125P MOLECULAR CLONING, EXPRESSION AND LOCALISATION OF THE RAT SOMATOSTATIN $sst_{2(b)}$ RECEPTOR SPLICE VARIANT IN THE RAT GASTRIC MUCOSA

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We have previously shown that the somatostatin (SRIF) receptor involved in the inhibition of gastric acid secretion in rat isolated gastric mucosa resembles the recombinant sst_2 receptor (Wyatt *et al.*, 1996). The aims of the present study were to identify and localise the sst_2 receptors in the rat stomach and to see if the $sst_{2(b)}$ splice variant, identified in the mouse (Vanetti *et al.*, 1992), also exists in the rat as suggested (Patel *et al.*, 1993).

Female Wistar rat (70-120g) gastric mucosae were isolated as previously described (Wyatt *et al.*, 1996). Total RNA was isolated over a cesium chloride gradient and messenger RNA (mRNA) was extracted using oligo(dT)-cellulose (Clontech). Reverse transcriptase-polymerase chain reactions (RT-PCR) were carried out on 2μg of mRNA using sst_2 receptor-specific primers. The products were cloned into the pCR II vector (Invitrogen) and sequenced using Sequenase v2.0 enzyme (Amersham). Using the conserved portion of the rat $sst_{2(a)}$ receptor construct with a hemagluttinin N-terminal epitope tag (Affymax), an equivalent rat $sst_{2(b)}$ construct was prepared and stably expressed in CHO-K1 cells. Radioligand binding studies were carried out as previously described (McKeen *et al.*, 1996). An $sst_{2(a)}$ receptor antibody was already available (Schindler *et al.*, 1997) and a selective anti-peptide antibody was raised against the unique C-terminus of the $sst_{2(b)}$ receptor in rabbits and used at 1:300 in all studies. Immunohistochemistry studies used 10μm slide-mounted sections of stomach from female rats perfused with 4% paraformaldehyde.

Two significant cDNA bands were seen following RT-PCR reactions on rat gastric mucosa. Upon sequencing, the largest band (848 base pairs, bp) was found to be identical to the previously cloned $sst_{2(a)}$ receptor (Kluxen *et al.*, 1992) while the smallest (527bp) was a novel sequence, the rat $sst_{2(b)}$ receptor, which had 86% homology with the mouse $sst_{2(b)}$ receptor. This sequence was deposited in the EMBL sequence database under Accession number X98234. Transfection studies provided two cell lines containing either the rat $sst_{2(a)}$ or $sst_{2(b)}$ isoforms at similar receptor densities (B_{max} values ~ 10 pmol/mg protein).

Western analysis using the selective $sst_{2(b)}$ antibody on membranes prepared from recombinant $sst_{2(b)}$ receptor-containing cells showed the receptor had an apparent molecular weight (MW) of about 75-90kD. Deglycosylation of membrane proteins from both the recombinant rat $sst_{2(a)}$ and $sst_{2(b)}$ receptor cell lines, using N-endoglycosidase F, resulted in a shift of the MW of the broad immunoreactive band from 75-90kD to 42kD. A differential distribution for the two receptor splice variants was observed in immunohistochemical studies. The $sst_{2(b)}$ receptor antibody strongly labelled parietal cells whereas the $sst_{2(a)}$ receptor was found to be present on cell membranes of structures towards the serosal surface of the stomach as well as on nerve fibres.

This study provides the first proof that the $sst_{2(b)}$ receptor exists in the rat. The functional properties of the recombinant $sst_{2(b)}$ receptor are described in detail elsewhere (Schindler *et al.*, this meeting). Both the $sst_{2(a)}$ and $sst_{2(b)}$ receptor proteins were shown to be glycoproteins of a similar size. Their differential distribution in the gastric mucosa is interesting as a mechanism whereby SRIF could exert differing functional effects in the same tissue by binding to different sst_2 receptor isoforms.

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126P SOMATOSTATIN-INDUCED REGENERATION OF CHO-K1 CELLS EXPRESSING RECOMBINANT HUMAN sst_4 RECEPTORS IS MEDIATED BY ACTIVATION OF MEK

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Somatostatin-14 (SRIF) not only inhibits cell growth (Lauder *et al.*, 1997) but can also stimulate proliferation (Kamiya *et al.*, 1993). The mechanism of the antiproliferative effect of SRIF has been proposed to involve the activation of tyrosine phosphatase (Buscail *et al.*, 1994), whereas the transduction pathway mediating the growth effect of SRIF has not been clearly defined. The SRIF sst_4 receptor type has been shown to activate the MAP kinase cascade (Bito *et al.*, 1994) which is an important pathway in mediating cell growth. We have therefore investigated the effect of the MEK inhibitor (PD 98059; Alessi *et al.*, 1995), as well as the tyrosine kinase inhibitors (genistein and lavendustin A) and the tyrosine phosphatase inhibitor, sodium orthovanadate on the proliferative effect of SRIF in CHO-K1 cells expressing human recombinant sst_4 receptors ($CHOsst_4$).

$CHOsst_4$ cells were grown to confluence on Thermanox™ coverslips in DMEM:F12 media supplemented with Glutamix I, 10% fetal calf serum (FCS) and 500μg ml^{-1} G418 sulphate. Using the method described by Fan & Frost, 1990, 11 parallel lesions (400 μm wide) were made across the monolayer. Regeneration of cells into the denuded area (in the absence of FCS) was measured with a Leica Q500 MC image analyser or by direct cell counting of cell suspensions from the whole coverslip using a Coulter™ counter. All values quoted are a mean ± se mean from 3 experiments with 4 replicates per test group. Statistical comparison was by analysis of variance followed by Tukey test using $P<0.05$ as the level of significance.

SRIF (0.1nM-1μM) caused a concentration-dependent (pEC_{50} 8.6±0.2) increase in the regeneration of cells into the denuded area after 24h (from 11.6±0.4% to 25.6±0.7%). This effect was due to an increase in proliferation since SRIF (100nM) significantly increased the cell numbers from $1.66\pm0.06\times10^5$ to $2.15\pm0.12\times10^5$. Neither genistein (50μM), lavendustin A (11nM), sodium orthovanadate (5μM) nor PD 98059 (2μM) affected the basal recovery (8.2±0.5%) of denuded $CHOsst_4$ cell monolayers. The SRIF (100nM)-induced regeneration (20.8±0.4%) was reduced to basal levels 7.6±0.4%, 9.3±0.5%, 9.9±0.5% and 7.6±0.5% in the presence of genistein, lavendustin A, sodium orthovanadate and PD 98059, respectively, as well as by pretreatment with pertussis toxin (100ng/ml) (8.6±0.7%). In contrast, bFGF (10ng/ml)-induced regeneration (20.7±0.4%), was unaffected by pertussis toxin pretreatment. Genistein, lavendustin A and sodium orthovanadate all abolished the increase in regeneration (21.1±0.3%) induced by bFGF (10ng/ml). However, PD 98059 caused a significant, but only partial inhibition of the response (15.1± 0.7%).

The results from the present study demonstrate that the transduction pathway mediating the proliferative effect of SRIF in $CHOsst_4$ cells is mediated entirely through pertussis toxin-sensitive G proteins and activation of MEK. This pathway is distinct from that activated by bFGF which is insensitive to pertussis toxin and only partially involves activation of MEK. However, both pathways are clearly dependent upon tyrosine phosphorylation.

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127P PPADS IS A PARTIAL AGONIST ON P2Y$_1$ RECEPTOR-MEDIATED CHANGES IN Ca^{2+}_i AS DETERMINED IN A FLUORESCENCE IMAGING PLATE READER (FLIPR)

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PPADS (pyridoxal-phospate-6-azophenyl-2',4'-disulphonic acid) has been described as a selective antagonist of ADP-induced responses at human P2Y$_1$ purinoceptors (Schachter et al 1996). FLIPR determines simultaneous, real-time, kinetic fluorescence intensities in a 96-well plate format. We have characterised the pharmacology of a human P2Y$_1$ receptor clone and demonstrated that PPADS has partial agonist activity at this receptor in a FLIPR functional screen measuring changes in intracellular calcium.

Recombinant human P2Y$_1$ receptors stably expressed in a 1321N1 astrocytoma cell line at 15000 cells per well (Schachter et al 1996) were incubated with the Ca^{2+} sensitive dye Fluo-3AM (4μM) at 37°C/5%CO_2 for 90 min and washed with Tyrodes buffer containing 2.5 mM probenecid. Antagonists were pre-incubated at 37°C/5%CO_2 for 30 min. Basal fluorescence was determined prior to agonist addition at 37°C by FLIPR.

For each response the peak increase in fluorescence was calculated and iteratively curve-fitted using a four parameter logistic model (Bowen and Jerman 1995).

Table 1: Functional (FLIPR) potencies at recombinant human P2Y$_1$ receptors transfected into a 1321N1 cell line

Drug	Agonist pEC_{50}	Antagonist pIC_{50}
PPADS	5.6 ± 0.2 (6)	5.6 ± 0.1 (11)
cibacron blue	< 4.0 (3)	5.8 ± 0.1 (6)
suramin	< 4.0 (3)	5.0 ± 0.1 (5)
DIDS	< 4.0 (3)	4.8 ± 0.2 (6)

Data are mean ± sem from (n) experiments
Antagonist potencies determined against 3 nM ADP

ADP caused a large (up to 45000 fluorescence intensity unit) increase in fluorescence , which peaked rapidly (6 - 8 sec) then returned to basal values over 3 - 4 min. The pEC_{50} for ADP was 8.5 ± 0.1 (n=15) in this system. Other agonists pEC_{50} values included: 2-methyl-thio-ATP 8.3 ± 0.2 (n=5); ATP 7.0 ± 0.2 (n=5); UTP 6.0 ± 0.2 (n=5); 5'adenylylimidophoshate (AMPPNP) 5.9 ±0.1 (n=5); AMP 5.5 ± 0.2 (n=5); UDP 5.4 ± 0.2 (n=5); and α,β methylene-ATP 5.4 ± 0.2 (n=5).

PPADS inhibited ADP responses in a concentration-dependant manner, but also stimulated Ca^{2+}_i release (Table 1) with intrinsic activity of 0.54 ± 0.03 (n=6). The pEC_{50} for PPADS matched its pIC_{50} generated against an EC_{50} concentration of ADP. Cibacron blue, suramin and 4,4'-diisothiocyanatostilbene-2,2'-disuphonic acid (DIDS), antagonised ADP (Table 1) and PPADS responses, but had no intrinsic activity in this system at the same concentrations. The 1321N1 cell line expresses an endogenous muscarinic M3 receptor. The pEC50 for the muscarine-induced response was 4.8 ± 0.2 (n=5). PPADS had a pIC50 < 4.0 against muscarine (100μM) induced increases in fluorescence.

We have characterised the pharmacology of the human P2Y$_1$ receptor expressed in 1321N1 astrocytoma cell line. Agonist potencies in a FLIPR functional assay were higher than previously reported (Schachter et al 1996). Under the conditions used in the FLIPR assay, PPADS is a partial agonist at the P2Y$_1$ receptor. This effect must be considered when interpreting PPADS activity in other functional systems. PPADS is a selective inhibitor of the Ca^{2+}_i release induced by the transfected P2Y$_1$ receptor over that caused by the endogenous M3 muscarinic receptor in this assay.

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128P FURTHER CHARACTERISATION OF P2X7 RECEPTORS ON NTW8 CELLS, A MOUSE MICROGLIAL CELL LINE

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Previous electrophysiological studies have shown that NTW8 cells (an immortalised murine microglial cell line) possess the $P2X_7$ receptor (Chessell *et al.*, 1997). This receptor differs from other P2X receptors, notably in biophysical properties, where repeated application of agonist can cause formation of large ligand-gated "pores" in the cell membrane. In this study $P2X_7$ receptors in NTW8 cells were further characterised using two biochemical techniques which we have previously shown can be used to assess $P2X_7$ receptor function (Michel *et al.*, 1997).

^{45}Calcium ($^{45}Ca^{2+}$) influx and influx of the DNA-binding dye, YO-PRO-1 were measured as described (Michel *et al.*, 1997). Studies were performed in buffer comprising (mM): sucrose 280, glucose 10, KCl 5, $CaCl_2$ 0.5 and Hepes 10 (pH 7.4, 22°C). Values shown are the mean±s.e.mean of 3-5 experiments.

At 22°C, ATP and dibenzoyl-ATP (DbATP) stimulated $^{45}Ca^{2+}$ influx into NTW8 cells. Significant $^{45}Ca^{2+}$ influx was detected within 30s and was maximal after 8min. Further studies were undertaken with a 16min incubation. Monovalent cations inhibited DbATP-stimulated $^{45}Ca^{2+}$ influx by >95% at a concentration of 140mM (pIC_{50} values for NaCl, KCl and choline chloride were 1.71±0.23, 1.40±0.24 and 1.19±0.11, respectively). DbATP (pEC_{50}=4.4±0.08) was the most potent agonist at stimulating $^{45}Ca^{2+}$ influx (6.01±1.5 fold over basal), whereas ATP was less potent (pEC_{50}=3.8±0.04). ADP and 2MeS-ATP also stimulated $^{45}Ca^{2+}$ influx but only at concentrations in excess of 300μM. Adenosine (30μM), UTP (1mM) and αβ-methylene ATP (100μM) did not stimulate $^{45}Ca^{2+}$ influx. $^{45}Ca^{2+}$ influx stimulated by 50μM DbATP was insurmountably blocked by pre-incubating cells for 20min with various P2 purinoceptor antagonists. Coomassie blue G was the most potent antagonist (pIC_{50}=6.1±0.05). Evans' blue, cibacron blue, pyridoxal phosphate (P5P) and suramin also antagonised DbATP-stimulated $^{45}Ca^{2+}$ influx, but were less potent (pIC_{50}=5.5±0.01, 5.1±0.01, 4.8±0.04 and 4.7±0.03, respectively).

DbATP and ATP also stimulated YO-PRO-1 influx into NTW8 cells. DbATP-stimulated YO-PRO-1 influx reached a maximum after 8min (197.6±7.53% of control). Further experiments were undertaken with 4min incubations. DbATP (pEC_{50}=4.4±0.07) was the most potent agonist at stimulating YO-PRO-1 influx, whereas ATP was less potent (pEC_{50}=3.3±0.08). ADP and 2MeS-ATP also stimulated YO-PRO-1 influx at concentrations in excess of 300μM. Adenosine (1mM), UTP (100μM) and CPA (100μM) did not stimulate YO-PRO influx. Substitution of the sucrose in the buffer with 140mM NaCl or 140mM KCl inhibited DbATP-stimulated YO-PRO-1 influx >85%, although the pEC_{50} values for DbATP were similar (4.6±0.35 and 4.4±0.11 in the presence of NaCl and KCl respectively). DbATP-stimulated YO-PRO-1 influx was non-competitively inhibited by pre-incubation with various P2 antagonists for 20min. Coomassie blue G and suramin were the most potent antagonists (pIC_{50}=6.7±0.10 and 6.4±0.15 respectively, against 50μM DbATP). Evans' blue, cibacron blue and P5P also inhibited DbATP-stimulated YO-PRO-1 influx (pIC_{50}=5.7± 0.19, 5.5±0.15 and 5.3±0.12 respectively).

In conclusion, this study demonstrates that $P2X_7$ receptor function can be quantified in NTW8 cells by measurement of either $^{45}Ca^{2+}$ or YO-PRO influx. DbATP-stimulated $^{45}Ca^{2+}$ and YO-PRO influx are both reduced by monovalent cations and can be blocked by a number of purinoceptor antagonists. Interestingly, suramin was more potent at blocking DbATP-stimulated YO-PRO influx than $^{45}Ca^{2+}$ influx. Finally, the rate of DbATP-stimulated YO-PRO influx in NTW8 cells was much more rapid than in CHO-K1 cells (Michel *et al.*, 1997).

