP	39 (16 – 83)	230 (23 – 1377)
Sodium salicylate	64,000 (752 – 117,300)	

Table 1: Effect of NSAIDs on PGE_2 and GMCSF release from human synoviocytes. 95% confidence intervals (95% CI) calculated using the Wilcoxon method.

Our results show cytokine induced PGE_2 production by human synoviocytes to be inhibited by a range of NSAIDs at concentrations consistent with inhibition of COX-2. Interestingly, our results also suggest that the production of GMCSF within synoviocytes is negatively regulated by COX-2 activity. This may partly explain why NSAIDs have little disease modifying activity in rheumatoid arthritis.

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156P SELECTIVITIES OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AS INHIBITORS OF PURIFIED OVINE COX-1 AND COX-2: EFFECTS OF HUMAN PLASMA

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Since the discovery of an inducible isoform of cyclo-oxygenase (COX-2) and the demonstration of its relevance in inflammation, many test systems have been developed to analyse the selectivities of non-steroidal anti-inflammatory drugs (NSAIDs) as inhibitors of COX-1 and -2. Here we report a fast and simple assay using purified ovine COX-1 and -2 in the presence of human plasma with which to study these selectivities.

Purified COX-1 and COX-2 were reconstituted to 10 U/ml in Tris buffer (50 mM, pH 7.5) containing adrenaline (5 mM) and hematin (1 μM) (Mitchell *et al.*, 1997) in the absence or presence of 3 % human plasma. The enzyme solutions were incubated for 30 min (37 °C) with either vehicle (0.1 % DMSO in Tris buffer) or one of the following drugs: aspirin (0.01 μM - 1 mM), ibuprofen (0.03 μM - 0.03 mM), indomethacin (0.1 pM - 0.1 mM), L-745,337 (0.1 μM - 0.1 mM), rofecoxib (0.01 μM - 0.1 mM), celecoxib (0.1 μM - 0.1 mM). Arachidonic acid (AA; 10 or 20 μM) was then added and after 15 min the reaction was stopped by addition (10 % v/v) of HCl 1M. The pH was then restored to 7.5 with 1 M NaOH. Prostaglandin E_2 levels were determined by RIA as a measure of COX activity.

In the absence of human plasma only aspirin and inodomethacin, of the drugs tested, inhibited COX-1 and -2 with IC_{50} values of (μM) 2.84 (1.3-5.8; 95% C.L.) and 3.3 (2-5.3), and, 2.14 (1.1-4.1) and 0.53 (0.2-1.5), respectively. In the presence of human plasma all the drugs tested inhibited COX-1 and COX-2 (Table 1). The traditional NSAIDs, aspirin, ibuprofen and indomethacin showed selectivity for COX-1 over COX-2. The newly developed compounds, L-745,337 (Chan *et al.*, 1955), rofecoxib and celecoxib, selectively inhibited COX-2. Celecoxib was the least COX-2-selective in this system and rofecoxib the most selective.

The test system we report here can provide rapid and simple assessments of the relative selectivities of NSAIDs. Clearly this could prove useful in screening novel compounds, especially if adapted to use human, purified COX-1 and COX-2 enzymes.

The "enabling" role played by the human plasma is particularly interesting. Obviously it may well be that plasma proteins bind the test compounds, the COX enzymes, the co-factors and/or the substrate, although it is unclear what effects such binding could cause. Clearly further experiments are required for this effect to be understood fully.

Table 1. Effect of NSAIDs on purified ovine COX-1 and COX-2

NSAIDs (n≥3)	IC_{50} (μM) (95% C.L.) COX-1	COX-2	COX-2/-1
Aspirin	1.65 (1.3-2.1)	5.34 (4.1-7.3)	3.24
Celecoxib	4.5 (3.7-5.7)	1.42 (1.2-1.6)	0.32
Ibuprofen	2.28 (1.8-2.9)	14.36 (9.1-23.8)	6.28
Indomethacin	0.0045 (0.001-0.01)	0.023 (0.01-0.04)	5.15
L-745.337	> 100	14.48 (6.9-31.9)	0.014
rofecoxib	> 100	1.25 (0.5-2.7)	0.003

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157P EXTRACELLULAR ATP ENHANCES RESPONSIVENESS OF RAT ALVEOLAR MACROPHAGES TO LIPOPOLYSACCHARIDES (LPS) TO INDUCE NITRIC OXIDE SYNTHESIS

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There is increasing evidence that purinergic mechanisms play a significant role in the control of inflammatory reactions (e.g. Di Virgilio et al., 1996). Studies on murine macrophages cell lines indicated that macrophages might be endowed with ionotropic receptors for ATP. In the present study it was tested whether extracellular ATP might affect NO synthesis in rat alveolar macrophages (AMΦ) which is mediated by the inducible form of nitric oxide synthase (iNOS) (Hey et al., 1995).

Rat AMΦ ($0.5*10^6$ cells per well) were cultured in DME/HAM-F12 medium containing 5 % FCS. After 20 h, the culture medium was renewed and the cells cultured for additional 10 h in the absence or presence of LPS and/or ATP. Thereafter, nitrite accumulated in the culture medium was determined. Data are given as means (± s.e.mean) of ≥ 6 experiments.

Control AMΦ accumulated 4.8 ± 1.8 nmol nitrite $(10^6 \text{ cells})^{-1}$ 10 h^{-1}. As shown in Fig. 1, addition of ATP (0.03 - 1 mM) to the culture medium increased nitrite accumulation by about 25-30 %. Addition of LPS (1 or 100 ng ml^{-1}) increased nitrite accumulation by 45±11 and 872±25%, respectively. However, presence of extracellular ATP (0.03 - 1 mM) enhanced markedly the stimulatory effect of the threshold concentration (1 ng ml^{-1}) of LPS (Fig. 1), but had no effect on the maximal effective concentration (100 ng ml^{-1}) of LPS (data not shown).

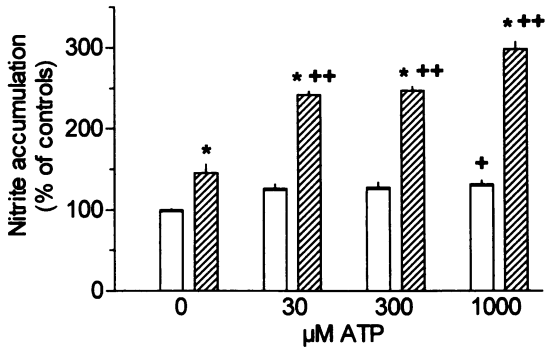

Fig. 1: Concentration-dependent effects of ATP on nitrite accumulation (expressed as % of controls, i.e. absence of LPS and ATP, of the respective cell preparation) by rat AMΦ cultured for 10 h in the absence (open columns) or presence (shaded colums) of 1 ng ml^{-1} LPS. Significance of differences from respective value in the absence of LPS, * $P < 0.001$; or absence of ATP, + $P < 0.05$; ++ $P < 0.001$

In conclusion, extracellular ATP alone has only minor effects on NO synthesis by rat AMΦ, but enhances the sensitivitiy to threshold concentrations of LPS to induce NO synthesis.

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158P THE EFFECT OF ENDOTHELIN ANTAGONISTS ON ENDOTHELIN-1 AND LIPOPOLYSACCHARIDE-INDUCED MICROVASCULAR LEAKAGE IN RAT AIRWAYS

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The leakage of plasma proteins from the microvasculature into airways tissue and the airway lumen is seen as an important factor in the development of inflammatory diseases of the lung. Endothelin-1 (ET-1) is a potent vasoactive agent whose effects are primarily mediated by two receptor subtypes, the ET_A and ET_B receptor. ET-1 has been shown to increase vascular permeability in the bronchi and intra-pulmonary airways in rats (Filep et al, 1991). Bacterial lipopolysaccharide (LPS) has been shown to cause the release of ET-1 in rats (Morise, 1994) and to elicit a reduction in plasma volume in rats, an effect which was inhibited by the ET_A receptor antagonist, FR 139317 (Allcock and Warner, 1997). In this study we have examined the effects of a selective ET_A receptor antagonist, PD 156707 (Reynolds et al, 1995), a mixed ET_A/ET_B receptor antagonist, bosentan (Clozel et al, 1994), and a selective ET_B receptor antagonist, BQ 788 (Ishikawa et al, 1994), on ET-1 and LPS-induced microvascular leakage (MVL) in rat airways.

Male Wistar rats (250-350g) received PD 156707 (0.001-30 mg kg^{-1}), bosentan (0.001-30 mg kg^{-1}), BQ 788 (0.01-3 mg kg^{-1}) or relevant vehicle i.v. 30 min prior to ET-1 (1 nmol kg^{-1} i.v.) or LPS (30 mg kg^{-1} i.v.). Animals received Evans blue dye (20 mg kg^{-1} i.v) 1 min prior to ET-1 or LPS. All i.v. administrations were made via the tail vein under brief isofluorane anaesthesia. The vehicles used were saline (0.9%) for PD 156707, distilled water for bosentan and 3% pluronic F-68 in 5% sodium hydrogen carbonate for BQ 788. The timings and doses used for ET-1 and LPS administration were determined in preliminary studies. Animals were euthanised 10 min after ET-1 or 15 min after LPS administration. The lungs were perfused with saline until free of blood, dissected free and the parenchyma scraped from the intra-pulmonary airways (IPA). The IPA and bronchi were placed in formamide at 37°C for 16h to extract Evans blue dye. The absorbances of the resulting extracts were determined against standard concentrations of Evans blue at 620nm. Results are expressed as concentration of Evans Blue (ng mg^{-1} of tissue) after leakage at time 0 had been subtracted. Statistical analysis was by one way analysis of variance with a correction for multiple comparisons using Dunnett's critical values.