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129P GENE-RELATED PEPTIDE 8-37 TO ITS RECEPTORS ON RAT L6 SKELETAL MYOCYTES

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Calcitonin gene-related peptide (CGRP) increases cyclic AMP in rat L6 myocytes (Poyner *et al.*, 1992). The receptors have a high affinity for the antagonist CGRP8-37 and so correspond to the putative CGRP1 subclass (Quirion *et al.*, 1992). The conformation of the C-terminal residues of CGRP8-37, particularly a β-bend between G^{33} and S^{34}, may be critical for high affinity binding of this antagonist (Boulanger *et al.*, 1996; Wisskirchen *et al.*, 1997). This study reports the results of an alanine scan of residues 30 to 37, in an attempt to elucidate the importance of specific amino-acids to high-affinity binding.

Experimental methods were as described previously (Poyner *et al.*, 1992). Concentration-response curves for human α–CGRP were constructed in the presence and absence of antagonists. Cells were pre-incubated with antagonist for 10 min, followed by a 5 min challenge with CGRP. Cyclic AMP was extracted and measured by a radio-receptor assay. Antagonists were used at 30nM (A36S, S34A), 0.3μM (CGRP8-37, F37S), or 1μM (the remainder; Table 1.) Dose-ratios were calculated for each antagonist. and used to calculate apparent pA2 values, by the Schild Equation. These values were compared with that for CGRP8-37. Statistical analysis was by one-way ANOVA followed by Dunnett's test.

CGRP stimulated cyclic AMP production in L6 cells with a pEC50 of 9.43±0.05 (n=31). All antagonists caused a parallel rightwards shift in the CGRP concentration-response curve. As they were only used at single concentrations, it was not possible to confirm competitive inhibition, but this has previously been demonstrated for CGRP8-37 (Poyner *et al.*, 1992). As can be seen in Table 1, replacement of K^{35}, G^{33}, V^{32}, and T^{30} by alanine all decreased affinity. Alanine occurs naturally in the sequence at position 36; substitution of this by serine tended to increase affinity, albeit not significantly.

This data indicates that the C-terminus of CGRP contains a number of amino acids which play a role in maintaining high affinity to the receptor. It remains to be determined whether these make direct contacts with the receptor or stabilise a favourable conformation of the ligand. It may also be possible to increase affinity by substitutions in the vicinity of S^{36}

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Table 1. Apparent pA2 values for CGRP8-37 analogues

Analogue	Appar. pA2	n	Analogue	Appar. pA2	n
CGRP8-37	8.11±0.16	3	S34A	8.36±0.09	4
F37A	7.90±0.20	3	G33A	7.10±0.02*	3
A36S	9.16±0.22	4	V32A	7.07±0.17*	4
K35A	6.17±0.15*	3	N31A	7.60±0.10.	4
			T30A	6.47±0.09*	3

Analogues indicate original residue, position and replacement.
*; Significantly different from CGRP8-37, $P<0.05$.
Apparent pA2 values are means ± s.e.m.

130P SELECTIVITY OF THE P-GLYCOPROTEIN SUBSTRATES RHODAMINE-123 AND DOXORUBICIN IN WILD-TYPE AND MDR1 cDNA TRANSFECTED LLC-PK1 CELLS

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P-glycoprotein (Pgp) is a transmembrane, energy-dependent efflux pump, involved in the transport of a variety of compounds out of the cell. It confers to the multidrug resistance (MDR) phenomenon and is expressed in many tumor cells, but also in normal tissues, e.g. the cerebral endothelial cells representing the blood-brain barrier.
The aim of this study was to investigate whether changes in transport of Rhodamine-123 (R123) and doxorubicin (dox), both Pgp substrates, could be used as an indicator for the modulation of Pgp functionality.

Pgp is encoded by the human MDR1 gene. Monolayers of kidney epithelial LLC-PK1 cells, both the wild-type and the MDR1 cDNA transfected cells (Schinkel et al., 1995), were used to investigate polarized transport of R123 and dox (1 μM). Differences in basal to apical transport compared with apical to basal transport, were estimated during 4 h. Inhibition of polarized transport for known Pgp inhibitors was determined after 3 h.

Both R123 and dox showed highly polarized transport using the MDR1-transfected cell line. Surprisingly, for R123 polarized transport was seen as well in the monolayers of the wild-type cells, indicating the presence of another active transporter for this molecule.

The inhibition by the Pgp inhibitors PSC 833 (2 μM) and cyclosporin A (10 μM); the organic cation and Pgp inhibitors verapamil (20 μM) and quinine (50 μM); and the organic cation transport inhibitor cimetidine (50 μM) on the polarized transport of R123 and dox was investigated.
Active transport of R123 in monolayers of the MDR1-transfected cells was inhibited by all drugs (20-50%), including cimetidine (30%). For dox, transport inhibition was found by quinine (15%) and the other drugs (50-70%), but not for cimetidine. In the monolayers of the wildtype cells the transport of R123 was not inhibited by PSC 833, while the use of the other inhibitors resulted in a decrease (30-80%, with 50% for cimetidine).

In conclusion, it seems that R123 is also subjected to active transport by the organic cation carrier as indicated by our studies and also by Masereeuw *et al.* (1997). This would indicate that R123, now often used as a marker for Pgp functionality, is not selective enough to study Pgp functionality in cell systems in which organic cation carriers are present. Furthermore, doxorubicin seems to be a more selective Pgp substrate and therefore useful for studies to Pgp in this cell system.

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131P AN INVESTIGATION INTO THE NMDA RECEPTOR SUBTYPE SELECTIVITY OF TWO NOVEL GLYCINE SITE ANTAGONISTS

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The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor is a ligand-gated cation channel. Molecular cloning has identified five genes encoding two types of NMDA receptor subunit, NR1 and NR2A-D. Alternative splicing of the NR1 subunit gene provides a further level of heterogeneity. Most functional native receptors are believed to be heteromeric complexes comprising both NR1 and NR2 subunits in as yet unknown stoichiometries. Recent interest in the glycine co-agonist site as a potential therapeutic target has yielded several novel, high affinity glycine site antagonists including MDL 105,519 and more recently, GV150,526A, (3-[2-(phenylaminocarbonyl) ethenyl]-4,6-dichloroindole-2-carboxylic acid sodium salt). Here, we investigate the subtype selectivities of these drugs by radioligand binding to cloned receptors expressed in human embryonic kidney (HEK) 293 cells.

HEK 293 cells were transfected with the appropriate NMDA receptor clones, cells collected 48 h post-transfection and [^{3}H] MDL 105, 519 radioligand binding was carried out by a filtration assay with an incubation time of 90 min at 4°C [Chazot et al., 1994]. Table 1 summarises the results of both the Dissociation Constants (K_D) for direct [^{3}H] MDL 105,519 binding and the Inhibitory Constants (K_I) for GV150,526A determined by displacement studies. Both compounds bound with high affinity to NR1 expressed alone with no significant difference between the NR1-1a and NR1-4b forms. There was no significant difference in K_Ds for [^{3}H]MDL 105,519 binding to NR1 alone, NR1/NR2A and NR1/NR2B binary combinations and adult rat forebrain membranes however, the GV 150,526A has a small but significantly lower affinity for NR1/NR2B compared to NR1/NR2A receptors ($p<0.05$) (Table 1).

In conclusion, these studies demonstrate that the major determinants for MDL 105,519 and GV 150,526A binding are present on the NR1 subunit. Furthermore, the N1 exon of the NR1 subunit does not effect their affinity and finally, neither the NR2A nor the NR2B subunit has a major effect on the binding affinity of these two glycine site antagonists.

Table 1 Subtype selectivity of MDL 105,519 and GV150526A

Preparation	MDL 105,519 K_D (nM)	GV150526A K_I (nM)
NR1-1a	2.9 ± 1.9	3.4 ± 1.5
NR1-4b	3.1 ± 2.4	5.0 ± 3.0
NR1-1a/NR2A	1.9 ± 1.0	1.5 ± 0.7
NR1-1a/NR2B	3.0 ± 2.0	7.2 ± 3.8
Forebrain	2.5 ± 0.3	1.8*

Results are mean ± SD for 2-4 separate determinations.

* Bunnemann, B., Carignani, C., Palea, S. *et al.*, (1996) *Soc. Neurosci. Abstr.* **22** 366.15.

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132P PROLONGED ACTIVATION OF $mGlu_2$ RECEPTORS ENHANCES FORSKOLIN-STIMULATED cAMP ACCUMULATION

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The metabotropic glutamate receptor $mGlu_2$ is classed as a group II mGlu receptor, and couples to inhibition of adenylyl cyclase activity (Tanabe *et al.*, 1992). Examination of its susceptibility to agonist-induced desensitization has not been reported. In this study we investigated the ability of $mGlu_2$ receptors stably expressed in Chinese Hamster Ovary (CHO) cells to undergo agonist-induced desensitization. Cells stably transfected with the rat $mGlu_2$ receptor ($mGlu_2$-CHO) or the rat $mGlu_5$ receptor ($mGlu_5$-CHO) in the plasmid pdKCR-dhfr were cultured in DMEM containing 10% fetal bovine serum, 25mM glucose, 1% (w/v) proline, 1000 units/ml penicillin, 1000 µg/ml streptomycin, 0.25 µg amphotericin B and 2mM glutamine. Cyclic AMP accumulation in intact cells was measured in the presence of the phosphodiesterase inhibitor 250 µM Ro201724, exactly as described previously (Mundell *et al.*, 1997).

The group II selective agonist (2s,1's,2's)-2-(Carboxycyclopropyl) glycine (L-CCG-1) inhibited 10µM forskolin-stimulated cAMP accumulation in $mGlu_2$-CHO cells in a dose-dependent manner (IC_{50} 150 ± 12 nM; n=4), but not in $mGlu_5$-CHO cells. The group II selective antagonist (2S)-α-ethylglutamate concentration-dependently reversed the inhibitory effect of 1µM L-CCG-1 (IC_{50} 842 ± 237 µM). $mGlu_2$-CHO cells were incubated in the presence or absence of 10µM L-CCG-1 for 2h, following which the cells were washed and exposed acutely to forskolin or forskolin plus 1µM L-CCG-1. In non-L-CCG-1-pretreated cells acute L-CCG-1 (1 µM) inhibited forskolin-stimulated cAMP by 95 ± 3% and in L-CCG-1-pretreated cells by 97 ± 2% (n=4).

In cells pretreated with L-CCG-1 for 2h, however, there was a marked increase in the response to forskolin. In non-L-CCG-1 pretreated cells the forskolin-stimulated cAMP concentration was 731 ± 78, whilst in 10µM L-CCG-1 pretreated cells, 1592 ± 283 pmol/mg protein/10 min; $P<0.05$, Student's t test for paired data, n=4). Basal cAMP accumulation was not altered by 10 µM L-CCG-1 pretreatment (298 ± 26 and 304 ± 16 pmol/mg protein/10 min in non-pretreated and pretreated cells, respectively). The L-CCG-1-induced increase in forskolin responsiveness was abolished by pretreatment of $mGlu_2$-CHO cells with pertussis toxin (25ng/ml; 18h) or co-application of 1mM (2S)-α- ethylglutamate.

These results indicate that $mGlu_2$-CHO receptors appear refractory to rapid agonist-induced desensitization, but that chronic activation of these receptors increases adenylyl cyclase responsiveness in a receptor- and pertussis toxin-sensitive G-protein-dependent manner.

We thank the MRC for financial support.

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133P EVIDENCE THAT HYPOXIA ENHANCES Ca^{2+} INFLUX THROUGH NICOTINIC ACETYLCHOLINE RECEPTORS IN THE HUMAN NEUROBLASTOMA SH-SY5Y

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Our recent studies have indicated that acute hypoxia enhances evoked release of [^{3}H]noradrenaline ([^{3}H]NA) from the human neuroblastoma SH-SY5Y (Wade *et al.*, 1997). This effect is selective in that release evoked by activation of nicotinic acetylcholine receptors (nAChRs) was enhanced by hypoxia, but not release evoked by solutions containing 100mM K^+. Here, we have investigated this action of hypoxia in greater detail.

Aliquots of SH-SY5Y cells in suspension were pre-loaded with [^{3}H]NA as previously described (Vaughan *et al.*, 1993) and perfused in a perifusion chamber at 1-2ml min^{-1}. Each aliquot was challenged twice (15min separation), using dimethylphenylpiperazinium iodide (DMPP; 30μM) as the agonist for nAChRs, and release of [^{3}H]NA determined by liquid scintillation counting (see Vaughan *et al.*, (1993) for details and solution compositions). Results are expressed as release evoked by the second challenge, S_2 , as a fraction of the first challenge, S_1, i.e. the S_2/S_1 ratio. Drugs and / or solution changes were made 4 min prior to, and during, the second challenge except for hypoxia , which was applied 9 min prior to, and during, the second DMPP challenge. Statistical significance was determined using unpaired Studet's t-tests.

In normoxic conditions, the S_2/S_1 ratio was 0.62±0.04 (mean ± s.e.m., n=16 experiments). This ratio was significantly greater (0.96±0.06, n=12, P<0.0002) in hypoxia (Po_2 *ca.* 30mmHg). In normoxia, the S_2/S_1 ratio was unaffected (0.61±0.05, n=4) by 3μM tetrodotoxin, suggesting a lack of involvement of Na^+ channels. By contrast, the S_2/S_1 ratio was significantly (P<0.0001) reduced in Ca^{2+}-free solutions (containing 1mM EGTA), to 0.02±0.01 (n=6). The S_2/S_1 ratio was also reduced by Cd^{2+} (200μM, sufficient to fully block L- and N-type Ca^{2+} channels in these cells; Reeve *et al.*, 1994) to 0.25±0.04 (n=7). This reduction was to a significantly lesser (P<0.001) degree than was seen in Ca^{2+} -free solutions, suggesting that Ca^{2+} influx through the nAChR pore contributed to DMPP-evoked [^{3}H]NA release. [^{3}H]NA release was also measured in the presence of 200μM Cd^{2+} under hypoxic conditions (Po_2 *ca.* 30mmHg, applied for 9 min prior to and during DMPP application). The S_2/S_1 ratio under these conditions (0.43±0.03, n=6) was significantly greater (P<0.02) than under normoxic conditions in the presence of Cd^{2+}.

Our results suggest that Ca^{2+} influx through the nAChR pore contributes significantly to [^{3}H]NA release from SH-SY5Y cells, and that this influx is enhanced under hypoxic conditions.

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134P PHOTOSENSITISATION OF PANCREATIC TUMOUR CELLS: EFFECTS OF MITOCHONDRIAL BENZODIAZEPINE RECEPTOR (MBR) LIGANDS

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The involvement of the mitochondrial benzodiazepine receptor (MBR) in the molecular mechanisms of δ-aminolaevulinic acid (ALA)-mediated photodynamic therapy (PDT) was examined in AR4 2J cells, a rat pancreatic tumour cell line, as a model system. Addition of exogenous ALA to the cultured cells led to their photosensitisation by the generation of photo-active protoporphyrin IX (PPix). In order to study the molecular basis of this effect AR4 2J cells were grown as a monolayer on small, poly-D-lysine coated coverslips which were inserted into a fluorimeter cuvette. Fluorescence emission from the cells was measured between 500 and 700 nm following excitation at 405 nm, the excitation maximum for PPix. Accumulation of PPix (emission maximum at 630 nm) displayed a steep dose-response curve for a 24 hour incubation at 37°C, the fluorescence signal increasing from 0.16 ± 0.02 in the absence of ALA to 0.43 ± 0.14, 1.47 ± 0.26, 2.21 ± 0.29 and 3.68 ± 0.33 in presence of 10, 50, 100 and 500 μM ALA, respectively (n = 6). The magnitude of PPix fluorescence also depended on the time of incubation, increasing from 0.13 ± 0.02 to 0.35 ± 0.02, 0.46 ± 0.02, 0.83 ± 0.05 and 2.21 ± 0.27 after a 2, 4, 8 and 24 hour incubation with 100 μM ALA, respectively (n = 6). Subsequent cellular photodestruction by exposure to light (λ > 400 nm) could be attributed to the production of singlet oxygen and hydroxyl radicals from the endogenous PPix (Kessel, 1977).

The mitochondrial benzodiazepine receptor is a high affinity binding site for dicarboxylic porphyrins, especially PPix, and has been implicated in the translocation of PPix and haem across mitochondrial membranes, presumably by way of an anion channel (Verma & Snyder, 1989). Further investigations showed that ligands of the MBR, such as the isoquinoline carboxamide PK11195, had a photoprotective effect on ALA-mediated PDT (Ratcliffe, & Matthews, 1995). Our results showed that the photoprotective effect was probably due to the interference of the MBR ligand PK11195 with the production of PPix from its precursors since the cellular PPix fluorescence produced by a fixed concentration of ALA (100 μM) was significantly reduced ($p < 0.01$) by the addition of 10 μM PK11195, from 2.21 ± 0.27 to 1.45 ± 0.14 for a 24 hour incubation with the MBR ligand (n = 4). Similar effects were seen when 10 μM dipyridamole, an alternative MBR ligand, was added to the incubation medium, causing a reduction of the fluorescence to 0.58 ± 0.15 (n = 4). The possibility that PK11195 blocks the transport of substrates between various cellular compartments is supported by changes in the fluorescence at 575 nm of an as yet unidentified metabolic product or precursor of the ALA-induced PPix which was significantly increased ($p < 0.05$) in the cell monolayers in the presence of 10 μM PK11195 *i.e.*, from 0.47 ± 0.09 to 0.62 ± 0.06 (n = 4), whereas the PPix fluorescence itself was decreased significantly ($p < 0.01$) from 2.21 ± 0.27 to 1.45 ± 0.14 (n = 4).

It can be concluded from these studies that the MBR is involved in the metabolic pathway of PPix production. MBR ligands are likely to exert their photoprotective effect by preventing the accumulation of phototoxic PPix and may therefore be used to control ALA-mediated PDT. Detailed knowledge of the kinetics and molecular mechanisms involved in the cellular production of phototoxic PPix is clearly of importance for the future use of ALA in cancer therapy.

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135P ACTION OF 5-HT$_{1B}$ RECEPTOR AGONISTS AT THE HUMAN HISTAMINE H$_1$ RECEPTOR EXPRESSED IN HEK-293 CELLS

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Sumatriptan and metergoline have been reported to have full, and methysergide partial, agonist activity at the 5-HT$_{1B}$ receptor (Miller *et al.*, 1992 and Gunning *et al.*, 1988). Sumatriptan and methysergide have also been reported to possess low affinity and low efficacy for the histamine H$_1$-receptor in rabbit femoral artery and guinea-pig ileum (MacLennan et al., 1991; Gunning *et al.*, 1988). Furthermore, in tissues expressing both 5-HT$_{1B}$ and H$_1$-receptors, amplification between these two receptor responses has been observed (MacLennan et al., 1991). In this study we have examined the agonist activity of the three 5-HT$_{1B}$ agonists at the human histamine H$_1$ receptor expressed in a human embryonic kidney cell line (HEK-293).

HEK-293 cells, transfected with the human H$_1$-receptor cDNA (specific ^{3}H-mepyramine binding; B_{MAX} 568 fmol/mg protein; K_D 2 nM; Walker *et al.*, 1997) were grown in 24-well plates in DMEM/F-12 medium containing 10 % FCS, 2 mM L-glutamine and 500 µg/ml G418. Cells were labelled with [^{3}H]-*myo*-inositol for 24 hours prior to the measurement of total [^{3}H]-inositol phosphate (IP) accumulation. This was performed by modifying the protocol described by Megson *et al.*, (1995) to include a centrifugation step to remove cell debris. All data are expressed as mean ± s.e.mean, n ≥ 3.

A concentration-dependent increase in the accumulation of IP was observed for sumatriptan and methysergide (Table 1). These responses were completely attenuated by pre-incubation with the H$_1$-receptor antagonist mepyramine (10µM; n = 3). However, no direct response was found with metergoline (Table 1). In addition, the maximal response for both sumatriptan and methysergide, when compared to histamine, was considerably lower (Table 1). All three 5-HT$_{1B}$ agonists shifted histamine concentration-response curves to higher agonist concentrations. Dissociation constants (K_B) for the binding of 5-HT$_{1B}$ agonists to the histamine H$_1$-receptor are shown in Table 1.

Table 1. Effect of 5-HT$_{1B}$ ligands on IP accumulation

	EC_{50} (µM)	% response*	K_B (µM)
Histamine	0.7 ± 0.1 (14)	100 (14)	
Sumatriptan	110.0 ± 40.8 (3)	3.8 ± 0.7 (3)	15.7 ± 7.1 (3)#
Methysergide	2.6 ± 1.2 (3)	6.6 ± 0.9(3)	7.6 ± 1.6 (3)#
Metergoline	N/A	N/A	0.48 ± 0.14 (3)

(*% maximal response compared to 100 µM Histamine; n values given in parenthesis; # calculated according to Stephenson, 1956).

In summary, the data presented demonstrates that both sumatriptan and methysergide are low affinity, low efficacy agonists at the human H$_1$ histamine receptor. Conversely, metergoline behaves as an H$_1$-receptor antagonist.

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136P FUNCTIONAL COUPLING OF HUMAN 5-HT$_{1A}$ RECEPTORS TO HUMAN G-PROTEIN COUPLED INWARD RECTIFIER K$^+$ CHANNELS EXPRESSED IN *XENOPUS* OOCYTES

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5-HT$_{1A}$ receptors couple to G-protein coupled inward rectifying K$^+$ channels (GIRK) in hippocampal pyramidal neurones (Andrade & Nicoll, 1987). Human 5-HT$_{1A}$ receptors have been shown to couple to human GIRK's in a membrane delimited manner when the respective mRNA's are co-injected into *Xenopus* oocytes (Schoots *et al.*, 1996). The aim of this study was to examine the pharmacology of this expression system to determine its validity as a model of h5-HT$_{1A}$ receptor function.

hGIRK1, hGIRK2 and h5-HT$_{1A}$ containing plasmids were injected in a 3:1 ratio of GIRK to 5-HT$_{1A}$ into the nucleus of excised and isolated *Xenopus* oocytes (30ng cDNA total). Two to six days later, two-electrode whole cell voltage clamping was used to determine the functional response of the cells.

Reversal of the K$^+$ concentration gradient by switching from a high Na$^+$ buffer (96mM Na$^+$) to a high K$^+$ buffer (96mM K$^+$) caused an inward current (I_K) in all cells tested (n=212). In 35/50 cells which did not subsequently respond to 5-HT, I_K was 30-70nA and was not blocked by addition of Ba^{2+} ions (200µM); Ba^{2+} is a GIRK channel blocker with no effect on endogenous oocyte K$^+$ channels. In the remaining 15 cells which were not responsive to 5-HT, I_K was 0.7-1.4µA and was blocked by Ba^{2+} suggesting the expression of GIRK channels which had a basal activity without 5-HT$_{1A}$ receptor expression.

Following reversal of the K$^+$ concentration gradient, addition of 5-HT (1µM) caused a further increase in inward current ($I_{5\text{-}HT}$) in some oocytes in the order of 0.36-4.5µA; I_K for these cells was 0.4-7.6µA. In four cells, a full sequential concentration response curve for 5-HT was determined with a resultant EC_{50} of 99 ± 21nM (mean ± SEM). Ba^{2+} (200µM) blocked I_K in 5-HT responsive cells by 55.0 ± 3.3% (n=8 cells). The 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) also elicited inward current (0.2-1.8µA) following reversal of I_K (n=8 cells). Responses to the agonists did not plateau but gradually desensitised following a rapid inward current. Fifteen minute washout periods were required to prevent tachyphylaxis during subsequent agonist application. Interestingly, in 2/8 cells tested with 8-OH-DPAT, the responses did not desensitise although tachyphylaxis still occurred.

Injection of pertussis toxin (2.5ng) resulted in a total abolition of 5-HT induced current and no change in I_K (n=6 cells) showing that the 5-HT$_{1A}$ receptor couples to the GIRK's through G_i/G_o G-proteins.

The 5-HT$_{1A}$ selective antagonist, WAY 100635 (10nM & 1µM) blocked the 5-HT (100nM) evoked inward current (n=4 cells) when both compounds were co-applied; WAY 100635 reduced $I_{5\text{-}HT}$ by 45.0 ± 10.8% (10nM) and 91.2 ± 4.6% (1µM). WAY 100635 (100nM) also reduced 8-OH-DPAT (100nM) evoked responses by 32.5 ± 6.8% (n=4).

Co-injection of human 5-HT$_{1A}$ cDNA with the cDNA for human GIRK1 and 2 subunits leads to functional expression of the receptors and channels which couple to each other in a pertussis toxin-sensitive manner. This system exhibits pharmacology characteristic of 5-HT$_{1A}$ receptors and could be used as a model to investigate the pharmacology of 5-HT$_{1A}$ receptors.

Thanks to Dr. A. Bach (BASF, Germany) for the h5-HT$_{1A}$ clone and to Dr. Douglas Fraser for assistance with cDNA preparation.

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137P FUNCTIONAL EXPRESSION OF HUMAN 5-HT_{1A} RECEPTORS BY LINKAGE TO HUMAN G PROTEIN-COUPLED, INWARDLY-RECTIFYING POTASSIUM CHANNELS TYPE K_{ir} 3.0 (GIRK) IN *XENOPUS* OOCYTES

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Inwardly-rectifying potassium channels (K_{ir}s) belong to one of five subfamilies (K_{ir} 1.0-K_{ir} 5.0; Chandy & Gutman, 1993). The G protein-coupled potassium channels belong to K_{ir} 3.0 (also known as GIRK). GIRKs couple in a membrane-delimited manner to neurotransmitter receptors including 5-HT_{1A}, dopamine D_2-like and opioid receptors.

In this study, human 5-HT_{1A} (h5-HT_{1A}) receptors were functionally expressed in oocytes from *Xenopus laevis* by injecting nuclei of mature oocytes with 25-150 ng of plasmid DNAs (pCIS2 and pcDNA1) coding for h5-HT_{1A} receptors and human GIRK1 (hGIRK1) and GIRK4 (hGIRK4). Recordings were made from day 5 post-injection using a two-electrode voltage clamp technique. Application of 1 μM 8-OH-DPAT to oocytes expressing functional h5-HT_{1A} receptors elicited very small outward currents (~5-10 nA) if the recordings were made in conventional extracellular saline (96 mM NaCl, 2 mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM HEPES; pH 7.5). The K^+ ion gradient was thus reversed by equilibrating oocytes in a high potassium-based saline (96 mM KCl, 2 mM NaCl) before recording agonist-evoked inward K^+ currents listed as the mean ± s.e.mean. Basal K^+ currents were always greater in injected oocytes (86.1 ± 16.1 nA; n=22) than in uninjected oocytes (26.1 ± 2.3 nA; n=10). Whilst the basal K^+ current in 7 uninjected oocytes was not altered by 300 μM $BaCl_2$, both the K^+ currents in 9 oocytes expressing only hGIRK channels and the K^+ currents evoked by 1 μM 8-OH-DPAT in oocytes expressing functional h5-HT_{1A} receptors were reversibly inhibited in a voltage-dependent manner by superfusion of 300 μM $BaCl_2$. The 5-HT_{1A} antagonist, *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)-cyclohexanecarboxamide (WAY 100,635), antagonised the 8-OH-DPAT-evoked response (n=4; IC_{50} = 56 ± 18 nM) in oocytes expressing functional h5-HT_{1A} receptors. This inhibition was specific for the h5-HT_{1A} receptor because the basal K^+ current in 9 oocytes expressing only hGIRK channels was unaffected by WAY 100,635 (10^{-9} to 10^{-4} M). 8-OH-DPAT-evoked responses were abolished by injection of 3 ng of pertussis toxin 3 hours earlier (n=6) and the basal K^+ current was maximally reduced by 80%. This block by pertussis toxin implicates the G_i/G_o subunits in signal transduction from the h5-HT_{1A} receptor.

Here, we found that 8-OH-DPAT-evoked responses from oocytes with h5-HT_{1A} receptors/hGIRK1 plus 4 combinations usually lay between 0.05 and 0.5 μA. In a complementary study, Watt *et al.* (1997) report functional coupling of h5-HT_{1A} receptors to hGIRK 1 plus hGIRK 2 that yielded responses to 8-OH-DPAT of 0.2-1.8 μA and similar sensitivities to $BaCl_2$, pertussis toxin and WAY 100,635.

In this pilot investigation, the responses evoked by 8-OH-DPAT were inhibited at three different loci, *viz.* (i) by WAY 100,635 at the 5-HT_{1A} receptor level, (ii) by pertussis toxin at the G protein level and (iii) by Ba^{2+} at the hGIRK protein channel level. To our knowledge, these data represent the first published quantitation of the potency of WAY 100,635 as an antagonist to human 5-HT_{1A} receptors coupled to human GIRKs as well as the functional sensitivity of this system to pertussis toxin.

Thanks to Dr. A. Bach (BASF, Germany) for the h5-HT_{1A} clone.

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138P INVESTIGATION OF PROTEIN KINASE C ISOENZYME EXPRESSION, DOWNREGULATION AND TRANSLOCATION IN CHO-K1 CELLS EXPRESSING H_1-HISTAMINE RECEPTORS

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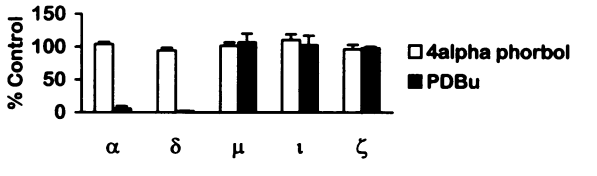
In a previous communication (Sanderson *et al.* 1996a), we reported that histamine (HA) can potentiate forskolin-stimulated cyclic AMP accumulation, as well as yielding a small cyclic AMP response on its own, in CHO-K1 (CHO-H1) cells, transfected with the bovine histamine H_1-receptor. Neither of these H_1-receptor mediated responses depended on the discharge of intracellular Ca^{2+} stores, or subsequent capacitative entry of extracellular Ca^{2+} (Sanderson *et al.*, 1996). However, the potentiation of the forskolin response was susceptible to inhibition by the protein kinase C (PKC) inhibitor, Ro 31-8220, and prolonged (24 hr) treatment of the CHO-H1 cells with the PKC activator phorbol 12,13-dibutyrate (PDBu, Sanderson and Hill, 1996). In this study, we have used Western blot analysis to examine which PKC isoforms are present in CHO-H1 cells, are down-regulated by PDBu, and therefore which are involved in the potentiation response.

Protein samples were prepared from confluent monolayer cultures of CHO-H1 cells, either as Triton X-100 detergent extracts from whole cells (PDBu treatments) or as cell membrane preparations (HA stimulations). Proteins were separated by 7.5% SDS-PAGE, transferred to nitrocellulose paper and immunodetected with monoclonal PKC isoform specific antibodies, using the ECL system. The developed blots were quantified densitometrically, using Molecular Analyst™ software. The quantified data are expressed as mean ± s.e.m., n=3, and analysed by paired Student's t-test.

Five PKC isoforms were readily detected in CHO-H1 cells, the conventional PKC isoform, PKC α, the novel isoforms PKC δ and PKC μ and the atypical isoforms PKC ι and PKC ζ. Treatment of CHO-H1 cells for 24 hr at 37 °C with PDBu (1 μM) but not its inactive analogue 4α phorbol (1 μM), caused the down-regulation of only PKC α and PKC δ (Fig 1).

Fig 1. Down-regulation of PKC isoforms by PDBu



Acute stimulation (5 min, 37 °C) of CHO-H1 cells with HA (100 μM) also increased the level of PKC α associated with cell membranes to 399 ± 64% of control values ($p< 0.05$). The regulation of PKC δ by H_1-receptor activation remains to be determined.

These results strongly suggest that at least one PKC isoform, PKC α, is involved in the potentiation of forskolin-stimulated cyclic AMP accumulation by bovine histamine H_1-receptors expressed in CHO-K1 cells.

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139P POTENTIATION OF ADENOSINE A_{2b} RECEPTOR-STIMULATED CYCLIC AMP ACCUMULATION BY HISTAMINE H_1 RECEPTORS IN HEK-293 CELLS

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In brain, stimulation of histamine (HA) H_1 -receptors significantly augments the cyclic AMP (cAMP) response to adenosine A_{2b}-receptors (Hill 1990). In this study we have transfected human H_1-receptor DNA into human embryonic kidney (HEK-293) cells, and examined whether these receptors can elicit a similar augmentation of the cAMP response to the adenosine A_{2b}-receptors endogenously expressed in this cell line (Cooper *et al.*, 1995).