This study shows that ET-1 and LPS both cause MVL into rat airways (4.96 increased to 8.96 and 2.62 increased to 34.91 ng mg^{-1} of tissue, $p < 0.05$ and $p < 0.001$ respectively) and that the ET_A antagonist, PD 156707, and the mixed ET_A/ET_B antagonist, bosentan, significantly inhibit ET-1-induced MVL whereas the ET_B receptor antagonist, BQ 788, at a dose greater than that shown to abolish the ET_B receptor mediated depressor response to ET-1 in rats (Ishikawa, 1994), significantly potentiated the response (See Table 1). This data suggests that ET-1-induced MVL into airway tissue is ET_A receptor mediated. When plasma leakage was induced by LPS, PD 156707 and bosentan significantly inhibited the response whereas BQ 788 was without significant effect (See Table 1). This data suggests that in part LPS-induced plasma leakage into airway tissue is caused by ET-1 and further suggests that this effect is ET_A receptor mediated.

Table 1 The effect of endothelin antagonists on ET-1 and LPS-induced microvascular leakage in rat airways

		ET-1 induced		LPS-induced	
Drug	Dose mg kg^{-1}	Vehicle treated	Drug treated (% change)	Vehicle treated	Drug treated (% change)
PD156707	10	-	-	34.9±4.7	18.2±2.8 (-48)*
	30	8.96±0.87	5.05±1.45 (-44)*	34.9±4.7	19.02±3.2 (-46)*
Bosentan	30	8.96±0.87	1.36±1.25 (-85)***	34.5±3.3	14.27±0.99 (-59)***
BQ788	3	7.5±1.07	14.56±2.83 (+94)**	28.7±3.0	23.29±2.32 (-15)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 4 - 6$.

LPS has been implicated in the pathophysiology of inflammatory diseases of the airways such as the Adult Respiratory Distress Syndrome (ARDS). The early phase of ARDS is characterised by MVL and oedema (Meduri, 1996). Plasma levels of ET-1 are raised in ARDS (Langleben, 1993). In these studies we have demonstrated a causal role for ET-1 in MVL and shown that it is responsible in part, for the leakage caused by LPS. We have demonstrated that this effect may be mediated via ET_A receptors. This suggests that an ET_A or a mixed ET_A/ET_B receptor antagonist may be of use in the treatment of the exudative phase of inflammatory airway diseases such as ARDS.

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159P NON-SPECIFIC BRONCHIAL HYPERRESPONSIVENESS, EARLY AND LATE ASTHMATIC RESPONSES AND CELL INFLUX IN A CONSCIOUS GUINEA-PIG MODEL OF ASTHMA

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Allergen inhalation in atopic asthmatics may result in early and late phase bronchoconstrictions, airway hyperresponsiveness (AHR) and airway leukocyte infiltration. Our previous studies have shown these 4 features of asthma in a conscious guinea pig model after challenge with a high dose of ovalbumin (Danahay & Broadley, 1997). However, two limitations of this model were a requirement for mepyramine to protect against fatal anaphylaxis, and AHR was not observed with all spasmogens (Lewis & Broadley, 1995). In this study, we expose sensitized guinea pigs to a *low* dose of antigen and determine airway responsiveness to a range of spasmogens.

Male Dunkin-Hartley guinea pigs (250-300g) were sensitized to ovalbumin (OA) (1ml i.p. of a suspension containing 10µg OA and 100mg $Al(OH)_3$ in normal saline). All procedures were started 14-21 days later. Airway function was recorded at regular intervals for 10 hours and at 24 hours after an inhalation of OA (100µg/ml for one hour) in conscious guinea pigs by whole body plethysmography and specific airway conductance (sGaw) measured (Danahay and Broadley, 1997). Each group (n=6) was exposed to: histamine 1mM, methacholine 1mM, 5-hydroxytryptamine (5-HT) 0.3mM or bradykinin 0.5mM by nose-only inhalation for 20s both 24h before and after inhalation of OA. Airway function was measured at intervals for 10 minutes. Control groups (n=6) received spasmogen 24 hours before and after normal saline exposure. Bronchoalveolar lavage was performed to determine airway cellular infiltration approximately 24h after antigen or saline exposure. All results were compared using Student's paired t-tests and shown as mean ± s.e.m.

Exposure to OA typically produced an immediate early phase bronchoconstriction seen as 55.6±13.1% reduction in sGaw. This resolved to baseline by 6 hours and was followed by a late phase bronchoconstriction (26.4±4.2% fall in sGaw) between 6 and 10 hours. Saline exposure produced no significant reduction in sGaw. Histamine, methacholine and 5-HT before saline or antigen challenge produced no significant bronchoconstriction. But, 24 hours after antigen exposures, they produced significant ($P<0.05$) bronchoconstrictions of 25.1±6.4%, 34.1±9.8% and 25.5±3.2% reductions in sGaw, respectively. No significant changes in the responses occurred 24 hours after saline exposure. Bradykinin 0.5mM produced bronchoconstrictions before antigen 7.6±4.3% or saline 20.5±4.5% and 24 hours after antigen produced a significantly larger bronchoconstriction (25.0±6.9%, $P<0.05$) not seen in the saline treated group (18.6±14.6%). OA produced a significant mean increase in total cell counts, macrophages and eosinophils when the spasmogens were histamine, methacholine and 5-HT ($5.0x10^6±0.4$, $3.0x10^6±0.2$ and $1.9x10^6±0.2ml^{-1}$), compared to the saline controls ($1.8x10^6±0.3$, $1.4x10^6±0.2$ and $0.2x10^6±0.04ml^{-1}$) respectively ($P<0.05$). With bradykinin as the spasmogen there was a further increase in total cell count and eosinophils after OA ($6.4x10^6±0.3$, and $2.8x10^6±0.2ml^{-1}$) compared to the other antigen treated groups and the corresponding saline control ($2.5x10^6±0.1$ and $0.5x10^6±0.03ml^{-1}$).

These results indicate that airway hyperresponsiveness in this guinea pig model is not spasmogen-specific. The increased cellular infiltration suggests a relationship between airway inflammation and AHR after antigen provocation. The increase in cell infiltration after bradykinin agrees with the observations by Rogers *et al.*, (1990).

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160P THE TIME COURSE OF SEPHADEX-INDUCED RAT LUNG INFLAMMATION AND A COMPARISON OF THE ACTIVITY OF VARIOUS ANTI-INFLAMMATORY DRUGS

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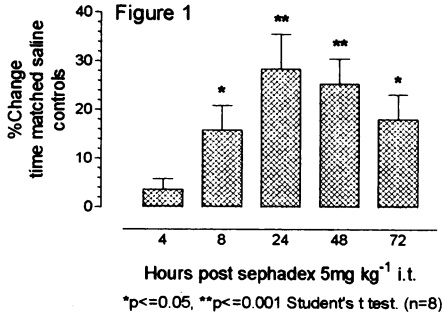
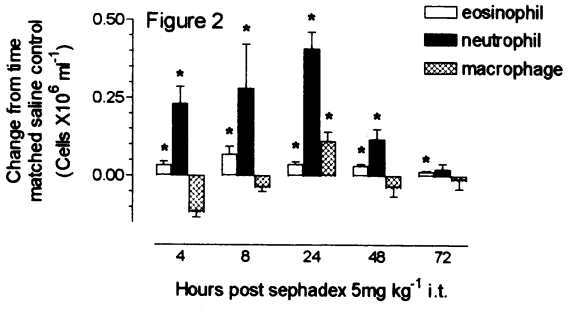
An important feature of the inflammatory response is the induction of permeability changes, leakage of plasma exudate into the interstitium and resultant oedema characterised by the presence of inflammatory cells such as eosinophils, neutrophils and macrophages which contribute to these changes. Sephadex beads are thought to provoke inflammation by acting as a non-specific trigger composed of crosslinked dextran to which Sprague-Dawley rats also have an endogenous hypersensitivity (Morrison *et al*, 1951). Following intra-tracheal instillation of Sephadex pathological changes occur leading to peribronchial and interstitial oedema which can be quantified simply by a gain in wet weight (Kallstrom *et al*, 1985: Cotgreave *et al*, 1988).
We have investigated the time course of oedema and inflammatory cell influx in BAL, and compared the inhibitory effect of Ebselen (a novel seleno-organic compound) with standard glucocorticoids (GCs).
Following instillation of swollen G200 sephadex (5mg kg^{-1}) to male Sprague-Dawley rats (300g) under halothane anaesthesia there was a time dependent increase in wet lung weight and inflammatory cell infiltration (see Figures 1 & 2).





GCs were administered 24 and 2 hours (p.o) before, and Ebselen 0, 4 and 12 hours (i.p) after sephadex (n=8 per dose group). Oedema was determined 24 hours post sephadex and the dose which gave a 50% inhibition of the maximum (ED_{50}) calculated.
All compounds inhibited lung oedema with the following rank order of potency, see table.

Drug	ED_{50} (mg kg^{-1})
Triamcinolone	0.19
Budesonide	2.31
Ebselen	4.64
Prednisolone	20.99

In conclusion, this model provides a robust test in which to evaluate potential therapies targeted at lung inflammation. Furthermore, the inhibitory activity of Ebselen was comparable with currently used GC therapies in this model.