Accumulation of [^{3}H]-cAMP and total [^{3}H]-inositol phosphates ([^{3}H]-IP) in cell monolayers and [^{3}H]-mepyramine binding to cell membranes were measured as previously described (Iredale *et al*, 1993; Megson *et al.*, 1995). All data are expressed as mean ± s.e.m., $n \geq 3$. Data from [^{3}H]-cAMP assays were analysed by two-way ANOVA and then by post-hoc Newman-Keuls tests.

Binding of the H_1-receptor selective radioligand [^{3}H]-mepyramine was undetectable in the untransfected HEK-293 parent cell line (HEK). Low and moderate levels of binding, respectively, were observed In the two transfected cell lines, H1j2 and H1j1 (Table 1), with K_D's consistent with published values (Table 1, Moguilevsky *et al.*, 1994). However, HA, at a maximally stimulating concentration (100 μM), gave [^{3}H]-IP responses in all three cell lines (Table 1). These responses were abolished by mepyramine (apparent K_B's: HEK, 9 ± 2; H1j1, 14 ± 5 nM) confirming that they were mediated by either endogenous (HEK) or transfected (H1j1, H1j2) human H_1-receptors.

The adenosine A_{2b}-receptor agonist, 5'-N-ethylcarboxamide-adenosine (NECA) gave strong [^{3}H]-cAMP responses in all of the three cell lines. HA alone gave no significant responses, but increased the response to NECA by 17% in H1j2, and 54% in H1j1 cells, $p<0.01$. (Table 1). This action of HA was sensitive to mepyramine (in H1j1 cells: apparent $K_B = 14 \pm 1$ nM), again suggesting mediation by H_1-receptors.

These results show that human H_1-receptors in HEK-293 cells can augment adenosine A_{2b} stimulated cAMP responses, as observed in brain. In the HEK-293 cells, the size of the augmentation seems to parallel the expression levels and G_q/G_{11}-coupled activity of the H_1-receptors.

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Table 1 Expression and Cell Signalling Responses of Human Histamine H_1-receptors in HEK-293 cells

	[^{3}H]-Mepyramine Binding		[^{3}H]-IP Response to Histamine	[^{3}H]-cAMP Response (fold over basal)		
Cell line	Bmax (fmol/mg)	K_D (nM)	100 μM HA (fold over basal)	10 μM NECA	100 μM HA	10 μM NECA + 100 μM HA
HEK	-	-	2.4 ± 0.2	17.2 ± 0.2	1.2 ± 0.2	17.4 ± 0.2
H1j2	45 ± 8	3 ± 1	5.7 ± 0.3	23.0 ± 0.2	1.2 ± 0.2	26.7 ± 0.2
H1j1	568 ± 69	2 ± 1	56.5 ± 3.5	30.8 ± 0.5	1.2 ± 0.5	47.0 ± 0.5

140P THE HUMAN ADENOSINE A RECEPTOR ACTIVATES THE MAP KINASE SIGNALLING PATHWAY IN TRANSFECTED CHO-K1 CELLS

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Recent studies have shown that the mitogen-activated protein (MAP) kinase signalling pathway can be activated by a variety of G_i/G_o protein-coupled receptors (for review see Sugden & Clerk, 1997). In this study we have investigated whether the G_i/G_o-coupled human adenosine A_1 receptor activates the MAP kinase pathway in transfected Chinese hamster ovary cells (CHO-A1).

CHO-A1 cells were grown in 6-well plate cluster dishes and serum-starved for 16 h, in DMEM/F-12 containing 0.1% bovine serum albumin, prior to the MAP kinase assay. MAP kinase activity was determined in CHO-A1 cell lysates (following 5 min agonist stimulation) using the epidermal growth factor receptor 662-681 peptide as a selective MAP kinase substrate (Heasley *et al.* 1994).

The selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA; 1 μM) stimulated a 6.5 ± 0.7 (n=3) fold increase in MAP kinase activity The MAP kinase response to CPA was concentration-dependent ($p[EC_{50}] = 8.13 \pm 0.03$; n=3) and time-dependent (peak activation occurring at 5 min). Pre-treatment with pertussis toxin (100 ng/ml for 16 h) completely inhibited the response to 1 μM CPA, indicating that G_i/G_o protein(s) couple the adenosine A_1 receptor to the MAP kinase pathway. Responses to 1 μM CPA were also attenuated by pre-treatment (30 min) with the MAP kinase kinase 1 (MEK1) inhibitor, PD 98059 (50 μM; 89 ± 4% inhibition; n=3). Recent studies have indicated that tyrosine kinase(s) and phosphatidylinositol 3-kinase (PtdIns 3-kinase) are involved in the activation of MAP kinase by G_i/G_o protein-coupled receptors (Sugden & Clerk, 1997). In this study, pre-treatment with the tyrosine kinase inhibitor, genistein (100 μM; 30 min) attenuated the MAP kinase response to 1 μM CPA (94 ± 10% inhibition compared to control responses; n=4). In contrast, daidzein (100 μM; 30 min), the inactive analogue of genistein, had no significant effect on the responses elicited by 1 μM CPA (96 ± 12% of control response; n=4). Furthermore, pre-treatment (30 min) of CHO-A1 cells with the selective PtdIns 3-kinase inhibitor, LY 294002 (30 μM) or wortmannin (100 nM) inhibited 1 μM CPA-induced MAP kinase activation by 40 ± 5% (n=6) and 55 ± 8% (n=4) respectively. Finally, pre-treatment with the selective protein kinase C inhibitor Ro 31-8220 (10 μM; 30 min) had no significant effect on CPA-induced MAP kinase activation (98 ± 5% of control response; n=3).

In summary, we have shown that the transfected human adenosine A_1 receptor stimulates MAP kinase activity in CHO-A1 cells. The signalling pathway appears to involve tyrosine kinase, PtdIns 3-kinase and MEK1 activation. These observations are consistent with the known mechanisms for G_i/G_o protein-coupled receptor-mediated activation of MAP kinase (Sugden & Clerk, 1997).

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141P FUNCTIONAL EVIDENCE FOR A ROLE OF INTRACELLULAR ANGIOTENSIN II IN A7r5 CELLS

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Recent studies suggest a role of intracellular angiotensin II (A II) in the cardiovascular system which is not mediated by angiotensin plasma membrane receptors (De Mello, 1995; Haller et al., 1996). These effects are mediated by binding to an intracellular site which is coupled to different signal transduction systems than those known to be mobilized by AT_1 receptor subtype (Haller et al., 1996). In the present study, we investigated a possible role of intracellular angiotensin II in the fetal rat aorta cell line A7r5 by measuring membrane currents and by $^{45}Ca^{2+}$ flux experiments.

In the patch clamp study, (a holding potential by 40 mV, temperature 22° C) the extracellular application of A II (10^{-6} M) did not evoke any change in membrane currents (n=5). In contrast, the intracellular application of A II (10^{-6} M) evoked an apparent inward current of 247 ± 74 pA (mean ± s.e.m, n=3), after establishment of the whole cell configuration (Cs^+ main internal ion of intracellular solution). The inward current after $5HT_2$ receptor stimulation (10^{-5} M) and the outward current after P_2 purinoceptor stimulation with UTP (10^{-3} M) were inhibited by angiotensin II (10^{-6} M) diffused into the cell via the patch pipette, by 94 ± 2% (n=3) and 76 ± 3 % (n=4), respectively (table 1). Inclusion of the well characterized AT_1 receptor blocker, losartan (10^{-6} M) together with angiotensin II in the intracellular solution, partly restores the response to extracellular serotonin application (75%), but not to UTP (28 %, table 1).

A second functional evidence comes from experiments in which intracellular Ca^{2+} stores were loaded with $^{45}Ca^{2+}$. In saponin permeabilized cells, angiotensin II (10^{-6} M) potentiates the IP_3 (10^{-5} M) inducible Ca^{2+} mobilization with 23 ± 4 % (n=6, T=22° C). Since in these conditions, angiotensin II itself did not mobilize Ca^{2+} this indicates that it can act synergistically on phospholipase C coupled hormonal responses.

Table 1

treatment	current (pA)
5HT (10^{-5} M)	320 ± 70 (n=5)
5HT + Intracell AII	20 ± 15 (n=3)
5HT+ Intracell AII + losartan	240 ± 45 (n=3)
UTP (10^{-3} M)	-752 ± 74 (n=6)
UTP + Intracell AII	-180 ± 70 (n=4)
UTP + Intracell AII + losartan	-210 ± 55 (n=4)

These preliminary results show an intracellular role for angiotensin II in A7r5 smooth muscle cells. Intracellular angiotensin II acts as a modulator of membrane ions fluxes, as well as influencing IP_3-mediated calcium release from intracellular stores.

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142P CHARACTERISATION OF A [^{35}S]GTPγS BINDING ASSAY FOR CHEMOKINE CXC 1 AND 2 (IL-8 α AND β) RECEPTORS EXPRESSED IN CHO CELLS

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Chemokines (CKs) are a group of cytokines which act as leukocyte chemoattractants. They can be grouped into CC- and CXC-CKs depending on the relative positions of the first two of four conserved cysteine residues (Power & Wells, 1996). The major leukocyte target of the CXC-CKs is the neutrophil, which possesses two CXC receptors (CXCRs): CXCR1, which is specific for interleukin-8 (IL-8), and CXCR2, which responds to IL-8 and a number of other CXC-CKs (Power & Wells, 1996). Studies on recombinant cell lines have shown that both of these receptors induce increases in cytosolic Ca^{2+}, which are sensitive to pertussis toxin (PTX), indicating a role for Gi/Go G-proteins (Gerard & Gerard, 1994). We report the characterisation of a [^{35}S]GTPγS binding assay in membranes from Chinese hamster ovary (CHO) cells stably expressing heamagglutinin (HA)-tagged human CXCR1 and CXCR2.

CHO-CXCR1 (25 µg protein/ml) or -CXCR2 (50 µg/ml) membranes were equilibrated to 30°C for 20 min in assay buffer (100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES, pH 7.4) in the presence of GDP (10 µM). Agonist was added and followed 10 min later by [^{35}S]GTPγS (100 pM). The total assay volume was 250 µl. The reaction proceeded for 20 min before termination by rapid filtration, washing (4 x 1 ml ice-cold water) and determination of bound radioactivity using a scintillation counter. Non-specific binding was determined in the presence of 100 µM GTP. EC_{50} values and maximal effects were determined by fitting the data with a four parameter logistic equation.

Firstly, optimum assay conditions were determined. Increasing concentrations of GDP (3 nM - 100 µM) caused a concentration-dependent decrease in [^{35}S]GTPγS binding. In both membranes, optimal IL-8 stimulation of [^{35}S]GTPγS binding was obtained at 10 µM GDP. Binding was independent of agonist pre-incubation time up to 120 min. Varying the incubation time with [^{35}S]GTPγS (2 - 80 min) showed that IL-8-stimulated binding was linear up to 20 min. Binding also increased linearly with membrane protein concentration over the range 5 - 100 µg/ml.

IL-8 induced concentration-dependent increases in [^{35}S]GTPγS binding; pEC_{50} values were 8.64±0.11 (CXCR1; n=9) and 6.15±0.05 (CXCR2; n=7); the maximal increases in binding were 266±17% (CXCR1) and 600±48% (CXCR2) of basal (basal = 100%). Binding was also stimulated by GROα, another CXC-CK, which had pEC_{50} values of 6.17±0.13 (CXCR1; n=3) and 6.59±0.05 (CXCR2; n=3). The maximal effects were not significantly different from those of IL-8 (255±20% and 613±115% of basal, respectively; $p>0.05$, Student's t-test). These potency orders are consistent with those previously reported for other responses mediated by these receptors (e.g. Loetscher *et al.*, 1994). IL-8, up to 10 µM, was without effect in membranes prepared from PTX-treated cells (100 ng/ml; 16 hrs) or in membranes from untransfected CHO cells. These results indicate that wild-type CHO cells lack IL-8 receptors and that the responses are mediated by the transfected CXCRs, coupled to Gi/Go proteins.

Thus, in these cells, CXC-CKs induce [^{35}S]GTPγS binding via the transfected CXCRs in a manner which is consistent with their known pharmacology. Interestingly, in the CXCR1 membranes PTX treatment markedly decreased the basal [^{35}S]GTPγS binding perhaps suggesting constitutive activity of CXCR1 in these membranes.

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143P INFLUENCE OF EXTRACELLULAR Ca^{2+} ON AGONIST-STIMULATED PHOSPHOINOSITIDE HYDROLYSIS IN BABY HAMSTER KIDNEY CELLS EXPRESSING mGluR1α

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It has recently been demonstrated that the rat type 1α metabotropic glutamate receptor (mGluR1α) and a homologue of this receptor, the salmon bifunctional receptor (sBimR), are activated by both glutamate and extracellular Ca^{2+} (Ca^{2+}_e) when expressed in *Xenopus* oocytes (Kubokawa *et al.*, 1996). The aim of the present study was to investigate the effects of varying $[Ca^{2+}]_e$ on the response of baby hamster kidney (BHK) cells recombinantly expressing either mGluR1α (BHK-mGluR1α) or M_3-muscarinic receptors (BHK-m3) to agonist stimulation and to determine whether the effects of $[Ca^{2+}]_e$ on mGluR1α activation are receptor- or cell type-specific.

Cells were cultured in DMEM (Glutamax-1) supplemented with 5% dialysed foetal calf serum (dFCS), 50 μg ml^{-1} gentamicin, 0.5 mg ml^{-1} G418 and 1 μM methotrexate (BHK-mGluR1α); or 5% dFCS, 50 μg ml^{-1} gentamicin and 300 μg ml^{-1} hygromycin B (BHK-m3). [^{3}H]-inositol monophosphate ($InsP_1$) accumulation was measured in cells incubated in the presence of 1 μCi ml^{-1} *myo*-[^{3}H]-inositol for 48 h prior to agonist stimulation in the presence of 10 mM LiCl. The $InsP_1$ fraction was resolved by Dowex ion-exchange chromatography as previously described (Challiss *et al.*, 1993). Data are presented as means ± s.e.mean for n>3 separate experiments. Statistically significant differences between concentration-effect curves were defined using two-way ANOVA.

Varying Ca^{2+}_e from 0-4 mM had little effect *per se* on basal $InsP_1$ accumulation in either cell-line (in BHK-mGluR1α, nominally Ca^{2+}-free, 5972± 1320; 4 mM Ca^{2+}, 6408± 1432 d.p.m. mg^{-1} protein). In the presence of 4 mM Ca^{2+}_e quisqualate (Quis; 30 μM) and methacholine (MCh; 300 μM) caused, 8.9 ± 2.8 and 20.6 ± 2.9 fold increases in BHK-mGluR1α and BHK-m3 cells respectively. In both cell-lines, maximal agonist-stimulated $InsP_1$ responses were markedly decreased under nominally Ca^{2+}-free conditions (to 28 ± 6; and 33 ± 2% respectively of the response observed in 4 mM Ca^{2+}_e). The concentration-effect curve for Quis-stimulated $InsP_1$ accumulation was influenced by increasing $[Ca^{2+}]_e$ from 1.3 to 4 mM (with a decrease in EC_{50} and an increase in maximal response). In contrast, MCh responses in BHK-m3 cells were unaffected by increasing $[Ca^{2+}]_e$ from 1.3 to 4 mM (P>0.05). This difference between the Ca^{2+}_e dependencies of responses in BHK-mGluR1α and BHK-m3 cells was more dramatically revealed when partial agonist responses were investigated. Thus, the concentration-effect curve for **1S**,3**R**-ACPD in BHK-mGluR1α cells was significantly affected by increasing Ca^{2+}_e from 1.3 to 4 mM (P<0.01), with the maximal $InsP_1$ response increasing from 46 ± 6 to 66 ± 4% relative to the Quis-stimulated response. In contrast, the concentration-effect curve for arecoline in BHK-m3 cells was unaffected by this change in $[Ca^{2+}]_e$ (P>0.05).

These data indicate that the effects of varying $[Ca^{2+}]_e$ on the responses to agonist stimulation of mGluR1α and M_3-mAChRs differ when the receptors are expressed in a common cell background, suggesting receptor-specific rather than cell-specific differences. Modulation of mGluR1α activity by changes in Ca^{2+}_e may be of physiological and patho-physiological significance (Erecinska & Silver, 1992).

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144P METHACHOLINE-STIMULATED [^{35}S]-GPTγS BINDING AND Ca^{2+} MOBILISATION IN CHO-CELLS EXPRESSING M_1 AND M_3 CHOLINOCEPTORS

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Agonist-stimulated [^{35}S]-GTPγS binding in cell-membrane preparations has proved to be a valuable approach to assess stimulus-response relationships at the level of the G protein (Lazareno & Birdsall, 1993; Traynor & Nahorski 1995). However, limitations of such approaches in membranes are that more distal signal transduction is often absent and promiscuity of receptor-G protein interaction is likely. Here we have used a permeabilized cell preparation in which M_1/M_3 receptor signalling via phosphoinositide hydrolysis to calcium release is maintained.

CHO-m1 and -m3 cells were cultured under standard conditions. Harvested cells were permeabilized using 50 μg ml^{-1} β-escin in an intracellular-like buffer (ICB) as described by Wojcikiewicz *et al.* (1990). [^{35}S]-GTPγS binding was assessed in ICB using β-escin permeabilised cells to initiate the reaction according to the method of Lazareno *et al.* (1993). In parallel $^{45}Ca^{2+}$-release assays were performed (Wojcikiewicz *et al.*, 1990).

[^{3}H]-NMS saturation binding to whole cell suspensions revealed similar levels of receptor expression in CHO-m1 and -m3 (B_{max} 2.72 ± 0.17; 2.55 ± 0.15 pmol mg^{-1} protein (n=5) respectively). Optimum assay conditions for [^{35}S]-GTPγS binding in permeabilized CHO-m1 and -m3 cells were found to be 60 min incubations at 30°C with 1.2 nM [^{35}S]-GTPγS in the presence of 10 μM GDP. Upon stimulation with a maximal concentration of methacholine (MCh; 1 mM), increases in [^{35}S]-GTPγS binding above basal were observed after 15 min, with maximum stimulations in CHO-m1 and -m3 (79 ± 4, 68 ± 9 fmol mg^{-1} protein that is 1.28 ± 0.04, 1.31 ± 0.04 fold stimulation over basal respectively (n=3)) occurring at 60 min. MCh-stimulated $^{45}Ca^{2+}$-release in permeabilized cells gave rise to a similar maximal release of $^{45}Ca^{2+}$ in CHO-m1 and -m3 cells (84 ± 4; 87 ± 2% of ionomycin-releasable pool, respectively (n=4)). Comparison of [^{35}S]-GTPγS binding and $^{45}Ca^{2+}$-release concentration-effect relationships revealed lower EC_{50} values for intracellular $^{45}Ca^{2+}$ mobilisation indicating signal amplification (see Table).

Comparison of mean EC_{50} values (± s.e.mean) for methacholine.

Cell line	[^{35}S]-GTPγS binding - log M (n=4)	$^{45}Ca^{2+}$-release - log M (n=4)
CHO-m1	5.31±0.14	6.26±.020
CHO-m3	5.17±.0.07	6.57±.0.01

M_1- and M_3-muscarinic acetylcholine receptors link preferentially to phosphoinositidase C (PIC) activation (Caulfield, 1993). The signal amplification seen between the G protein and release of intracellular calcium may occur after PIC activation at the level of inositol 1,4,5-trisphosphate accumulation and/or the opening of intracellular release channels. We conclude that a single permeabilized cell system can be effectively used to investigate agonist-mediated stimulus-response relationships at the level of G protein activation by receptors and more distal events such as calcium-release.

E.C.A. is a Wellcome Trust Prize Student.

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145P EFFECTS OF LITHIUM ON LIPID PRODUCTS OF AGONIST-STIMULATED PHOSPHOLIPASE C AND D ACTIVITIES IN CHO-m1 CELLS

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Lithium is an uncompetitive inhibitor of the enzyme inositol monophosphatase; this ability to disrupt the phosphoinositide cycle may provide a mechanistic explanation of the anti-manic action of this ion (Nahorski *et al.*, 1991). Agonist-stimulation of M_1-muscarinic receptors expressed in Chinese hamster ovary cells (CHO-m1) causes a prolonged, non-desensitizing activation of phospholipase D (PLD) activity (Sherriffs *et al.*, 1997). Here we have compared the effects of lithium on agonist-stimulated phospholipase C (PLC) and PLD activities in CHO-m1 cells.

To assess PLD activity, monolayers of CHO-m1 cells were labelled with $^{33}P_i$ (2 μCi well^{-1}) for 24 h in phosphate-free medium and pre-incubated ±butan-1-ol (50 mM) prior to agonist challenge (Sherriffs *et al.*, 1997). To determine CMP-phosphatidate (CMP.PA) accumulation, cells were incubated with [^{3}H]-cytidine (0.1 μCi well^{-1}) for 1 h and experiments performed as described previously (Jenkinson *et al.*, 1994). Data are expressed as mean ± s.e.mean for n≥3 experiments.

In agreement with previous studies (Jenkinson *et al.*, 1994), Li+ (5 mM) had no effect on the initial (0-5 min) accumulation of Ins(1,4,5)P_3 stimulated by methacholine (MCh; 1 mM), but dramatically and time-dependently decreased the plateau (5-30 min) response. In $^{33}P_i$ -labelled cells, MCh (1 mM) stimulated a rapid increase in phosphatidic acid (PtdOH) accumulation to a new elevated plateau at 15 min (basal, 1437 ± 272; +MCh 15 min, 9261 ± 1664 d.p.m. well^{-1}). A similar response was observed in the presence of Li+ (5 mM)(basal, 1847 ± 397; +MCh 15 min, 9308 ± 1191 d.p.m. well^{-1}). The increase in PtdOH can arise through phosphorylation of PLC-derived diacylglycerol or directly via PLD activity. In the presence of butan-1-ol (50 mM), PLD-derived PtdOH accumulates as phosphatidylbutanol (PtdBut). Under these conditions, MCh stimulated, after a brief lag, a linear increase in PtdBut for at least 30 min (basal, 313 ± 37; +MCh 30 min, 15117 ± 496 d.p.m. well^{-1}). In contrast to effects on phosphoinositide turnover, Li+ enhanced the early (1-15 min) accumulation of PtdBut, however construction of concentration-effect curves for Li+ action indicated that this enhancement of agonist-stimulated PtdBut accumulation was only observed at high (>5 mM) Li+. In [^{3}H]-cytidine-labelled CHO-m1 cells, MCh (1 mM) stimulated a dramatic accumulation of CMP.PA in the presence of Li+ (5 mM)(basal, 1324 ± 45; +MCh 30 min, 10128 ± 1032 d.p.m. well^{-1}). The initial agonist-stimulated increase in CMP.PA was unaffected by the presence of butan-1-ol (50 mM), however at later times (15-45 min) accumulation of this intermediate was decreased by as much as 50%.

These data suggest that whilst Li+ can have profound effects on phosphoinositide turnover, it only affects agonist-stimulated PLD activation in CHO-m1 cells at supra-therapeutic concentrations (>5 mM). The inhibitory effect of butan-1-ol on MCh-stimulated CMP.PA accumulation in the presence of Li+ also suggests that PLD-derived PtdOH becomes a quantitatively significant source of this intermediate with time. Why Li+-mediated effects on phosphoinositide cycle intermediates are not reflected by changes in PtdOH, even when the contribution of PLD is excluded, remains to be resolved.

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146P COUPLING OF THE HUMAN CGRP RECEPTOR VIA PERTUSSIS TOXIN SENSITIVE G-PROTEINS

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We have identified a novel, single membrane spanning protein, Receptor Activity Modulating Protein (RAMP), that enables the orphan receptor CRLR (Calcitonin-Receptor-Like-Receptor, Aiyar *et al.*, 1996) to behave as a CGRP (Calcitonin Gene Related Peptide) receptor when co-expressed in *Xenopus* oocytes or HEK293T cells. RAMP also increases the CGRP response of the endogenous oocyte CGRP receptor more than 100-fold. Here, we examine coupling of native and exogenous CGRP receptors to G-protein regulated potassium channels in *Xenopus* oocytes.

m'G(5')pp(5')GTP capped mRNA encoding RAMP, CRLR, and the potassium channels CIR (Kir3.1) and GIRK1 (Kir3.4) were injected into *Xenopus* oocytes (20 to 100ng per oocyte) and whole-cell currents were recorded (at 25°C) using two microelectrode voltage-clamp 3 to 7 days post-injection. Oocytes were voltage-clamped in ND96 solution (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES, pH 7.5, and potassium currents were recorded in a high potassium solution (in mM): 90 KCl, 1 $MgCl_2$, 5 HEPES, pH 7.5. Measurements refer to changes in holding current recorded at a membrane potential of -60mV. In several experiments pertussis toxin (Sigma) was injected into oocytes (1 to 2.5ng per oocyte) 5 to 8 hours prior to recording. Human αCGRP was made by GlaxoWellcome. Data are mean ± s.e.mean.

In uninjected oocytes and in oocytes expressing the potassium channels CIR and GIRK1, switching from ND96 to high potassium solution led to a small inward shift in holding current (Table 1). However, even in the absence of CGRP, co-expression of RAMP1 or of RAMP1 plus CRLR with the potassium channels activated a large inward potassium current (Table 1), which was completely blocked by addition of 1mM $BaCl_2$ to the extracellular solution. A current-voltage plot for the potassium current showed strong inward rectification. Subsequent application of 100nM CGRP led to a small (6.2 ± 1.8%, n=8) increase in potassium current amplitude.

RNA injected	Potassium current (nA)
uninjected oocyte	97.5 ± 26.6, n=8
CIR + GIRK1	159.1 ± 70.9, n=7
RAMP + CIR + GIRK1	406.4 ± 73.9, n=16
RAMP + CRLR + CIR +GIRK1	504.5 ± 64.0, n=22

Injection of pertussis toxin or co-expression of transducin (which binds G-protein βγ sub-units) led to marked inhibition of potassium current amplitude (64.3±10.3% and 74.2±5.5% inhibition respectively, n=5) in oocytes expressing RAMP or RAMP plus CRLR. We conclude that the human CGRP receptor can signal constitutively through a pertussis toxin-sensitive G-protein to activate G-protein regulated potassium channels in *Xenopus* oocytes.

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147P INTERACTIONS OF THE A_1 ADENOSINE RECEPTOR WITH MULTIPLE G_i-FAMILY G PROTEINS: STUDIES WITH A_1 ADENOSINE RECEPTOR $G_i1\alpha$ FUSION PROTEINS

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The A_1 adenosine receptor (A_1AR) mediates inhibition of adenylate cyclase via interactions with members of the PTX-sensitive G_i subfamily of heterotrimeric G proteins. Recently, we reported the use of PTX-resistant mutant forms of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ to study specificity of coupling between the α_{2A}-adrenoceptor and such $G_i\alpha$s (Wise *et al.* 1997a). We then generated a fusion protein between the α_{2A}-adrenoceptor and PTX-insensitive (C351G) $G_i1\alpha$ in order to study agonist-induced GTP turnover in a system where the levels of expression of receptor and cognate G protein were accurately determined (Wise *et al.* 1997b).

In the present work we have generated fusion proteins between the A_1AR and PTX-resistant versions of $G_i1\alpha$, $G_i2\alpha$ and $G_i3\alpha$ by in-frame fusion of the N-terminus of the relevant G protein to the C-terminus of the receptor. Addition of the A_1 agonist CPA (10 μM) to membranes of PTX-treated HEK293T cells expressing individual A_1AR-$G_i\alpha$ fusion proteins resulted in stimulation of GTPγS binding activity (Table 1). Elevation of GTPγS binding by CPA was found to be dose-dependent, with EC_{50} values calculated to be 23.4 ± 2.2, 25 ± 2.2 and 17.4 ± 1.7 nM for A_1AR-$G_i1\alpha$, -$G_i2\alpha$ and -$G_i3\alpha$ fusion proteins, respectively.

	[35S]GTPγS Binding	
Fusion Protein	Basal (cpm)	CPA (10 μM) (cpm)
$A_1G_{i1}\alpha$C351G	2230(±41)	10639(±158)
$A_1G_{i2}\alpha$C352G	2471(±48)	12163(±154)
$A_1G_{i3}\alpha$C351G	616(±86)	7994(±882)

GTPγS binding was determined by incubating membranes (5μg/well pre incubated with 40μM GDP) with varying concentrations of CPA or 0.6mM GTP to define non-specific binding and 0.3nM [^{35}S]GTPγS in assay buffer containing 10mg/l saponin for 30min at 18°C whilst mixing. CHO cells stably expressing each of the A_1AR-G_i fusion proteins also displayed CPA-mediated stimulation of GTPγS binding activity following PTX pretreatment. CPA inhibited forskolin amplified adenylate cyclase activity in a dose related manner with IC_{50} values of 1.6 ± 0.05, 1.2 ± 0.03 and 1.8 ± 0.08 μM for CHO cells stably expressing A_1AR-$G_i1\alpha$, -$G_i2\alpha$ and -$G_i3\alpha$ fusion proteins, respectively. For cAMP measurements, cells were seeded in 96 well plates for 24 h and then incubated in 300 μM IBMX for 30 min at 37°C. Forskolin (30μM) and varying concentrations of CPA were then added to the wells for 30 min at 37°C. The cAMP was extracted using 0.1M HCl for 1h at 4°C and neutralised with 0.1M $KHCO_3$. The cAMP content was measured using a Biotrak Kit (Amersham).

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b) Wise, A., Carr, I.C. and Milligan, G. (1997) Biochem. J. 325, 17-21.

148P INDUCED EXPRESSION OF THE ORL1 RECEPTOR USED TO STUDY SIGNALLING EFFECTS

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The ecdysone inducible expression system (Invitrogen) (No *et al.*, 1995) has been used to investigate the effects of altering the expression of the opioid receptor ORL1 on signalling.

CHO cells containing the ecdysone inducible system were stably transfected with the ORL1 receptor. 10μM muristerone A (mur A) was used to induce expression for 20 hours and clones were selected using a ^{3}H nociceptin SPA binding assay. Cells were stimulated with 0, 5, 10, 15, or 20μM mur A for 30hours and assayed for ^{3}H nociceptin binding, [^{35}S]GTPγS binding and cAMP. Membranes were prepared in 20mM HEPES 10mM $MgCl_2$ pH7.4 and then used in the [^{35}S]GTPγS and the ^{3}H nociceptin binding assays. For the GTPγS membranes (10μg/well pre incubated with 40μM GDP) were mixed with nociceptin (0.2μM) or 0.6mM GTP to define non-specific binding and 0.3nM [^{35}S]GTPγS in assay buffer containing 10mg/l saponin for 30min at 18°C whilst mixing. ORL1 binding was performed using membranes (10μg/well) incubated with [^{3}H] nociceptin (0.5-0.71nM), SPA beads (1mg/well) in assay buffer (50mM HEPES, 10mM $MgCl_2$, 1mM EDTA pH7.4) for 60min at 18°C whilst mixing. The plates were then spun at 1500g for 5min and bound radioactivity measured. Inhibition of forskolin stimulated cAMP was also measured. For this cells were seeded in 96 well plates and the next day were incubated with 300μM IBMX for 30min at 37°C. Forskolin (30μM) and nociceptin (0.1μM) were then added to the wells for 10mins at 37°C. The cAMP was extracted using 0.1M HCL for 1hour at 4°C and neutralised with 0.1M $KHCO_3$. The cAMP content was measured using a Biotrak Kit (Amersham).

From the initial experiments 20μM mur A and 30hours induced the highest expression level. Mur A increased the specific binding from 67(±58)dpm to a maximal of 2685(±181)dpm ($P<0.05$). The [^{35}S]GTPγS results are shown in table 1.

mur A (μM)	Basal (cpm)	nociceptin (0.2μM, cpm)	PTX (50ng/ml, cpm)
0	1720(±101)	1703(±85)	1988(±66)
20	3861(±112)	5702(±113)	1816(±181)

Mur A had no effect on the basal level or the forskolin stimulated cAMP levels but the inhibition of the forskolin response by nociceptin was increased from 14(±24)% to 57(±11)% in a concentration dependant manner. All results are the mean of three experiments and are shown ± the s.e.mean.