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161P PHOSPHODIESTERASE ISOENZYME PROFILES IN NEUTROPHILS FROM ASYMPTOMATIC COPD AND NORMAL HORSES

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Equine chronic obstructive pulmonary disease (COPD) is an allergic disease of horses characterised by bronchoconstriction and neutrophil recruitment to the lungs (Robinson et al., 1996). Inhibition of cyclic nucleotide phosphodiesterase (PDE) may reduce both bronchoconstriction and inflammatory cell activation in allergic airway disease (Spina et al., 1998). There are seven diverse PDE families and tissue localisation of the PDE isoenzymes differs (Torphy, 1998).

Initially inhibitors selective for PDEIII, III/IV, IV and V were used at a single, high (10^{-5}M) concentration to identify the isoenzymes present in neutrophils from 5 asymptomatic COPD horses and 5 healthy controls. Concentration response curves were then constructed for the PDEIV and PDEIII/IV isoenzyme inhibitors, as well as the non-selective inhibitor, theophylline.

cAMP and cGMP hydrolysing PDE activity in equine neutrophils was measured by calculating the rate of conversion of [^{3}H]cAMP to [^{3}H]adenosine and [^{3}H]cGMP to [^{3}H]guanosine, respectively, using Dowex resin chromatography.

The total cAMP PDE activity was similar in neutrophils from asymptomatic COPD and normal horses (146.0 ± 10.2 vs 156.2 ± 7.1 pmol/min/mg, respectively; mean ± sem). Addition of a PDEIV (CDP840) or a mixed PDEIII/IV (zardaverine) inhibitor caused a significant reduction in enzyme activity in both groups of horses ($p<0.01$; ANOVA followed by Dunnett's test). The PDEIII inhibitor, siguazodan, caused a smaller, although significant ($p<0.05$), reduction in PDE activity, but only in the COPD group. The total amount of cGMP PDE activity in equine neutrophils was very low (5.5 ± 0.6 and 6.8 ± 0.6 pmol/min/mg for asymptomatic COPD and normal horses, respectively; mean ± sem) and was significantly reduced ($p<0.01$) by zaprinast only in the COPD group.

CDP840, zardaverine and theophylline caused concentration dependent inhibition of neutrophil PDE activity (Table 1).

Table 1: IC_{50} values for inhibition of PDE activity in neutrophils from asymptomatic COPD and normal horses

Horses (n=5)	Inhibitor	IC_{50} (mean ± sem)
Normal	CDP840	8.8×10^{-9} ± 0.1M
	Zardaverine	8.2×10^{-7} ± 0.3M
	Theophylline	6.8×10^{-3} ± 0.4M
Asymptomatic COPD	CDP840	7.3×10^{-9} ± 0.2M
	Zardaverine	8.6×10^{-7} ± 0.9M
	Theophylline	5.6×10^{-3} ± 0.2M

This study shows that there is no difference in total PDE activity in neutrophils from asymptomatic COPD and normal horses. PDE IV appears to be the main isoenzyme in equine neutrophils and PDE activity is inhibited with equal potency in neutrophils from asymptomatic COPD and normal horses.

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162P HYPOCHLOROUS ACID-INDUCED MODIFICATION OF DNA IN HUMAN RESPIRATORY TRACT EPITHELIAL CELLS: POTENTIATION OF OXIDATION AND CHLORINATION REACTIONS BY NITRITE

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Hypochlorous acid (HOCl) is generated at sites of inflammation and plays an important role in the immune defence against micro-organisms. However, it also possesses a variety of deleterious effects, which can lead to aggravation of tissue injury. Nitrite (NO_2^-), a major end product of nitric oxide ($NO^\bullet$) oxidation has been found to react with HOCl (Eiserich, *et al.*, 1997), resulting in the formation of nitryl chloride (NO_2Cl), a putative nitrating and chlorinating agent. In the present study we examined the effect of HOCl alone, and in combination with NO_2^-, upon DNA contained within human respiratory tract epithelial cells.

The human bronchial epithelial cell line (HBE1) was exposed to sodium nitrite (0.005-1.0 mM; 0.5-5 hr), to HOCl (0.1-1.0 mM; 10 min) or to a dual exposure of both species (NO_2^-: 1.0 mM; 2 hr followed by HOCl 100 μM; 10 min). Following exposure, cells were washed thoroughly and lysed to enable DNA extraction. DNA isolation from cells, hydrolysis and derivatization of bases and analysis by GC-MS were carried out as previously described (Spencer, *et al.*, 1996; Jenner *et al.*, 1998). DNA strand breaks were detected by exposing cell lysates to alkaline pH and monitoring the rate of strand unwinding using the fluorescent dye ethidium bromide, which binds selectively to double-stranded DNA. Cell viability was monitored using the MTT assay. Statistical analysis was by one-way ANOVA with post-hoc Tukey-Kramer test.

No decrease in cell viability was observed at any exposure condition. Exposure to NO_2^-, at physiological pH, resulted in significant DNA strand breakage and concentration-dependent increases in the levels of hypoxanthine and xanthine, the deamination products of adenine and guanine respectively, but no increases in any oxidised base modification products. By contrast, exposure to increasing concentrations of HOCl resulted in increases in several DNA base modification products, in particular oxidized pyrimidines, and a greater percentage of strand breakage. Dual exposure led to the generation of significantly higher levels of oxidised pyrimidines and 5-Cl-uracil than seen after exposure to HOCl alone (Table 1). However, there was no corresponding rise in DNA strand breaks.

Modified base product	Baseline	NO_2^- (1mM; 2hr)	HOCl (0.1mM)	Dual Exposure
5-Cl-Uracil	0.01 ± 0.00	0.01 ± 0.00	0.09 ± 0.01*	0.13 ± 0.01#
Thymine Glycol	0.67 ± 0.04	0.73 ± 0.06	2.89 ± 0.19*	5.08 ± 0.32#
Hypoxanthine	0.13 ± 0.01	3.59 ± 0.34*	0.25 ± 0.01*	1.96 ± 0.08#
Xanthine	0.24 ± 0.02	3.82 ± 0.31*	0.97 ± 0.06*	2.31 ± 0.11#

Table 1: Levels of modified base products (nmol/mg DNA). Values are mean ± S.D.: n=8). * indicates significant difference over baseline ($p < 0.001$); # indicates significant difference over HOCl exposed cells ($p < 0.001$).

The data indicate that both NO_2^- and HOCl, species relevant to lung disease, can cause significant damage to cellular DNA. Each species produces a unique pattern of damage which may be used to identify damage by this species *in vivo*. In addition, exposure of cells to HOCl in the presence of NO_2^- potentiates HOCl mediated DNA damage, presumably by the formation of nitryl chloride.

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163P EFFECT OF PDE3 AND PDE4 INHIBITORS ON PROLIFERATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM HEALTHY AND ASTHMATIC DONORS

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We have previously shown that PDE3 activity is elevated while PDE4 activity is reduced in monocytes from asthmatic subjects compared to healthy subjects (Landells et al., 1997). In contrast, lipopolysaccharide-induced TNFα release by monocytes from healthy and asthmatic subjects was inhibited by the PDE4 selective inhibitor rolipram, but not the PDE3 selective inhibitor siguazodan (Landells et al., 1998).

To evaluate the relative contribution of PDE3 and PDE4 on cell function, we have investigated the combined effect of rolipram and siguazodan on proliferation of human peripheral blood mononuclear cells (HPBMC) using cells from healthy and asthmatic subjects. Healthy subjects had no history of atopic disease or asthma. Asthmatic subjects were taking β-agonist therapy only. HPBMC were isolated from peripheral venous blood by density gradient centrifugation. Proliferation was induced by phytohaemagluttinin (2μg/ml) and assessed by [3H]-thymidine incorporation (0.1μCi/well), added 24hr prior to cell harvesting at 72hr.

HPBMC proliferation was significantly inhibited at 10μM by rolipram ($P < 0.05$) and the combination of rolipram and siguazodan ($P < 0.05$) but not by siguazodan ($P > 0.05$) in the healthy and asthmatic groups (see Table 1). The combination of rolipram and siguazodan produced a significantly greater inhibition of proliferation than rolipram alone ($P < 0.05$). There was no difference between healthy and asthmatic groups in the response to any of the PDE inhibitors used.

Table 1. Effect of PDE inhibitors on HPBMC proliferation.

Drug	Healthy	Asthmatic
Rolipram	63.5 ± 8.1	59.6 ± 9.0
Siguazodan	96.8 ± 3.6	84.7 ± 4.3
Rolipram plus Siguazodan	45.2 ± 11.1	39.0 ± 6.9

(All values mean % control (± s.e. mean) at 10μM; n=5)

These results indicate that PDE3 may have a contributory role in the control of inflammatory cell function which has implications for the development of selective PDE inhibitors for the treatment of asthma.

We are grateful to Rhone-Poulenc Rorer for the support of L.J. Landells.

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164P INHIBITION OF VINCRISTINE UPTAKE INTO P-GLYCOPROTEIN-CONTAINING VESICLES BY THE RESISTANCE MODIFIER, XR9051

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Multidrug resistance (MDR) is a major obstacle to effective anticancer chemotherapy. One important mechanism is decreased accumulation of cytotoxic drugs in the tumour cells due to drug efflux mediated by P-glycoprotein (Pgp). Agents (resistance modifiers) that inhibit this process may be of great clinical benefit. Unfortunately many of them, e.g. verapamil, have other undesirable pharmacological effects so new agents are needed. XR9051, a novel derivative of a diketopiperazine, reverses Pgp-mediated resistance to various cytotoxic drugs at submicromolar concentrations but has no effect in assays for resistance mediated by the multidrug resistance associated protein (Dale et al, 1998). A convenient *in vitro* means for analysing interaction of resistance modifiers with Pgp-mediated transport is provided by following drug accumulation into inside-out membrane vesicles prepared from the plasma membranes of Pgp-expressing resistant cells (Horio et al, 1988; Neo et al, 1997). Here we report the inhibition of the ATP-dependent and ATP-independent uptake of tritiated vincristine by cold vincristine and by XR9051.