These results show that as the mur A concentration is increased there is an increase in the expression level of ORL1, an increase in the basal and nociceptin stimulated [^{35}S]GTPγS binding and increases in the nociceptin inhibition of cAMP.

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149P COMPARATIVE STUDY OF TRANSFECTED P2Y RECEPTORS: EFFECT OF THE SURAMIN ANALOGUE NF023, PERTUSSIS TOXIN AND PROTEIN KINASE C ON THE PHOSPHOLIPASE C RESPONSE

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Studies on endothelial cells have shown that $P2Y_1$ and $P2Y_2$ receptors are both coupled to phospholipase C (PLC) but that the responses were differentially affected by antagonists, pertussis toxin (PT) and protein kinase C (PKC) (e.g. Patel et al, 1996). Using cloned receptors transfected into 1321N1 cells we have recently reported on the differential sensitivity of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors to antagonists (Charlton et al, 1996a,b). Here we extend these studies on cloned receptors by examining a further antagonist (suramin analog NF023; Ziyal et al, 1994) and regulation by PT and PKC.

The 1321N1 clonal cell lines transfected with turkey $P2Y_1$, human $P2Y_2$ and human $P2Y_4$ receptors were obtained and cultured as described in Charlton et al (1996a,b). Formation of total [^{3}H]inositol phosphates ([^{3}H]$InsP_x$), an index of PLC activity, was measured as described in Charlton et al (1996a). PT (100 ng/ml) was incubated with cells for 16h prior to stimulation.

Concentration response curves to 2-methylthio ATP (2MeSATP) with the $P2Y_1$ transfectants gave an EC_{50} of 0.054 μM in the absence of NF023 and of 0.15, 0.30, and 0.59 μM in the presence of 10, 30 and 100 μM NF023 respectively. Responses in the presence of 100 μM NF023 failed to reach a maximum, so values were obtained by extrapolation; on Schild plot this gave an apparent pA_2 of 5.33. By contrast these concentrations of NF023 failed to have a significant effect on either the maxima or EC_{50} values when $P2Y_2$ transfected cells were stimulated with UTP. With $P2Y_4$ transfected cells there was a paradoxical decrease in EC_{50} for UTP in the presence of 100 μM NF023 (1.42 ± 0.46 and 0.31 ± 0.15 μM in the absence and presence of the antagonist respectively; $P<0.05$). The effect of PT was examined on the time course of [^{3}H]$InsP_x$ accumulation; there was no change in the responses of the $P2Y_1$ or $P2Y_4$ transfected cells, while the responses of the $P2Y_2$ cells were reduced by 20.6 ± 3.8 and 27.7 ± 0.6 % at 10 and 20 min stimulation respectively ($P<0.05$). The EC_{50} values for UTP with $P2Y_2$ cells were unchanged by PT but the size of response was diminished at all concentrations of UTP. The presence of the PKC inhibitor Ro 31 8220 (10 μM) or the activator phorbol myristate acetate (PMA: 100 nM), both for 10 min before and during stimulation with agonist had a similar effect on each of the transfected cell lines. Ro 31 8220 enhanced the responses to between 117 and 135 % of control, but this was not significant when pooled across experiments. TPA reduced the response to about 40 % of control, and this was significant at the $P<0.05$ level.

These results show that NF023 has some ability to discriminate between the 3 P2Y receptor types tested. Further, a differential response to PT was apparent in the cloned receptors, with some parallels with the differences seen in native endothelial receptors. The differential modulation by PKC in endothelial cells was not present when the receptors were transfected into 1321N1 cells.

Charlton, S.J. et al (1996a) *Br. J. Pharmacol.* 118, 704-710.
Charlton, S.J. et al (1996b) *Br. J. Pharmacol.* 119, 1301-1303.
Patel, V., Brown, C. & Boarder, M.R. (1996) *Br. J. Pharmacol.* 118, 123-130.
Ziyal, R. et al (1994) *Drug Dev. Res.* 31, 336.

150P COMPARATIVE STUDY OF TRANSFECTED P2Y RECEPTORS: REGULATION OF TYROSINE KINASES AND MITOGEN-ACTIVATED PROTEIN KINASES BY $P2Y_1$ AND $P2Y_2$ RECEPTORS

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Tyrosine kinase and p42/p44 mitogen activated protein kinase (MAPK) pathways are commonly activated by G protein-coupled receptors. We have shown that these pathways are implicated in the regulation by P2Y receptors of vascular endothelial cell prostacyclin production and smooth muscle proliferation and that $P2Y_1$ and $P2Y_2$ receptors are differentially coupled to their respective functional responses in endothelial cells (e.g Patel et al, 1996). To facilitate the study of this differential regulation of cell function by $P2Y_1$ and $P2Y_2$ receptors we report here on the regulation of tyrosine kinase and MAPK by these 2 receptors when transfected into a common host cell.

We used clonal cell lines of 1321N1 cells transfected with turkey $P2Y_1$ and human $P2Y_2$ receptors, as described previously (Charlton et al, 1996). Phospholipase C (PLC) was measured by accumulation of [^{3}H]inositol phosphates ([^{3}H]$InsP_x$), tyrosine protein phosphorylation by western blot with the PY20 phosphotyrosine antibody, p42 and p44 MAPK phosphorylation by western blot with antibody specific for the tyrosine phosphorylated MAPK proteins, and p42/p44 MAPK activity by kinase assay with [^{32}P]ATP and a nonapeptide substrate.

Using the phosphotyrosine western blot there was no widespread detectable change in tyrosine phosphorylation as a result of stimulating the $P2Y_1$ transfectants with 2-methylthio ATP (2MeSATP; 10 nM - 30 μM) or the $P2Y_2$ transfectants with UTP (30 nM - 500 μM) for between 30 s and 20 min. When submaximal concentrations (50 μM) of the tyrosine phosphatase inhibitor pervanadate were used to raise tyrosine phosphorylation then stimulation of the $P2Y_2$ cells with UTP (100-300 μM) gave a substantial and widespread reduction in the level of tyrosine phosphorylation. Ionomycin (1-3 μM) had a similar effect on the pervanadate raised tyrosine phosphorylation. However, this did not occur when $P2Y_1$ transfected cells were stimulated with 2MeSATP. Contrasting with this, western blots specific for the tyrosine phosphorylated forms of p42 and p44 MAPK were enhanced in a dose dependent manner by agonists acting on both $P2Y_1$ and $P2Y_2$ transfected cells. Using the peptide kinase assay for MAPK we found that 2MeSATP stimulated $P2Y_1$ cells with a log EC_{50} of -7.71 ± 0.16 and UTP stimulated $P2Y_2$ cells with a log EC_{50} of -6.41 ± 0.21. This compares with a log EC_{50} of -7.55 ± 0.17 and -5.85 ± 0.06 for stimulation of $P2Y_1$ and $P2Y_2$ cells respectively when measuring [^{3}H]$InsP_x$ accumulation.

These results suggest that, in contrast to native endothelial receptors, stimulation of neither $P2Y_1$ or $P2Y_2$ transfected receptors generate a widespread increase in tyrosine phosphorylation, while the $P2Y_2$ receptor alone is coupled to a reduction in tyrosine phosphorylation. Both p42 and p44 MAPK are activated on stimulation of each receptor; this response has essentially the same EC_{50} as does the stimulation of PLC.

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151P SIMPLIFICATION OF THE G-PROTEIN ($G_{o\alpha}$) GPTγS BINDING ASSAY

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^{35}S-GTPγS binding is a commonly used assay in G-protein studies and usually involves separation of bound and unbound ligand by filtration on nitrocellulose (NC) filters. These membranes have certain limitations for high throughput testing. The aim of the present study was to investigate the utility of glass fibre (GF/B) filters in this assay. Purified $G_{o\alpha}$ subunit was obtained from Chiron Technologies (Emeryville, US). The ^{35}S-GTPγS binding assay mixture, for total binding (TB),: contained 25mM HEPES (pH7.5), 1mM EDTA, 5mM $MgCl_2$, 0.1mM dithiothreitol (DTT), 100mM NaCl, 0.025% lubrol and 2.5ng $G_{o\alpha}$. The reaction was initiated by the addition of 0.6nM ^{35}S-GTPγS (~500,000cpm) and the samples incubated for 30min at RT on an orbital shaker. Assays were set up in 96 deep (1ml) well plates (for GF/B) or in Multiscreen (Millipore) plates for NC. Non-specific binding (NSB) was determined by inclusion of 100μM GTP. Incubation was terminated by vacuum filtration on a Multiscreen plate manifold or on a GF/B plate harvester (Packard). Prior to use GF/B filters were pre-treated with 0.3% polyethylenimine (PEI) or DTT. The NC plates were washed with 5x150μl of buffer (25mM TrisHCl pH7.5, 1mM EDTA, 25mM $MgCl_2$, 100mM NaCl) whilst the GF/B plates were treated with, unless otherwise stated, 3x1ml of buffer. Filter plates were dried (50^{0}C, 30min) and counted (Topcount, Packard). In agreement with previous reports (Sternweis & Robishaw, 1984) a high level of specific binding was obtained with NC (TB=3140±124cpm, NSB=175±38cpm). In contrast, GF/B plates displayed a much narrower signal (TB=8431±1203cpm, NSB=4762±504cpm). Increasing PEI to 1% did not significantly lower NSB (TB=8117±1213cpm, NSB=4323±492cpm). However, doubling the number of washes improved signal (TB=8417±364cpm, NSB=2401±174cpm). Pre-treating the GF/B filters with 0.1mM DDT further lowered NSB (TB=5639±323cpm, NSB=491±38cpm) to that comparable with NC. Raising [DDT] to 1mM produced no additional drop in NSB (TB=5498±391cpm, NSB=404±29cpm). GTP, GTPγS, GppNHp and GDP potently inhibited ^{35}S-GTPγS binding with IC_{50} values of: 17, 1,1.7, and 7.6nM, respectively. GMP showed weak affinity (IC_{50}: 390μM). Suramin, a reference G-protein inhibitor, also attenuated ^{35}S-GTPγS binding (IC_{50}: 13μM). In general, these results with GF/B were similar to those obtained using NC plates and comparable to published data (Sternweis & Robishaw, 1984; Beindl etal., 1996).

In conclusion the data show that GF/B membranes can be used in the G-protein ^{35}S-GTPγS binding assay without compromising signal. Furthermore, since these plates are more amenable to semi-automation this method would be particularly useful for high volume testing.

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152P ALTERED EXPRESSION OF INOSITOL 1,4,5-TRISPHOSPHATE ($InsP_3$) RECEPTOR SUBTYPES AND Ca^{2+} STORES ORGANISATION IN DEVELOPING RAT VASCULAR SMOOTH MUSCLE

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Intracellular Ca^{2+} released from the sarcoplasmic reticulum (SR) in response to $InsP_3$ binding to its receptor may play a role in the normal development (Shiraishi *et al*, 1995) as well as contraction of developing smooth muscle cells. The neonatal rat portal vein is a good model for studying vascular smooth muscle as cells only begin to develop after birth (Ody *et al*, 1993) Our aim was to examine the relative expression of type 1 and type 3 $InsP_3$ receptors and SR organisation in neonatal compared to adult rat portal vein and aorta. In order to relate alterations in $InsP_3$ receptor expression to function, $InsP_3$-induced contraction was measured.

Portal veins and aortae were obtained from male Sprague Dawley rats of 3-5 days old (weight 10.4 ± 0.2 g, n=22) and 5-6 weeks old (weight 313 ± 5 g, n=20). Membrane preparations from neonatal and adult rats were subjected to Western blotting using type 1 and type 3 $InsP_3$ receptor specific antibodies. The ultrastructure of the SR in developing smooth muscle cells was examined in an electron microscope using osmium ferricyanide staining. For $InsP_3$ induced contraction experiments, 200 μm wide strips of neonatal and adult portal vein were attached to a sensitive force transducer and permeabilised with β-escin. Immediately following permeabilisation, a maximal response with pCa4.5 was obtained. The SR was loaded using a Ca^{2+} containing mock intracellular buffer and 100 μM $InsP_3$ added.

Western blot analysis of smooth muscle membrane preparations probed with anti-type 3 and anti-type 1 $InsP_3$ receptor antibody showed a specific band at approximately 220 kDa. Densitometric analysis of the specific bands showed levels of type 3 $InsP_3$ receptor were increased 20 times in the neonatal portal vein compared to adult, and increased 8 times in the neonatal aorta compared to adult. In contrast, analysis of type 1 $InsP_3$ receptor expression showed a twofold decrease in the neonatal rat portal vein compared to adult, and in aorta levels of type 1 $InsP_3$ receptor were unchanged in neonatal compared to adult samples. Osmium ferricyanide stained sections revealed the distribution of SR in the adult portal vein at the periphery of the cell, close to the plasma membrane, however in the neonatal portal vein proportionally more SR was distributed in the centre of the cell, at the nuclear poles. In both the neonatal and adult aorta, the SR was distributed predominantly at the centre of the cell, around the nuclear poles. In permeabilised portal vein, the $InsP_3$-induced contractions were significantly increased in neonates compared to adults when expressed as a percentage of pCa4.5 maximal contractions (neonate 31 ± 3%, adult 16 ± 2% of maximum, mean ± sem, $P < 0.005$, n=5).

These results indicate that alterations in $InsP_3$ receptor subtypes expression and intracellular calcium stores distribution in neonatal compared to adult vascular smooth muscle may have a role in the developmental process. These alterations could also contribute to differing contractile properties during development

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153P EVIDENCE FOR INVERSE AGONISM OF SR141716A AT HUMAN RECOMBINANT CANNABINOID CB_1 AND CB_2 RECEPTORS

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SR141716A is a selective antagonist for the CB_1 receptor (Rinaldi-Carmona *et al.*, 1994). In the mouse bladder and guinea-pig myenteric plexus-longitudinal muscle, CB_1 receptor-mediated inhibition of neurogenic contractions are antagonized by SR141716A but which also increases the twitch contraction amplitude (Pertwee *et al.*, 1996; Pertwee & Fernando, 1996). These effects suggest that either SR141716A is antagonizing endogenous agonist or that it is an inverse agonist. To investigate further the actions of SR141716A we have studied its effects in Chinese hamster ovary (CHO) cells expressing human recombinant CB_1 and CB_2 receptors, by examining the binding of guanosine 5'-O-(γ-[^{35}S] thio) triphosphate ([^{35}S]GTPγS).

Cells were grown in Hams F-12 medium with 10% fetal calf serum, 500 μg ml^{-1} G418 and harvested using 0.5 mM EDTA (4Na). Cells were homogenized then centrifuged at 50,000 x g for 10 min at 4 °C. The [^{35}S]GTPγS binding assay was carried out in 10 mM Hepes buffer with 100 mM NaCl, 32 mM MgCl, 320 μM GDP, 10 μG protein and 1.0 nM [^{35}S]GTPγS in a final volume of 250 μl. Non-specific binding was determined using an excess of GTP (10 μM). Assay tubes were incubated at 37°C for 45 min, filtered and washed 3 times with Hepes buffer and the radioactivity counted. Saturation and competition radioligand binding studies with [^{3}H]CP55,940 were performed as described by Felder *et al.* (1995).

In cells expressing hCB_1 and hCB_2 receptors (Bmax 8.4±2.3 and 84.8±12.6 pmol mg^{-1} protein; n=3) the affinities of ligands were appropriate for the labelling of these receptors (Table 1; see Felder *et al.*, 1995). The cannabinoid receptor agonists WIN55,212-2 and CP55,940 increased the incorporation of [^{35}S]GTPγS (Table 1) in both hCB_1 and hCB_2 receptors. SR141716A inhibited basal [^{35}S]GTPγS binding at each subtype whereas cannabinol (3 μM) had no effect on basal binding in hCB_1 receptors but reduced binding at hCB_2 receptors.