Vesicles were prepared from drug-sensitive (H69) and drug resistant cells (H69/LX4) using the sucrose gradient method of Ishikawa (1988) as described by Neo (1997). The ATP assay medium contained 250mM sucrose, 10mM TRIS-HCl (pH7.4), 10mM $MgCl_2$, 1mM ATP, 10mM phosphocreatine, 100μgml^{-1} creatine kinase, 100nM ^{3}H-vincristine and any drug being tested. For ATP-free conditions, ATP and components of the regenerating system were replaced by 10mM NaCl. Vesicles were added to start the assay and a sample taken at 6min into ice cold stop solution (250mM sucrose/10mM TRIS-HCl (pH 7.4) and filtered immediately through nitrocellulose filters (Whatman, 0.2μm) presoaked in polyethylenimine (3%v/v). Preliminary experiments demonstrated rapid uptake, complete within six minutes. Counts were corrected for background and converted to pmoles of vincristine using standards. For each drug concentration, triplicate determinations were made for three different vesicle preparations. ATP-dependent uptake was calculated as the difference in the uptakes in the presence and absence of ATP with inhibitor present in both determinations.

In the absence of inhibitor, the vesicles showed approximately 20pmoles vincristine (mg protein)$^{-1}$ ATP-dependent uptake and both ATP-dependent and independent uptake decreased with increasing concentrations of the inhibitor. The ATP-sensitive uptakes were fitted with a hyperbolic inhibition function (non-linear least squares unweighted) with maximum uptake and K_D of 16±2 mole (mg protein)$^{-1}$ and 1.5±0.7 μM (mean±s.e.m., n=3) for cold vincristine and 18±3 pmole (mg protein)$^{-1}$ and 0.20±0.09μM for XR9051. These results confirm that XR9051 is a potent inhibitor of P-glycoprotein-mediated vincristine transport in vesicles, its affinity comparing favourably with the 3μM reported for verapamil in a similar assay (Horio et al, 1988).

We would like to thank Mrs. Wendy Gibson for her excellent assistance.

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165P PHYSIOLOGICAL MICROASSAY OF TISSUE GLUTATHIONE STATUS *IN VITRO*

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The tripeptide glutathione (GSH; γ-glutamylcysteinylglycine) is an abundant tissue thiol which plays a major role in thiol maintenance, detoxification and antioxidant defence (Meister, 1991). We have developed a rapid platereader-based microassay at 37 °C and pH 7.4 which may be employed to determine either GSH and the homodimer oxidation product, GSH disulfide (GSSG) or total GSH, i.e. GSH+0.5 GSSG. We then applied this assay to study the acute effect of a GSH-depleting agent, L-buthionine-(*SR*)-sulfoximine (BSO) (Ito *et al.*, 1997) on tissue GSH status in rats.

Male Sprague-Dawley rats (320-350 g) received either normal saline or BSO (220 mg kg^{-1} i.p.) and were exanguinated 5 h later following anaesthesia with halothane. Liver samples were homogenised in 5 % w v^{-1} 5-sulphosalicylic acid (SSA, 5 ml g^{-1} tissue) + EDTA (0.25 mM), centrifuged and the supernatant analysed. Plasma derived from blood collected in EDTA, was added to 10 % w v^{-1} ice-cold SSA (2:1) + EDTA (0.5 mM), centrifuged and the supernatant analysed. For the measurement of GSSG, thiols were first derivatised (1 h) with 2-vinylpyridine (2-VP) in the presence of triethanolamine (TEA) (2 µl neat 2-VP and 6 µl neat TEA per 100 µl sample) (Griffith, 1980). GSH was assessed photometrically in a platereader at 37 °C in a GSH reductase / 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) recirculating assay essentially as described by Tietze (1969). The 100 µl assay well comprised (final concentration): 70 µl DTNB (0.6 mM); 10 µl ß-NADPH (0.5 mM); 10 µl sample; 10 µl GSH reductase (2.5 U ml^{-1}). Reagents were dissolved in sodium phoshate buffer (125 mM, pH 7.4) containing EDTA (6.3 mM). The recirculating assay was initiated after a 10 min incubation at 37 °C by the addition of GSH reductase and the initial rate determined from the absorbance increase measured every 10s at 405 nm over 1 min. The content of hepatic GSH or GSSG is expressed as µmol g^{-1} dry weight. Data are mean±s.e. mean.

Initial reaction rate was linear with GSH standards (Figure 1). Total GSH in liver from control rats was 17.9±2.1 µmol g^{-1} (n=6). This was reduced by approximately 40 % to 10.5±0.8 µmol g^{-1} by BSO treatment (n=5) (P<0.02). Plasma total GSH was similarly reduced from 15.6±0.6 µM in control animals (n=6) to 9.3±1.0 µM (n=5) in BSO-treated animals (P<0.01). The change in hepatic total GSH was associated with a fall in GSH from 17.7±2.1 µmol g^{-1} in control animals to 10.3±0.8 µmol g^{-1} in BSO-treated animals (n=5-6, P<0.02) while hepatic GSSG was unchanged (control: 0.10±0.02 µmol g^{-1}; BSO: 0.08±0.02 µmol g^{-1} (P>0.05)).

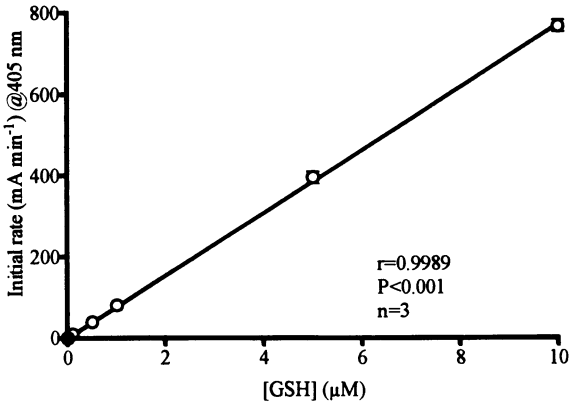

Figure 1. Standard curve for GSH determination

In conclusion, this microassay allows the sensitive determination of both plasma and tissue GSH status under physiologically relevant conditions with both economy of time and materials.

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166P EFFECT OF PROCESS PARAMETERS ON THE DOPAMINE AND LIPID PEROXIDATION:POTENTIAL OF WELDING FUMES AS MARKERS FOR NEUROTOXICITY

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There is growing concern over the neurotoxic effects of chronic occupational exposure to fumes generated by welding processes, containing manganese and iron compounds. Elevated iron levels in the brain have been linked to increased lipid peroxidation, dopamine depletion and predisposition to the development of Parkinson's disease. Although it is established that manganese produces the related syndrome of manganism, a recent study suggested that low levels of manganese ingestion might actually be neuroprotective, inhibiting oxidative processes catalysed by iron [1]. This creates a dilemma for toxicological evaluators seeking to recommend welding processes which reduce the risk of neurotoxicity. In this study, we have used welding fume samples, generated by model processes, to evaluate their potential to release solutes which promote oxidation of dopamine and peroxidation of brain lipids in cell free assays.

Fume samples were generated by metal inert gas welding of mild steel plates in a conical welding chamber. The particulates generated were collected onto a 0.15m diameter glass fibre filter. The study compared samples from processes using solid and flux cored wires, normal and reverse polarity systems, and the influence of using CO_2 or argon as the inert gas shield. Samples were suspended in Dulbecco's minimal essential medium without phenol red at a concentration of 10 mgml $^{-1}$. Suspensions were shaken at 37°C for 2 hours Samples were centrifuged at 10 000g for 15 minutes and the supernatant removed for assay. For dopamine oxidation, dopamine was added to the sample at a final concentration of 10 mgml $^{-1}$, and oxidation of a 200 µl aliquot measured as an increase in absorbance at 550 nm after 10 minutes. For lipid peroxidation, a 100µl aliquot was mixed with an equal volume of fresh brain homogenised in 4 volumes of medium, and incubated at 37°C with shaking for 1 hour. 1 ml of 0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25M HCl was added and the mixture boiled for 15 minutes. After removal of precipitate by centrifugation at 1000 g for 10 minutes, the absorbance of 200 µl aliquots was measured at 550 nm. Both assays were performed in triplicate on three separate occasions.

All the fumes tested significantly increased the rate of dopamine oxidation (mean control 0.089 (±0.005); test range 0.131 - 0.223 (±0.001 - 0.056), p≤ 0.05 Anova and Bonferroni t-test). Conversely, all fumes significantly reduced lipid peroxidation levels compared to controls (mean control 0.12 (± 0.018); test range 0.09 - 0.1 (±0.003-0.004), p≤ 0.05 Anova and Bonferroni t test).

We conclude that real welding fume solutes can profoundly affect dopamine oxidation rate, which is consistent with a neurotoxic action. However, their action to inhibit lipid peroxidation may indicate that this effect is too selective to be due to soluble iron alone, and implicates manganese/iron ratios in the solutes as worthy of future monitoring.

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167P NICORANDIL: POTASSIUM CHANNEL OPENER OR NITROVASODILATOR IN HUMAN ARTERIES?