Table1 Affinity and efficacy of ligands at hCB_1 and hCB_2 receptors.

		pK_i	pEC_{50}	Maximum
CP55,940	hCB_1	8.59±0.13	7.81±0.03	116±20
	hCB_2	8.77±0.02	8.44±0.09	40±2
WIN55,212-2	hCB_1	7.49±0.25	6.35±0.13	114±21
	hCB_2	8.76±0.08	8.32±0.0	22±8
SR141716A	hCB_1	7.71±0.13	8.34±0.05	-56±4
	hCB_2	5.94±0.19	5.90±0.03	-70±9
Cannabinol	hCB_1	6.93±0.26	-	-
	hCB_2	7.11±0.11	6.28±0.03	-52±6

pK_i values from competition binding. pEC_{50} and maximum effects (% over basal) are from [^{35}S]GTPγS binding. Values are the mean±s.e.mean of 3-5 estimates.

These results suggest the effects of SR141716A at hCB_1 and of SR141716A and cannabinol at hCB_2 receptors are due to inverse agonism rather than antagonism of endogenous agonists. First, cannabinol had no effect on the basal incorporation of [^{35}S]GTPγS at a concentration almost 30 fold higher than its affinity for the receptor. Second, PMSF (50 μM), an irreversible inhibitor of anandamide synthase, had no effect on the basal incorporation of [^{35}S]GTPγS in either hCB_1 or hCB_2 implying anandamide is not present in these assays.

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154P STIMULATION OF $G_{q/11}$-COUPLED RECEPTORS IS LINKED TO HYPERTROPHIC RESPONSE IN RAT NEONATAL VENTRICULAR CARDIOMYOCYTES

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Activation of $G_{q/11}$-coupled receptors leads to formation of inositol phosphates [IP] with subsequent stimulation of protein kinase C [PKC]; it has been suggested that this pathway is capable of increasing rate of protein synthesis (Sugden & Bogoyevitch, 1996). To test this hypothesis we assessed in neonatal rat ventricular cardiomyocytes the effects of stimulation of 4 $G_{q/11}$-coupled receptors [R] (α_1-adrenergic R by noradrenaline [NA], ET_A-R by endothelin-1 [ET-1], thromboxane A_2 [TXA_2]-R by U 46619 and AT_1-R by angiotensin II [ANG II] on IP-formation and rate of protein synthesis.

Isolation of neonatal cardiomyocytes, IP-formation (determined as accumulation of total [^{3}H]-IP's in [^{3}H]-myo-inositol prelabelled cells during a 60 min incubation at 37°C in HANKS buffered saline solution that contained 10 mM LiCl) and rate of protein synthesis (determined as [^{3}H]-phenylalanine incorporation during a 24 hours incubation) were performed as recently described (Pönicke et al., 1997). In some experiments cells were preincubated for 24 hours with pertussis toxin [PTX] (250 ng/ml) or 10μM phorbol-12-myristate-13-acetate [TPA]. All data are given as means ± SEM of n experiments.

Stimulation of all four R led to concentration-dependent increases in IP-formation and rate of protein synthesis; pEC_{50}- and E_{max} (maximal increase above basal)-values are given in the table. The NA-effects were antagonized by 1μM prazosin, the U 46619-effects by 1 μM SQ 29548, the ET-1 effects by 1μM BQ-123 and the ANG II-effects by 1μM losartan, but also by 1μM BQ-123 (Pönicke et al., 1997).

	Protein-Synthesis		IP-Formation	
	pEC_{50}	E_{max}	pEC_{50}	E_{max}
Noradrenaline	6.7 ± 0.1	40 ± 3	6.5 ± 0.2	292 ± 50
U 46619	6.0 ± 0.2	33 ± 5	5.5 ± 0.1	128 ± 35
Endothelin-1	8.4 ± 0.1	52 ± 4	9.8 ± 0.1	130 ± 14
Angiotensin II	7.0 ± 0.2	29 ± 3	7.5 ± 0.2	42 ± 7

n=6-12; E_{max}= maximal increase in % above basal

Pretreatment of the cardiomyocytes with PTX decreased the ET-1 effects on protein synthesis by 39 ± 5 % (n=9) and abolished the ANG II-effect but did not affect NA- and U 46619-effects on protein synthesis; on the other hand, pretreatment of the cells with TPA that desensitizes PKC as well as treatment with the specific PKC-inhibitor Gö 6850 (Toullec et al., 1991) suppressed effects of all four agonists on protein synthesis.

We conclude that, in rat neonatal cardiomyocytes, stimulation of $G_{q/11}$-coupled receptors increases rate of protein synthesis; this involves PKC stimulation. The NA- and U 46619-effects are PTX-insensitive, while the ET-1 effect is partly PTX-sensitive, and the ANG II-effect is brought about by ET-1 in a PTX-sensitive manner.

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155P SELECTIVE SOMATOSTATIN (SRIF)-INDUCED DESENSITISATION OF HUMAN RECOMBINANT SST_4 RECEPTORS EXPRESSED IN CHO-K1 CELLS

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Using the technique of microphysiometry, we have shown (Smalley et al., 1997) that SRIF and the SRIF agonist, L-362855 (Raynor et al., 1993) cause concentration dependent increases in extracellular acidification rates (EAR) in CHO-K1 cells expressing human recombinant sst_4 receptors ($CHOsst_4$ cells). Although SRIF was approximately 30 times more potent than L-362855, the SRIF-induced maximum was only 50% of that to L-362855. We have investigated the mechanism responsible for the lower SRIF maximum.

Approximately 500,000 $CHOsst_4$ cells were plated into microphysiometer cups 18h before experimentation. Cells were perfused with a bicarbonate free DMEM (pH 7.4) solution at a rate of 120μL/min. EARs were measured as previously described (Smalley et al., 1997). The basal EARs were 100-300μV/s (0.1-0.3 pH unit/min). After an equilibration period of 1 hour, UTP (3μM) was added as standard. Unless otherwise stated, increasing concentrations of SRIF or L-362855 were added every 30 min. In some studies concentration-effect curves to L-362855 were obtained in the continuous presence of SRIF(0.1-1nM). Agonist responses were measured in μV/s or normalised as a percentage of the response to UTP. All values are the mean ± se mean from 3-6 experiments.

Both SRIF (pEC_{50} 9.69±0.23) and L-362855 (pEC_{50} 8.34±0.10) caused concentration-dependent increases in EAR with respective maxima of 19.6 ± 0.9 and 43.0 ± 7.9% of the response to the initial UTP challenge. In the continuous presence of SRIF (0.1, 0.3 or 1nM), concentration-effect curves to L-362855 were displaced to the right with a progressive reduction in the maximum response. In the presence of 0.1nM and 0.3nM SRIF, the pEC_{50} for L-362855 was 7.13±0.60 and 6.70±0.07 with maxima of 30.9±7.4 and 19.1±2.4 % of the response to UTP respectively. In the presence of SRIF (1nM), the maximum response to L-362855 was only 6.7±0.1% of the response to UTP. SRIF (0.3nM) had no effect on the increase in EAR produced by UTP (3μM) [190±14μV/s and 196±14μV/s in the absence and presence of SRIF respectively].

Exposure of cells to a single maximally effective concentration of SRIF (30nM) or L-362855 (300nM) produced similar increases in EAR (74.5±12.6% and 63.0±10.1% of the response to UTP). A second challenge, 30 min later showed a marked reduction in responses to SRIF (9.8±1.2%) but not to L-362855 (52.0±4.3%). Concentration-effect curves to SRIF and L-362855 were therefore obtained by combining the data from cells exposed to only single agonist concentrations (0.03-300nM). Both SRIF (pEC_{50} 9.20±0.06) and L-362855 (pEC_{50} 7.99±0.29) produced concentration-dependent increases in EAR with similar maximal increases in EAR (89.4±12.5 and 78.8±17.2% of the response to UTP, respectively).

The results suggest that the higher affinity agonist SRIF, but not L-362855, induces a specific desensitisation of sst_4 receptors expressed in CHO-K1 cells. The mechanism involved in this desensitisation is as yet unknown but may involve either SRIF-induced receptor internalisation or receptor phosphorylation (Hipkin et al.,1997).

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156P EFFECTS OF TYPE SELECTIVE INHIBITORS ON CAMP PHOSPHODIESTERASE ACTIVITY AND INSULIN SECRETION IN THE CLONAL β CELL LINE BRIN BD11

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Our previous studies showed that the predominant cAMP phosphodiesterase (cAMP-PDE) in rat and human islets is PDE3 (Shafiee-Nick et al., 1995). In view of the cellular heterogeneity of islets the present work attempts to characterise the cAMP-PDE in a glucose-responsive, insulin secreting β-cell line, BRIN BD11 maintained in RPMI 1640 and used at passages 27-32 (McLenaghan et al. 1996). Cells were homogenized and assayed for cAMP-PDE as described for islets (Shafiee-Nick et al.,1995) and insulin secretion studied as described previously (McLenaghan et al 1996), in the presence and absence of drugs. Org 9935 (0.02 - 5 μM) a potent and selective PDE3 inhibitor, inhibited PDE by a maximum of 39 ± 2% (control 495 pmol $min^{-1}ml^{-1}$) in the pellet, but not the supernatant fraction (IC_{50} ~0.2μM). A large concentration of zaprinast (75 μM) inhibited cAMP-PDE by about 30%, suggesting the presence of PDE1 (Ca^{2+} - calmodulin activated). Rolipram (0.05 - 10 μM), the PDE4 inhibitor, inhibited cAMP concentration dependently, in the soluble fraction, with a maximum inhibition of around 30%. These data suggest a quantitative difference in the expression of the different PDEs between BRIN BD11 cells and islets, in which PDE3 accounts for about 70% of the PDE activity. In the presence of 16.7 mM glucose, Org 9935 and another PDE3-selective agent SK&F 94836 each augmented insulin secretion. (e.g. insulin secretion, ng 10^6 cells $^{-1}$ 20 min^{-1}, mean ± SEM, control 1.6 ± 0.1, SK&F 4936 10 μM 2.3 ± 0.2 $P < 0.01$). Zaprinast up to 50 μM had no effect on insulin secretion. Rolipram (10 μM), which was without effect on insulin secretion in islets (Shafiee-Nick et al., 1995) also increased insulin secretion (2.6 ng 10^6 $cells^{-1}$ 20 min^{-1}, $P < 0.05$ vs control) by BRIN-D11 cells. In the presence of low glucose concentrations (5.6 mM) the adenylyl cyclase activator forskolin (1-25 μM) produced a concentration-dependent augmentation of insulin secretion (e.g. insulin secretion, ng 10^6 $cells^{-1}$ 20 min^{-1}, mean ± SEM, forskolin (1μM) 2.3 ± 0.2, forskolin (5 μM) 3.9 ± 0.6, forskolin (25 μM) 5.3 ± 0.4 $P < 0.01$). SK&F 94836, in a concentration which was without effect on insulin secretion in 5.6 mM glucose, markedly augmented the effects of a high concentration of forskolin (insulin secretion, ng 10^6 cells $^{-1}$ 20 min^{-1}, mean ± SEM, forskolin + SKF 94836 - 18.0 ± 0.4, $P < 0.01$ vs control). As found previously in islets, the data are consistent with the functional importance of PDE3 in relation to insulin secretion. However, the greater proportion of PDE1, together with the ability of rolipram to augment insulin secretion suggest important differences from islets.

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Shafiee-Nick et al. (1995) *Br. J. Pharmacol.* 115, 1486-1492.

157P NOCICEPTIN ACTIVATION OF ERK1/ERK2 FOLLOWING EXPRESSION OF THE ORL1 RECEPTOR IN CHO CELLS

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Agonist stimulation of a variety of G protein coupled receptors has been shown to enhance the enzymatic activity of the extracellular signal related kinases (ERK1/ERK2) (also known as p42/p44 MAP kinase) and the c-Jun N terminal kinase (JNK), subtypes of the mitogen activated protein kinase cascade (MAPK) (Coso, *et. al.*, 1996). In the present study we have examined the ability of the recently characterised ORL1 opioid like receptor to mediate activation of ERK1/ERK2 in CHO cells stably transfected with this receptor.

In order to measure ERK1/ERK2 activity CHO cells stably expressing the ORL1 receptor were quiesced for 24h in serum free medium prior to assay. Cells were treated with nociceptin (0.001nM to 100nM) for ten minutes. For inhibitor studies pertussis toxin (PTX) was added to 50ng/ml for the final 16h quiescence period, and 50μM PD098059 (MEK1/MEK2 inhibitor) was added to the cells for 30 minutes prior to stimulation with 0.1μM nociceptin. Following drug treatment cell lysates were prepared at 4°C in 300μls lysis buffer (25mM Tris/HCL, 40mM p-nitrophenol, 25mM NaCl, 10%(v/v) ethylene glycol, 10μM dithiothreitol, 0.2%(v/v) Tween 20 at pH7.5) containing proteinase inhibitor used according to manufacturer's instructions (Boehringer). The cell lysates were homogenized by passing 15 times through 26 gauge needle and centrifuged at 10000g for 5mins @ 4°C. 15μl of supernatant was assayed for MAPK activity using the Amersham p42/p44 MAP kinase assay system and following the instructions therein (Kazuhiko, et. al., 1996).

Nociceptin (0.1μM) mediated a rapid increase in ERK1/ERK2 activity reaching maximum activity 5 minutes after nociceptin stimulation and returning to basal after 30 minutes. Nociceptin mediated a concentration dependent increase in ERK1/ERK2 activity with an EC_{50} of 0.28(0.17-0.45)nM. This was not significantly different from that obtained for inhibition of forskolin stimulated cAMP with nociceptin EC_{50} = 0.15(0.08-0.27)nM. The stimulation of MAPK activity induced by 0.1μM nociceptin was suppressed by 89% in the presence of 50μM PD098059, indicating that the response is predominantly mediated by MEK1/MEK2. The nociceptin stimulation was also abolished by pretreatment of the cells with 50ng/ml PTX suggesting that ERK1/ERK2 activation is as a consequence of ORL1 receptor coupling to the PTX sensitive Gi-subfamily of heterotrimeric G proteins.

In conclusion, nociceptin stimulation of the ORL-1 receptor results in activation of ERK1/ERK2 MAP kinase in a PTX-sensitive manner.

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158P SERUM DEPRIVATION ATTENUATES ALLERGEN-INDUCED MAST CELL DEGRANULATION

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Mast cells play an important role in hypersensitivity type I responses. Antigenic stimulation of a mast cells via the high-affinity receptor for IgE (FcεRI) triggers the release of various inflammatory mediators from its granules. The initial events of the FcεRI signal transduction cascade involve tyrosine phosphorylation of the β- and γ-subunits of the receptor and the protein tyrosine kinases $pp53/56^{lyn}$ (Lyn) and $p72^{syk}$ (Syk) (Benhammou, 1997). In this study, the effects of serum deprivation on the activation of a rat mast cell line, RBL-2H3, were investigated.

Cells were incubated for 90 minutes at 37°C in tyrode's buffer in the presence (control) or absence (deprivation) of 10 %(v/v) fetal bovine serum. Cells were primed with IgE directed against dinitrophenyl (DNP) and activated with human serum albumin-conjugated DNP (DNP-HSA, 100 ng/ml). After 30 min, the release of β-hexosaminidase was determined as an indicator for degranulation (Fischer *et al.*, 1995). Cells were stimulated with antigen (100 ng/ml DNP-HSA) and lysed after 0.5, 1, 2 or 5 min. Homogenates were immunoprecipitated using monoclonal antibodies to phosphotyrosine (PY), the FcεRI-β or -γ subunits, Lyn or Syk kinase. Tyrosine phosphorylation was determined by SDS-PAGE protein separation and Western blot analysis using anti-PY antibody and chemiluminescence detection. Membrane integrity (retention of calcein; Lichtenfels *et al.*, 1994) and mitochondrial activity (hydrolysis of MTT to formazan; Mosmann, 1983) were used as parameters for cell viability. Membrane expression of FcεRI was determined via flow cytometry using FITC-labeled IgE.

Serum deprivation resulted in a significant decrease of β-hexosaminidase release (Table 1). This impaired degranulation was associated with a decreased protein tyrosine phosphorylation at 0.5 to 5 min after FcεRI activation. Immunoprecipitation with antibodies to the FcεRI-β or -γ subunits, Lyn or Syk kinases, indicated that tyrosine phosphorylation of these proteins was impaired by deprivation. Both cell viability and membrane expression of FcεRI were not affected by serum deprivation (Table 1).