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Nicorandil (N) possesses structural features in common with both potassium channel activators and nitrovasodilators. Animal studies have produced conflicting evidence as to which of these mechanisms is of greater importance in mediating vasodilation (Berdeaux et al, 1992). Few comparable studies have been undertaken in human blood vessels. The aim of this study was to investigate mechanisms of vasodilation to N in human radial (RA) and mammary (MA) arteries.

Vessel segments were obtained from patients undergoing coronary artery bypass graft surgery. 2-3mm rings were studied in classical organ baths. Vessels were constricted to their individual EC_{50} values to phenylephrine. Relaxation to N(10^{-7}-10^{-4}M) was studied before and after treatment with vehicle (V), the ATP dependent potassium channel blocker glibenclamide (G10μM) the soluble guanylate cyclase inhibitor oxadiazole - [4,3-a] quinoxalin-1-one (ODQ10μM), G+ODG, or the calcium dependent potassium channel blockers charybdotoxin and apamin (C50nM+A500nM). The area under the curve (AUC) for each dose response curve to N and the % decrease after treatment was calculated.

Relaxation to N was endothelium independent. RA was more sensitive to N than MA (Table 1). Neither V nor C+A attenuated relaxation to N. ODQ caused a significant attenuation of relaxation to N in RA and MA, whereas G had a significant effect only in RA (Tables 1 & 2). Effects of G and ODQ were additive.

These results suggest that in human arteries, the contribution of nitrovasodilation and ATP dependent potassium channel opening to vasodilatation to N is dependent on the artery studied.

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GO'D held a Wellcome Vacation Scholarship.

Table 1: Relaxation to N pre and post G and ODQ

	MA Pre-G	Post-G	MA Pre- ODQ	Post ODQ	RA Pre-G	Post G	RA Pre-ODQ	Post ODQ
N 10^{-6}M	8+2	7+2	7+3	7+3	27+8	18+3	10+3	9+2
N 10^{-5}M	27+8	29+7	20+3	11+4	72+12	48+10	65+12	20+3
N 10^{-4}M	95+5	82+8	109+9	25+5	121+9	93+9	129+13	80+8

Table 2: % change in area under curve for relaxation to N

	V	C+A	G	ODQ	ODQ + G
MA	+4+7	0+13	-6+5	-80+5*	-85+3*
RA	+5+10	-9+12	-35+5*Δ	-53+4*Δ	-81+4*

* significant reduction in AUC ΔRA significantly different to MA $p<0.05$ unpaired t tests, data mean + SE $n \geq 6$

168P COMPARISON OF FUNCTIONAL PROPERTIES OF HUMAN SAPHENOUS VEIN PREPARATIONS HARVESTED BY MINIMALLY INVASIVE TECHNIQUES

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In vessels isolated for grafting purposes the surgical techniques affect the functional properties of the preparation, which in their turn might influence the short and long-term patency of the graft. In the present study we investigated the vasoconstrictor and, -dilator characteristics of saphenous vein remnants harvested by means of the new skin-bridging methods, and compared them with those of conventionally dissected vein segments, in vitro.

Saphenous vein remnants were obtained after aortocoronary bypass surgery from patients subjected to conventional (CV, n=6), mediastinoscope-assisted (MV, n=4) or endoscope-assisted venectomy (EV, n=5).The preparations were placed immediately in University of Wisconsin (UW) solution at 4°C for 2 days. Subsequently, each piece of SV was cut into 4 rings of approximately 5 mm length, after adhesive fat and connective tissue had been removed. The rings were mounted between two L-shaped stainless steel hooks, in 8 ml organ baths filled with oxygenated Krebs-Henseleit solution of 37°C (pH 7.4). Each preparation was fixed, via a silk thread, to an isometric force transducer. Each ring was subjected to a pretension of 40 mN which was maintained throughout the experiment. After an equilibration period of 60 minutes the vascular rings were primed and tested for viability, by exposing them twice to an isotonic KCl solution (KCl) of 123.8 mM.. The responses induced by the second KCl challenge were taken as maximal and those to the α_1-adrenoceptor agonist phenylephrine (Phe) were expressed as a percentage of the maximum. One hour after the second KCl-induced contraction, a cumulative concentration-response curve (CRC) was constructed for Phe (1 nM- 0.1 mM). For the construction of the CRC's of the nitric oxide (NO)-donor sodium nitroprusside (SNP) (1 nM- 0.1 mM) and acetylcholine (ACh) (1 nM- 0.1 mM), the venous preparations were precontracted with Phe (10 μM). The responses of the preparations to acetylcholine were taken as evidence for the presence or absence of functional endothelium. Appropriate controls were run simultaneously in different ring preparations obtained from the same vascular segments. The storage of the segments in UW solution for maximally 48 h had no detectable effect on the pharmacologically induced responses. The absolute values of the contractile responses to KCl were 27±5.0 mN, 42±7.7 mN and 35±13 mN for the CV (n=6), MV (n=5) and the EV (n=4) harvested vein preparations, respectively. The cumulative addition of the α_1-adrenoceptor agonist phenylephrine (1 nM- 0.1 mM) caused concentration-dependent contractions in the venous segments obtained via the three examined surgical methods. The sensitivity (pD_2: CV 6.0±0.1 n=6, MV 5.9±0.2 n=5, EV 6.0±0.2 n=4), and the maximal responses (E_{max}: CV 99±3 n=6, MV 104±6 n=5, EV 96±7 n=4), to Phe were similar for preparations obtained via the three surgical methods. Neither the maximal responses (E_{max}: CV 129±4 n=6, MV 106±5 n=5, EV 127±4 n=4) to SNP, nor the sensitivity (pD_2: CV 6.7±0.1 n=6, MV 6.2±0.1 n=5, EV 5.9±0.4 n=4) proved significantly different ($p>0.05$), for the differentially harvested vein segments. In the three groups of preparations the addition of ACh to the organ bath evoked only a weak dilator response (E_{max}: CV 11±5 n=6, MV 11±4 n=5, EV 5±6 n=4).

The fact that SNP caused a relaxation below baseline-values (>100%) in all precontracted preparations may indicate the presence of myogenic tone. It is concluded that the new minimally invasive harvesting techniques (MV, EV), compared to the conventional method (CV), can be applied without significant additional loss of vascular reactivity.

169P MECHANISM OF REACTIVE OXYGEN SPECIES-INDUCED AORTIC VASOCONSTRICTION AND DETERIORATION OF FUNCTIONAL INTEGRITY

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Reactive oxygen species (ROS) are involved in a variety of disease states which can have direct or indirect cardiovascular implications. This study was performed to investigate the mechanism of ROS-induced vasoconstriction and the influence of ROS on the functional integrity of isolated rat aorta. ROS were generated by means of electrolysis (30 mA, during 0.5, 1, 2 and 3 min.) of the organ bath fluid. ROS induced a transient (60 min.) vasoconstriction and the maximally induced contraction was dependent on the duration of electrolysis (in mN: 1.18 ± 0.11, 2.72 ± 0.51, 3.70 ± 0.39, 4.72 ± 0.36 (n=6, $P<0.05$) for 0.5, 1, 2 and 3 min. of electrolysis respectively). Dimethyl sulfoxide (0.5%) diminished the ROS-induced (3 min., 30 mA) vasoconstriction almost completely, indicating a major role for hydroxyl radicals. Pretreatment of the aortic rings with the dual cylclooxygenase/lipoxygenase inhibitor meclofenamate (10 μM), completely prevented the electrolysis (30 mA, 3 min)-induced contraction (n=6, $P<0.05$). Indomethacine (10 μM) and the lipoxygenase inhibitor esculetin (10 μM) partially attenuated contraction (1.08 ± 0.13 and 3.31 ± 0.53 mN, for indomethacine and esculetin, respectively vs. 5.99 ± 0.7 mN for vehicle (ethanol 0.02%), n=6, $P<0.05$). The PLA_2 inhibitor oleyloxyethyl phosphoryl choline was able to reduce the ROS-induced (3 min., 30 mA) contraction by approximately 70% (1.85 ± 0.25mN, n=6, $P<0.05$). However the specific $cPLA_2$ inhibitor trifluoromethyl ketone (50 μM) proved ineffective in this respect (n=6). By using the specific mitogen-activated protein (MAP) kinase inhibitor PD98059 (1 mM) it was shown that the activation of extracellular regulated kinase (ERK), a MAP kinase contributes to ROS-induced (3 min., 30 mA) vasoconstriction (2.1 ± 0.18 mN, n=8, $P<0.05$). The effects of ROS on the functional integrity of the aortae, was investigated in particular with respect to receptor and non-receptor mediated responses. Electrolysis periods of 0.5 and 1 min. induced a modest leftward shift of the concentration response curve fo methoxamine (pD_2: 5.50 ± 0.11 and 6.03 ± 0.48 vs. control: 5.21 ± 0.17, n=6, ns), and longer periods of electrolysis additionally lowered the maximum response to (E_{max}) methoxamine (4.1 ± 0.43 vs. control 7.67 ± 0.71 mN, n=6, $P<0.05$). Methacholine-induced vasorelaxation proved diminished in aortae subjected to electrolysis (approx. 95% in control and 5% in electrolysis treated preparations, n=4, $P<0.05$) whereas the relaxation to sodium nitroprusside (10 μM) was nearly complete in all preparations (98 % in control and 97% in electrolysis groups). KCl-induced contractions proved attenuated only after longer periods of electrolysis for 2 and 3 min. (in % of 3rd KCl contraction before treatment: 95 ± 1, 70 ± 2 and 40 ± 1 for control, 2 and 3 min. respectively).
From these results we may conclude that ROS induce an eicosanoid and ERK MAP kinase-mediated vasoconstriction in isolated rat thoracic aorta. In addition, exposure to ROS leads to a deterioration of functional integrity characterized by endothelial dysfunction and decreased contractile function.

170P LOSS OF NITRIC OXIDE SYNTHASE AND SOLUBLE GUANYLATE CYCLASE PROTEIN IN RAT CORONARY MICROVASCULAR ENDOTHELIAL CELLS AFTER CULTURE

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Rat coronary microvascular endothelial (CMVE) cells show a decrease in nitric oxide (NO) and soluble guanylate cyclase (sGC) activity during cell culture. This change is unlikely to be due to a disruption in receptor-dependent pathways; agonist-induced increases in intracellular calcium being unaffected by the culture process (Lang *et al.*, 1996). Previous studies have also shown a significant loss of endothelial nitric oxide synthase (eNOS) mRNA from the CMVE following culture (Bayraktutan *et al.*, 1998). In the present study, we used Western blotting to examine the effect of culture on the expression of eNOS and sGC protein by rat CMVE. The CMVE of male Wistar rats (250-350g) were isolated using the method of Piper *et al.* (1990) and grown in culture for varying times up to confluence (5-7 days post isolation). At the appropriate times, cells were lysed (mM: Tris-HCl, 20, EDTA, 2, EGTA, 10, Triton-X100, 0.1%, dithiothreitol, 1, PMSF, 1, leupeptin, 10g/ml, pepstatin A, 10g/ml, pH 7.5), the particulate fraction removed and the samples concentrated (Centriprep-30, Amicon, USA). The lysates were then run on polyacrylamide denaturing gels and blotted onto a PVDF membrane. The membranes were incubated with 1/1000 dilutions of either anti-eNOS monoclonal (Transduction Laboratories, USA) or anti-sGC (β_1 subunit) polyclonal antibodies (Calbiochem, UK). Antibody binding was detected using horseradish peroxidase linked secondary antibodies (Biorad, UK) before substrate incubation and exposure to photographic film. Each experiment was repeated four times. In freshly isolated CMVE cells, the eNOS (figure 1.a) and sGC (figure 1.b) proteins produced prominent blots. However, after only twelve hours in culture, there was a significant reduction in these protein levels, to the extent that an eNOS protein was undetectable and the sGC barely so. After 24 hrs the sGC also became undetectable. The less dramatic loss of sGC compared with the eNOS suggests there may be a link between these two events; the reduced production of NO leading to the loss of a stimulus for sGC expression. The findings of the present study indicate that the loss of eNOS and sGC activity following culture in CMVE occurs at least at the translational level.

This work was supported by the UK Medical Research Council.

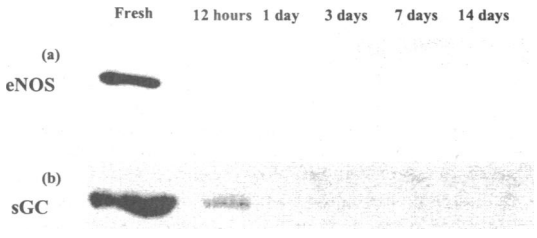

Figure 1. Western blot analysis with (a) anti-eNOS antibody showing eNOS and (b) anti-sGC in freshly isolated rat CMVE and the effect of culture thereon.

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171P ANTIOXIDANT VITAMINS C AND E INHIBIT HOMOCYSTEINE-INDUCED PRODUCTION OF SUPEROXIDE ANIONS BY CULTURED PORCINE AORTIC ENDOTHELIAL CELLS

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A moderate elevation in plasma homocysteine (HC) levels is now recognised as an independent risk factor for cardiovascular disease, though the exact mechanism is unknown (Durand *et al.*, 1998). It has been proposed that HC-mediated endothelial cell damage via the overproduction of oxygen free radicals may be involved in this process (Loscalzo, 1996). The present study investigates the effect of HC, and antioxidant vitamins C and E on cultured porcine aortic endothelial cell (PAE) superoxide anion (O_2^-) production. PAE were isolated and grown in culture as previously described (Lang *et al.*, 1991). Confluent monolayers of PAE were incubated for 24 hours at 37°C either in the presence of culture medium alone, HC alone (1mM), or HC with either vitamin C (10μM) or vitamin E (10μM). Following these incubations the PAE were trypsin (0.05% w/v) digested and then harvested for measurement of O_2^- production using lucigenin-chemiluminescence. Following estimations of cell numbers using a Coulter Counter, the PAE were added to an aliquot of Hepes (20mM)-buffered physiological saline (pH 7.4) of the following composition (mM): NaCl 138; KCl 5.3; NaH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 15 and $CaCl_2$ 1.5 at 37°C. Lucigenin was added to a final concentration of 500mM. The cells were then placed into the warmed (37°C) chamber of a custom built luminometer, with output (in mV) displayed on a Macintosh computer via a maclab apparatus. The total intracellular O_2^- production by these cells was then measured following the addition of 1% triton X - 100. The integral for the response represents the total O_2^- produced and was normalised to cell number. All data are expressed as mean ± standard error of the mean ($n \geq 8$), compared using one way analysis of variance followed by Student-Newman-Keuls test and considered significant when $p<0.05$. No changes in baseline chemiluminescence was observed in the absence of triton X – 100. In the PAE incubated with culture medium alone, O_2^- production was 30.39±2.0 mV.s/10^6 cells in the presence of triton X - 100. This level was significantly ($p<0.001$) increased following incubation with HC (67.12±4.2 mV.s/10^6 cells). This increase was completely inhibited ($p<0.001$) by concomitant incubation with vitamin C (29.93±1.9 mV.s/10^6 cells) or vitamin E (27.60±2.84 mV.s/10^6 cells). These data demonstrate that exposure to HC increases the intracellular production of O_2^-, an increase that is completely inhibited by the antioxidant vitamins C and E. These observations provide a possible mechanism for the endothelial dysfunction that is associated with hyperhomocystenaemia and demonstrate that antioxidant vitamins may be useful interventions.

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172P RECOVERY OF RELAXATION TO ACETYLCHOLINE IN MOUSE ISOLATED CAROTID ARTERIES TEN DAYS AFTER ENDOTHELIAL DENUDATION *IN VIVO*

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Previous studies of endothelial re-growth following denudation with wire filaments or balloon catheterisation have been primarily carried out in large animals such as rabbits or pigs (Azuma *et al.*, 1995; Borg-Carpra *et al.*, 1997). A murine model of endothelial re-growth has now been developed in which the endothelial cell layer is removed from the left common carotid artery using a fine PTFE filament. Histological studies have shown that in this model, endothelial re-growth is complete after ten days (Jackson, 1996), but the effect of endothelial regeneration on vascular responses has not been investigated.

In this study, the effect of endothelial denudation on relaxation to the endothelium-dependent relaxant acetylcholine (ACh; 10^{-9}M-10^{-5}M) and the endothelium-independent relaxant diethylamine NONOate (10^{-9}M-10^{-5}M) was investigated in isolated arterial segments pre-constricted with phenylephrine (3μM) and 5-HT (3μM). Endothelial denudation was carried out in the left common carotid artery of C57BL/6J mice (~20 weeks old, anethsetised with 70 μg g^{-1} sodium pentobarbitone) using a fine nylon filament as previously described (Jackson, 1996). Mice were sacrificed 1 or 10 days after surgery and both the left and right carotid arteries removed. The arteries were cut into 2 mm segments and mounted in a wire myograph for recording of changes in isometric tension. Arterial rings were set to a resting tension of ~9mN and maintained at 37°C in oxygenated Krebs buffer. In some experiments production of endothelium-derived nitric oxide (NO) was inhibited by incubating the tissues with the NO synthase inhibitor Nω-nitro-L- arginine (100μM pre-incubated for 5 mins). As preliminary experiments indicated that there was no significant difference in the responsiveness of left and right carotid arteries, right carotid arteries were used as time-matched controls for the injured vessels.

All data are expressed as mean ± s.e.mean. and differences between mean values were calculated using 2-way ANOVA ($\alpha=0.05$). ACh evoked concentration-dependent relaxation of pre-constricted segments from control vessels. The IC_{50} and maximum relaxation values for ACh-evoked relaxation in control tissues at 1 and 10 days post-surgery were 17.7 ± 5.4nM and 96.3 ± 1.4% (n=4), and 22.2 ± 6.5nM and 89.4 ± 8.1% (n=6), respectively. In contrast, ACh failed to relax pre-constricted segments from injured arteries at 1 day post-surgery (maximum relaxation 1.93 ± 4.6%; n=4). However, at 10 days post-surgery ACh-evoked relaxation was observed although the relaxation to this endothelium-dependent agonist was significantly reduced compared to control values (IC_{50} of 0.25 ± 66μM and maximal relaxation 58.3 ± 16.6%; n=6; P=0.004). Pre-incubation with Nω-nitro-L-arginine abolished relaxation to ACh in both control and injured arteries at 10 days post-surgery, reducing the maximal relaxation to 1.8 ± 4.9% and 11.3 ± 8.3% (n=3; P>0.05), respectively.

In contrast to ACh, the NO donor diethylamine NONOate relaxed arteries at both day 1 or day 10 post-surgery. The IC_{50} and maximum relaxation values for relaxation to diethylamine NONOate in control tissues at day 1 and day 10 were 1.3 ± 0.3μM and 95 ± 4.5% (n=3) and 0.16 ± 0.02μM and 89.5 ± 13.3% (n=4), respectively. The corresponding values for injured arteries were 2.3 ± 0.6μM and 101.3 ± 5.6% (n=3), and 63.8 ± 17nM and 99.4 ± 10.5 % (n=4), respectively.

In conclusion, these data indicate that in this mouse model of endothelial injury, endothelial re-growth appears to be accompanied by restoration of endothelial cell function.

F. Plane is a Wellcome Trust Career Development Fellow and this work is supported by the BHF.

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173P A STUDY OF RELAXATION MECHANISMS ACTIVATED BY THE NOVEL NITRIC OXIDE DONOR, DIETHYLAMINE NONOate, IN THE RAT ISOLATED MESENTERIC ARTERY

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Nitric oxide-evoked relaxation of rat isolated mesenteric arteries appears to be mediated by the stimulation of soluble guanylyl cyclase and direct activation of smooth muscle cell BK_{Ca} channels (Plane *et al.*, 1996; Mistry & Garland, 1998). Our previous studies indicated that endogenous release of NO by endothelial cells can influence which mechanism predominates during relaxation (Plane *et al.*, 1996). In these experiments, relaxation was induced with the NO donor 3-morpholinylsydnoneimine chloride (SIN-1). As this agent is known to have effects in addition to the generation of NO (Feelisch, 1991), we have employed the novel NO donor, diethylamine NONOate (DEA NONOate; Maragos *et al.*, 1991), to show if a similar modulatory influence occurs in the rat isolated mesenteric artery.

Male Sprague-Dawley rats (250-300 g) were stunned and killed by cervical dislocation. Segments of third order mesenteric artery (ID_{100} = 233 ± 6.9 μM; n=30) were mounted in a Mulvany-Halpern myograph under a normalised tension for isometric recording of tension changes. The tissues were maintained at 37°C in oxygenated Krebs buffer. All data are expressed as mean ± s.e. mean. Differences between mean values were calculated using Students t-test.

Diethylamine NONOate (0.01 μM-10 μM) evoked concentration-dependent relaxation in phenylephrine (3 μM) stimulated arterial segments. Relaxation was not significantly different between endothelium intact (n=11; pD_2 = 6.7 ± 0.2) or endothelium denuded segments (n=12; pD_2 = 6.8 ± 0.6). Maximum relaxations were 97.1 ± 0.5 % and 94.0 ± 1.6 %, respectively. Diethylamine alone (1-30 μM) failed to evoke relaxation in either intact or denuded arterial segments (n=4).

DEA NONOate-evoked relaxation was significantly attenuated by ODQ (10 μM; 10 mins; $P<0.05$), an inhibitor of soluble guanylyl cyclase, in both endothelium-intact (n=7; pD_2 = 5.8 ± 0.4; R_{max} = 92.7 ± 3.6 %) and endothelium-denuded segments (n=5; pD_2 = 5.6 ± 0.3; R_{max} = 94.4 ± 3.1 %). Furthermore, addition of both ODQ and charybdotoxin together caused a further rightward shift in the concentration response curve to DEA NONOate of both endothelium-intact (n=9; pD_2 = 5.1 ± 0.4; R_{max} = 91.8 ± 2.8 %) and denuded (n=6; pD_2 = 5.0 ± 0.4; R_{max} = 92.7 ± 2.8 %) arteries.

Pre-incubation of endothelium-intact arteries with the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (100 μM; 30 mins), did not effect relaxation to DEA NONOate (n=7; pD_2 = 7.1 ± 0.3; R_{max} = 99.2 ± 2.1 %). The subsequent addition of the guanylyl cyclase inhibitor, ODQ (10 μM; 10 mins), however, produced a significant inhibition of DEA NONOate-evoked relaxation (n=3; pD_2 = 5.6 ± 0.2; R_{max} = 94.0 ± 1.3 %; $P<0.05$). However, the combined application of ODQ and charybdotoxin (50 nM; 10 mins) failed to cause any further inhibition of DEA NONOate-evoked relaxation (n=7; pD_2 = 5.3 ± 0.6; R_{max} = 98.2 ± 1.6 %).

These data indicate, that in the rat mesenteric artery relaxation to the novel nitric oxide donor, diethylamine NONOate apparently occurs by a combination of pathways. These pathways include an ODQ sensitive stimulation of soluble guanylyl cyclase and direct activation of charybdotoxin sensitive smooth muscle cell BK_{Ca} channels. However, in contrast to studies with the nitric oxide donor SIN-1, basal release of nitric oxide from an intact endothelium does not appear to alter the predominant relaxation pathway.

This work was supported by the MRC and Wellcome Trust.

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174P AGE, PERTUSSIS TOXIN AND AGONIST-INDUCED VASOCONSTRICTION OF THE RAT AORTA

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We have shown that vasoconstrictor responses of the rat tail artery to agonists such as noradrenaline (NA) and serotonin (5-HT) decrease with age and that in the tail arteries from young - but not old - rats pertussis toxin (PTX) lowers the vasoconstrictor response to agonists (Robert *et al.*, 1998; Spitzbarth-Régrigny *et al.*, this meeting). This suggests that the age-related fall in the vasoconstrictor response to agonists corresponds to a loss of a PTX-sensitive component in senescent animals. In order to determine whether this is a general phenomenon common to other arteries we investigated the effects of age and PTX on agonist-induced vasoconstriction in the rat aorta.

Thoracic aortas were dissected out from male 3-, 12- and 24-month old WAG/Rij rats (n = 4 - 6 per group) under sodium pentobarbitone anaesthesia (60 $mg.kg^{-1}$, i.p). Aortic rings (2-mm long) were de-endothelialised and mounted between two stainless steel hooks in an organ bath (1.5 ml) filled with HEPES-buffer solution (37°C, 100% O_2). Rings were treated with PTX (1 $\mu g.ml^{-1}$, 2 h) or not (control) at a passive tension of 2 g. Cumulative dose-response curves to NA (10^{-9} to $3x10^{-6}$ M) and 5-HT (10^{-7} to $3x10^{-5}$ M) were performed by recording isometric tension (g). Results are expressed as pD_2 and E_{max} (means ± s.e.m.).

Significant differences between pD_2 and E_{max} ($P < 0.05$) were determined by one- or two-way ANOVA and the Bonferroni test.

Age and PTX had no effect on responses to NA (3-month pD_2 7.18 ± 0.22, E_{max} 2.38 ± 0.19 g). Age had no effect on E_{max} but a minor significant effect on pD_2 for 5-HT, 3-month 5.53 ± 0.07, 12-month 5.79 ± 0.05 (+ 5%), 24-month 5.76 ± 0.06 (+ 4%) (both $P < 0.05$ *versus* 3-month). PTX had no effect on pD_2 for 5-HT but slightly lowered E_{max} in aortas from 3- and 12-month old animals, 3-month 2.14 ± 0.09, PTX 1.78 ± 0.11 (- 17 %), 12-month 2.33 ± 0.13, PTX 2.03 ± 0.05 (- 13%) both $P < 0.05$, 24-month 2.08 ± 0.14, PTX 1.99 ± 0.12 (- 10%, $P > 0.05$).

These data indicate that the PTX-sensitive G_i-protein-mediated component of agonist-induced vasoconstriction in aortas is of far less importance than in the tail artery.

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175P PROTEIN TYROSINE KINASE IS INVOLVED IN THE VASOCONSTRICTOR RESPONSE TO ENDOTHELIN-1 OF THE HUMAN INTERNAL MAMMARY ARTERY

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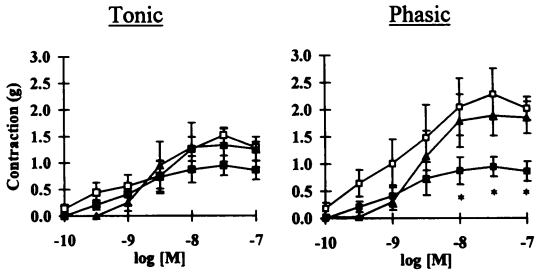
The trophic effect of endothelin-1 (ET-1) involves cross-talk between the G-protein-coupled receptors and a tyrosine kinase pathway (Simonson & Herman, 1993). We investigated whether a non-receptor tyrosine kinase pathway is involved in the constrictor effect of ET-1 in human arteries by evaluating the impact of a tyrosine kinase inhibitor (genistein; Akiyama *et al.*, 1987) on ET-1 induced vasoconstriction of the human internal mammary artery.

Human internal mammary arteries (n = 15) were harvested intra-operatively and placed in HEPES-buffer solution (HBS) at 4°C. Less than 3 hours later, arterial segments were cleansed of connective tissue, cut into rings (2-mm long), de-endothelialised and mounted between two stainless steel wire hooks in an organ bath (1.5 ml) filled with HBS (37°C, 100% O_2). Passive tension was increased stepwise from 1 to 3 g with 30 min equilibration at each step. A cumulative dose-response curve to ET-1 (10^{-10} to 10^{-7} M; n = 5) was performed in the absence or presence of genistein (10^{-5} M; n = 5) or its inactive structural analogue, daidzein (10^{-5} M; n = 5). Isometric force (g) was recorded. Results are expressed as means ± s.e.m. Significant differences between results obtained in the presence *vs* absence of genistein or of daidzein were determined by one-way ANOVA and the Bonferroni test (* $P < 0.05$).

ET-1 produced a dose-dependent increase in tension with a phasic and a tonic component. Genistein inhibited the ET-1-induced phasic but had no effect on tonic contraction (Figure 1). Daidzein had no effect on either (Figure 1).

Figure 1: ET-1 (10^{-10} to 10^{-7} M) dose-response curves in absence (unfilled squares) or in presence of genistein (filled squares) or daidzein (filled triangles).



We suggest that cross-talk between tyrosine kinase and G-protein-coupled receptor pathways is the basis of the phasic contraction induced by ET-1 in the human mammary artery.

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176P EVIDENCE FOR ENHANCED ENDOTHELIUM-DEPENDENT VASODILATION *IN VITRO* IN THE OBESE ZUCKER RAT INDEPENDENT OF NITRIC OXIDE AND PROSTANOIDS

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The obese Zucker rat, a model of metabolic Syndrome X, exhibits raised oxidative stress (Laight *et al.*, 1998). However, despite evidence that reactive oxygen species impair nitric oxide (NO)-mediated vascular endothelial function (McQuaid *et al.*, 1997), agonist-stimulated endothelium-dependent vasorelaxation in isolated aortic smooth muscle of the obese Zucker rat has been shown to be either similar or enhanced relative to its non-obese littermate, the lean Zucker rat (Auguet *et al.*, 1989; Growcott *et al.*, 1995). We have now investigated this phenomenon by characterising endothelium-dependent vasorelaxation in obese and lean Zucker rats *in vitro*.

13-week old obese and lean, male Zucker rats were anaesthetised with sodium pentobarbitone (60 mg kg^{-1} i.p.) and killed by cervical dislocation. Thoracic aortic rings were mounted under isometric conditions in Krebs-Henseleit solution gassed by carbogen and warmed to 37 °C. Rings were precontracted with phenylephrine (PEP, 3 μM) in the presence or absence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 0.3 mM) and the cyclo-oxygenase inhibitor indomethacin (INDO, 10 μM). A concentration-response curve to acetylcholine (ACh) was then constructed. After washout, the rings were equivalently precontracted with the depolarising agent KCl (40 mM) and the concentration-response curve to ACh repeated. Data are mean±s.e. mean.

Vasodilation to ACh was similar in lean and obese Zucker rats (lean: area under the curve (AUC)=162.0±18.7 units; pD_2=7.24±0.14; E_{max}= 93.9±4.3 %; obese: AUC=142.0±14.2 units; pD_2=7.11±0.14; E_{max}=90.5±2.2 % (n=4, all $P>0.05$). However, obese Zucker rats had a significantly higher L-NAME- and INDO-insensitive vasorelaxation that was completely abolished by KCl (Figure 1) (lean: AUC=1.2±0.5 units; pD_2=6.70±0.06; E_{max}= 1.3±0.5 %; obese: AUC=6.8±1.0 units ($P<0.01$); pD_2=6.87±0.06 ($P>0.05$); E_{max}=9.3±0.9 % ($P<0.01$) (n=4)). Papaverine (100 μM) elicited 100 % vasorelaxation in all KCl-precontracted preparations

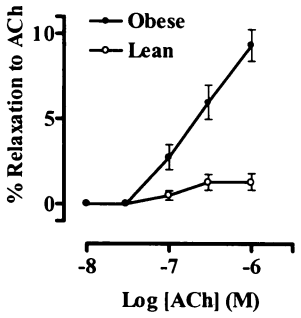

Figure 1. Relaxation to ACh in the presence of INDO and L-NAME.

Our data therefore suggest that the obese Zucker rat exhibits a significant non-NO, non-prostanoid component of endothelium-dependent vasodilation. Since this may be blocked by depolarisation, the alternative mediator may be an endothelium-derived hyperpolarising factor(s) (see Garland *et al.*, 1995). The availability of such additional pathways of endothelium-dependent vasodilation in the obese Zucker rat may conceivably contribute to the maintenance of normal endothelial function in oxidant stress.

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

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177P EFFECT OF TYROSINE KINASE INHIBITORS ON CYTOKINE-STIMULATED ENDOTHELIN PRODUCTION IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

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We have previously reported that endothelin-1 (ET-1) mRNA expression and peptide production in human vascular smooth muscle cells (HVSMCs) are markedly increased by exposure to cytokines and lipopolysaccharide (Woods *et al.*, 1998). Pro-inflammatory cytokines have been shown to mediate their actions via phosphorylation of tyrosine kinase (Iwasata *et al.*, 1992; Akarasereenont *et al.*, 1994). In this study we have attempted to determine if a similar mechanism of signal transduction is involved in cytokine stimulation of ET-1 production from saphenous vein (SV) HVSMCs.

SV was obtained from patients undergoing coronary artery bypass graft surgery. Explants of HVSMCs were grown in DMEM supplemented with 2mM glutamine, penicillin (100U.ml^{-1}), streptomycin (100μg.ml^{-1}) and 15% foetal calf serum (37°C; 5% CO_2; 95% air). HVSMCs were identified by α-actin staining. Cells in culture grown on 96 well plates were treated with a mixture of tumour necrosis factor-α (10ng.ml^{-1}) and interferon-γ (1000U.ml^{-1}) for 48h in the presence or absence of herbimycin or genistein. Medium was removed and ET-1 levels were measured by specific sandwich ELISA (R&D Systems). Cell viability was assessed by the ability of cells to reduce MTT to formazan (Mosmann, 1983).

Herbimycin (1nM-10μM) or genistein (1nM-1mM) inhibited cytokine stimulated ET-1 production from HVSMCs with IC_{50} values of 0.91μM (0.71-1.18μM) and 58.7μM (47.2-72.9μM) respectively (Figure 1). Neither herbimycin nor genistein affected HVSMC viability during the incubation period.

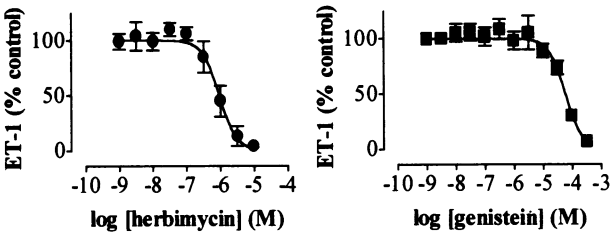

Figure 1. Effect of herbimycin and genistein on cytokine stimulated ET-1 production in HVSMCs. Data represents mean±s.e.m of n=5 donor patients in triplicate.

These results indicate that the signal transduction events leading to cytokine stimulation of ET-1 peptide release is partly mediated via phosphorylation of tyrosine kinase. Further studies are necessary to elucidate which other pathways may be involved.

Supported by a grant from the European Commission. TDW holds a British Heart Foundation Lectureship (BS/95003). JAM is a Wellcome Career Development Fellow.

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178P THE ROLE OF VASCULAR SMOOTH MUSCLE CELLS IN SERUM AND CYTOKINE-STIMULATED ENDOTHELIN-1 PRODUCTION IN INTACT RAT AORTA

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Endothelin-1 (ET-1) mRNA expression and peptide production in human vascular smooth muscle cells (VSMCs) are increased by exposure to cytokines and lipopolysaccharide (Woods *et al.*, 1998). VSMC culture provides useful information but differs in important aspects from VSMCs *in situ*. Here we have determined, in an intact rat blood vessel, whether ET-1 production can also be stimulated, and the relative contribution of VSMCs and endothelium to ET-1 production.

Rats (male Wistar 200-250g) were killed by sodium pentobarbitone and the aorta removed and carefully cut into rings. In some preparations the endothelium was removed by gentle abrasion with a scalpel. Rings were then placed in DMEM supplemented with 2mM L-glutamine, penicillin (100U ml^{-1}) and streptomycin (100μg ml^{-1}) (37°C; 5% CO_2; 95% air) in the presence or absence of cytokines (tumour necrosis factor-α 10ng.ml^{-1} and interferon-γ 1000U.ml^{-1}) and/or 10% foetal calf serum (FCS). Medium was removed at 1, 4, 24 and 48h, acidified with HCl, and ET-1 concentrated over disposable C18 columns. ET-1 levels were subsequently measured by specific sandwich ELISA (R&D Systems). The continuing presence of functional endothelium was tested after 48h by the ability of acetylcholine (10^{-6}M) to relax rings contracted with phenylepherine(10^{-5}M) (Furchgott and Zawadzki, 1980). Tissue viability was measured with the Cytotoxicity Detection Kit (LDH), Boehringer Mannheim.

FCS markedly stimulated ET-1 production. Thus at 48h in the endothelium intact group, it was 52.3±10 pg/mg ET-1 compared to 7.0±2.6 (n=3) pg/mg in tissues without FCS. Endothelial removal reduced ET-1 secretion to 8.9±2.8 and 2.3±0.3 pg/mg with or without FCS respectively. Cytokines had no significant effect on ET-1 production in any group.

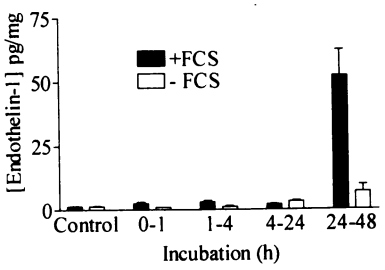

Figure 1. Effect of FCS on ET-1 production in rat aorta with intact endothelium, in organ culture. Mean ± s.e.m. of tissues from 3 rats.

These results show that in intact contractile vessels under culture conditions endothelial cells release the majority of ET-1. However, approximately a third of production may be from VSMCs underlining the potential of these cells to produce ET-1.

Supported by a grant from the St Bartholomew's Joint Research Board and Mr GM Rees. TDW holds a British Heart Foundation Lectureship (BS/95003).

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