Table 1 treatment	% β-hex release	calcein fluoresc.	formazan OD	FcεRI fluoresc.
control	45.3 ± 0.6	422 ± 12	1.24 ± 0.04	35.1 ± 1.4
deprivation	21.6 ± 0.8*	419 ± 8	1.22 ± 0.07	36.0 ± 1.1

(*mean ± s.e.mean are given of at least 3 independent experiments of triplicates, $p<0.01$ vs. control, t-test)

The present results demonstrate that serum deprivation impairs antigen-mediated degranulation of RBL-2H3 mast cells and suggest that serum factors play a role in tyrosine phosphorylation of the FcεRI signal transduction pathway.

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(Supported by GLAXO WELLCOME Ltd., The Netherlands)

159P BRADYKININ CAUSES INHIBITION OF METHACHOLINE-INDUCED BRONCHOCONSTRICTION *IN VIVO* IN MICE

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The generation of bradykinin is thought to play a relevant role in inflammatory diseases such as asthma. During the past decade several mice models have been developed to study the characteristics of asthma. Studies on mice isolated trachea have shown that bradykinin exerts a relaxant response *in vitro* (Van Heuven-Nolsen et al., 1997). The aim of this study was to examine the effects of bradykinin on airway resistance in mice *in vivo*. Thus, the dose-response relationship and the receptor-type was investigated. Furthermore, we evaluated whether the inhibition of the methacholine-induced bronchoconstriction resulted from the possible release of tachykinins, nitric oxide, catecholamines or prostaglandins.

Male Balb c mice were anaesthetised with urethane (2 g/kg, i.p.) and received tubocurarine chloride (3.3 mg/kg, i.v.). Animals were ventilated with a small animal ventilator (frequency 200 strokes/min, tidal volume 0.15 ml) and placed in a body plethysmograph. Airway resistance was computed breath by breath from transpulmonary pressure, tidal volume and flow rate. Results are expressed as mean ± s.e. mean.

Baseline resistance in mice amounted 2.1 ± 0.2 cm H_2O/ml sec^{-1}, n=14. Intravenous (i.v.) administration of bradykinin did not cause a direct effect on airway resistance. Also pretreatment with propranolol (1 mg/kg, i.v.), atropine (1 mg/kg, i.v.) or indomethacin (5 mg/kg, i.v.) did not result in any effect of i.v. bradykinin on baseline airway resistance. Successive injections of methacholine 0.5 mg/kg, i.v., caused reproducible bronchoconstrictions in mice. The increase in airway resistance after methacholine amounted 20.1 ± 1.3 cm H_2O/ml sec^{-1}. I.v. injection of bradykinin (4-40 μg/kg) together with 0.5 mg/kg methacholine caused a dose-dependent inhibition of the methacholine-induced bronchoconstriction, with an ED_{50} value of 3.4 ± 0.4 μg/kg for bradykinin. The maximal inhibition of the bronchoconstrictor response to methacholine by bradykinin amounted 65.5 ± 2.0 %. The inhibition of the methacholine-induced bronchoconstriction by bradykinin (50.3 ± 1.0 %, n=5) could be prevented by treatment with the B_2 receptor antagonist HOE 140 (0.13 mg/kg, i.v.; 106.8 ± 8.7 %, n=5). Also pretreatment with either propranolol (1 mg/kg, i.v.), L-NAME (30 mg/kg, i.v.) or indomethacin (5 mg/kg, i.v.) completely reversed the inhibition of the methacholine-induced bronchoconstriction by bradykinin (100.7 ± 7.9 %; 92.5 ± 7.7 %; 103.1 ± 14.9 respectively, n=5 for all groups). The inhibition of the methacholine-induced bronchoconstriction after bradykinin was not affected by the NK_1 receptor antagonist RP 67580 (17.5 μg/kg, i.v.; 47.9 ± 9.3 %, n=5). In conclusion, the results of this study demonstrate that bradykinin causes a dose-dependent inhibition of the methacholine-induced bronchoconstriction *in vivo* in mice. This response is B_2-receptor mediated and at least, involves the activation of ß-adrenoceptors and the synthesis of nitric oxide and cyclo-oxygenase products.

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160P REGULATION OF HISTAMINE H_1 RECEPTOR GENE TRANSCRIPTION IN CULTURED HUMAN AIRWAY SMOOTH MUSCLE (HASM) CELLS

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Histamine H_1 receptor stimulation of inositol phospholipid hydrolysis can be modulated by a range of agents in HASM cells including β_2 adrenoceptor agonists and dexamethasone (Hardy et al 1996). In addition, when HASM cells were pre-exposed to 0.1mM histamine for periods over 4h or longer, we found the H_1-mediated inositol phosphate response to a second histamine challenge was reduced by 48 ± 3 %(n=4, $p<0.01$). Little is known about the longer term regulation of histamine H_1 receptor transcription in these cells. The aims of this study were therefore to investigate the potential for isoprenaline, dexamethasone and histamine itself to modulate H_1 receptor mRNA levels in HASM cells.

HASM cells were grown as previously described (Hardy et al 1996) and preincubated with histamine (100μM), dexamethasone (1μM), or isoprenaline (10μM) for 2hr, 12hr, or 24hr before mRNA extraction. Reverse transcription (RT) was then performed using random hexamers. cDNA (1μl) was then used as template for PCR amplification using primers based on the nucleotide sequence for the cloned human H_1-receptor. A parallel reaction was performed using primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to normalize the data. Initial experiments were performed to optimise cycle number for each reaction: 25 cycles of PCR were used for the histamine reaction and 21 cycles for GAPDH. PCR products were then denatured and applied in triplicate to filters on a dot blot apparatus. Each filter was hybridised as appropriate with a ^{32}P-labelled internal probe and product was quantified using a PhosphorImager.

Expression of H_1 receptor mRNA after 2hr incubation with dexamethasone was reduced by 45 ± 6% compared with control ($p<0.05$, n=3). Smaller (nonsignificant) reductions were seen following dexamethasone pretreatment for 12hr (23 ± 14%, n=3) or 24hr (30 ± 15%, n=5). No significant change was seen following histamine preincubation, although there was a trend towards a reduction at later time points (2hr: 20 ± 10% reduction, 12hr: 19 ± 8%, 24hr: 40 ± 21%, all n=3-5). Isoprenaline pretreatment also produced small but nonsignificant reductions in H_1 receptor mRNA levels (2hr: 38 ±13%, 12h: 22 ± 7%, 24h: 7 ± 37%, n=3-5).

In summary, this study demonstrates that dexamethasone pretreatment decreases histamine H_1 mRNA level after a 2hr incubation time. These data may help to explain the reduced inositol phosphate responses seen in HASM cells in response to histamine following dexamethasone pretreatment.

Hardy E., Farahani M., Hall I.P. (1996). *Br.J.Pharmacol*. **118**, 1079-1084

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161P PRODUCTION OF THE ISOPROSTANE, 8-ISO $PGF_2\alpha$, BY HUMAN PULMONARY ARTERY SMOOTH MUSCLE CELLS IS CYCLOOXYGENASE-2-DEPENDENT

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8-iso prostaglandin (PG) $F_{2\alpha}$ is the most abundant isoprostane *in vivo*, the production of which was initially described as independent of the enzyme cyclooxygenase (COX). However recent evidence has shown a role for COX-2, the inducible form of COX, in the formation of isoprostanes. In this study we have addressed the role of COX-2 in the production of PGE_2, a typical COX metabolite and the isoprostane, 8-iso $PGF_{2\alpha}$, by human pulmonary artery (PA) smooth muscle cells

Human PA obtained from lung cancer surgery was left in supplemented DME medium and the smooth muscle allowed to explant. After 4-6 weeks the smooth muscle formed a confluent monolayer which stained positive for smooth muscle α-actin. Western blots were carried out to characterise the isoform of COX expressed in the human PA smooth muscle cells. PGE_2 released into medium was measured by radioimmunoassay (Mitchell et al. 1993) and 8-iso $PGF_{2\alpha}$ was measured by ELISA (Cayman Chemical).

The inflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) stimulated release of both 8-iso $PGF_{2\alpha}$ and PGE_2 from human PA smooth muscle cells, but neither interferon γ (IFNγ) nor lipopolysaccharide (LPS) significantly increased the release of these eicosanoids (Figure 1). IL-1β was much more effective than TNFα in stimulating the production of both 8-iso $PGF_{2\alpha}$ and PGE_2. In each case approximately 50 fold more PGE_2 was released than 8-iso $PGF_{2\alpha}$. The stimulated release of both 8-iso $PGF_{2\alpha}$ and PGE_2 was completely inhibited by the COX-2 inhibitor L-745,337 (10μM). Western blots showed only very low amounts of COX

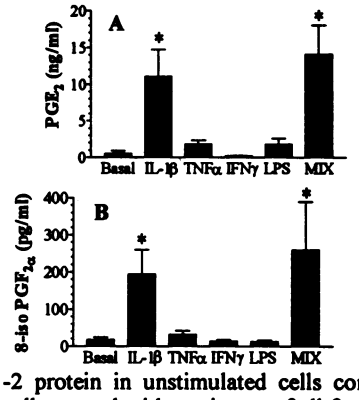

*Figure 1 shows the release of (A) PGE_2 and (B) 8-iso $PGF_{2\alpha}$ from human PA smooth muscle cells treated with cytokines (10 ng/ml) or LPS (10 μg/ml) alone or in combination (MIX). Data are the mean ± s.e.mean (n=5-12). *p<0.05 cf. basal (ANOVA).*

-2 protein in unstimulated cells compared with levels from cells treated with a mixture of all four inflammatory mediators used in this study.

These results provide further evidence for the production of isoprostanes by COX enzymes from human cells. Thus isoprostane levels in vivo may not be directly related to oxidative stress levels and their use as indicators of this should be approached with caution. Furthermore 8-iso $PGF_{2\alpha}$ has been shown to have potent biological actions as both a vasoconstrictor and vasodilator (Jourdan et al. 1997) and thus contribute to either pulmonary hypotension or hypertension under inflammatory conditions, including those seen in sepsis, when levels of cytokines are elevated.

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162P INCREASED TRACHEAL VASCULAR PERMEABILITY DUE TO A REPEATED CHALLENGE WITH DNS IN DNFB-SENSITIZED MICE: A ROLE FOR TACHYKININS

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Occupational asthma can be caused by low molecular weight compounds via an unknown IgE independent mechanism. Increased vascular permeability leading to oedema formation in the lung is one of the characteristics of occupational asthma. It has been reported that respiratory tract infections increase the susceptibility to neurogenic inflammation leading to increased vascular permeability in the rat trachea. Recently, a mice model for non-IgE mediated asthma has been developed using skin-sensitization with dinitrofluorobenzene (DNFB) and subsequent dinitrosulphonic acid (DNS) challenge. In this study the effect of a repeated challenge with DNS on tracheal vascular permeability was studied in the DNFB-model in mice. The possible involvement of sensory nerves was studied by the use of capsaicin and RP 67580, a neurokinin-1 (NK_1) receptor antagonist.

On day 0 and 1 male Balb/c mice were sensitized with DNFB (0.5%) as described by Buckley and Nijkamp (1994). On day 5 mice were challenged intranasally (i.n.) with DNS (0.6%, 50 μl). To assess tracheal leakage Evans Blue (EB, 1.25% in sterile saline) was injected intravenously (i.v.) at 22 h after the challenge. At 23 h 30 min a second challenge with DNS, capsaicin (10^{-10} mol/mouse, 50 μl) or vehicle was given i.n. In some DNFB-sensitized animals RP 67580 (10^{-9} mol/mouse, 50 μl) was injected i.v. 15 minutes before the second challenge. Shortly before the animals were sacrificied at 24 h, heparine (50 IU/mouse) was injected i.v. Thereafter, a blood sample was taken and the trachea removed. The amount of EB was extracted from the trachea in formamide at 40 °C overnight. EB content in extracts and plasma samples was measured spectrofotometrically at 620 nm. The amount of vascular leakage (μl/mg tracheal dry weight) was calculated by dividing EB content in trachea by EB content in 1 ml plasma.

In naive mice, a repeated challenge with DNS did not influence the tracheal vascular permeability. However, in DNFB-sensitized mice, a repeated challenge of DNS induced a significant increase of the vascular permeability compared to the control group (DNFB/DNS/veh: 0.81±0.07 & DNFB/DNS/DNS: 2.09±0.23 (μl/mg tracheal dry weight), n=8, $P<0.001$ ANOVA). Capsaicin given i.n. to DNFB-sensitzed and DNS-challenged mice also caused a significant increase (DNFB/DNS/veh: 1.30±0.20 & DNFB/DNS/caps: 2.12±0.30 (μl/mg tracheal dry weight), n=10, $P<0.001$ ANOVA). The increased vascular permeability due to a second DNS challenge in DNFB-sensitized mice could be completely abolished by RP 67580 (DNFB/DNS/veh/DNS: 1.97±0.13 & DNFB/DNS/RP67850/DNS: 0.84±0.05 (μl/mg tracheal dry weight), n=6, $P<0.001$ ANOVA).

In summary, this study indicates that a repeated challenge with DNS increases the vascular permeability in the trachea. This effect could be mimicked by capsaicin and abolished by a NK_1-receptor antagonist, indicating that sensory nerves and more specifically the NK_1 receptors are involved.

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163P RELATIVE POTENCIES OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON PURIFIED CYCLO-OXYGENASE-1 AND -2

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are a diverse group of drugs with the common ability to inhibit the enzyme cyclo-oxygenase (COX). COX is present in cells constitutively (COX-1) and/or after stimulation with inflammatory agents (COX-2). Currently, it is thought, that NSAIDs owe their beneficial anti-inflammatory effects to inhibition of COX-2 and their shared side-effects (eg gastric ulceration) to inhibition of COX-1 (see Mitchell et al., 1995). Indeed, a wide variety of assay system's have been used to assess the relative potencies of prescribed and novel NSAIDs on COX-1 and COX-2 enzymes. Here we describe a simple and rapid assay system for the assessment of relative potencies of NSAIDs on COX-1 and COX-2 using purified ovine enzyme (Cayman Chemical Company).

Experiments were performed in 96-well plates, in a final reaction volume of 200µl, containing combinations of epinephrine (5 mM) and hematin (1µM) in 50 mM Tris (pH 7.5). Different nonsteroidal drugs were added to individual wells and the reaction initiated by the addition of 2U/ml of COX-1 or COX-2. Plates were incubated at 37°C for 30 min before the addition of arachidonic acid (30µM) for a further 15min. The reaction was stopped by heating the plate to 100°C for 5min. The plate was then centrifuged at 3000g for 30 mins and supernatant removed for the measurement of prostaglandin E_2 by radioimmuoassay. The IC_{50} values for drugs which inhibited COX-1 or COX-2 are shown in table 1.

Drug	IC_{50}, COX-2 (µg/ml)	IC_{50}, COX-1 (µg/ml)	ratio: COX-2:COX-1
Nimesulide	2.9	9.8	0.3
Meloxicam	102	53	1.9
Piroxicam	535	143	3.2
Indomethacin	2.2	0.3	7.3
Diclofenac	1.5	0.2	7.5
Ketoprofen	21	0.3	70

Table 1. Relative potencies of nonsteroidal anti-inflammatory drugs on ovine purified COX-1 and COX-2. The ratio shown in the last column was calculated by dividing the IC_{50} value for a given drug on COX-2 by its value on COX-1. Thus, the lower the value in this column the more COX-2 selective the drug. Data represents the mean ± the standard error of the mean for n=3-9 experiments.

None of the drugs tested showed specificity for COX-2. However, the gastric sparing drugs, nimesulide and meloxicam were approximately equipotent inhibitors of COX-1 and COX-2. Piroxicam, indomethacin and diclofenac were 'moderately' COX-1 selective inhibitors, whereas ketoprofen showed a high level of COX-1 selectively.

The relative potencies of nonsteroidal anti-inflammatory drugs obtained with this assay are in keeping with their activity on COX-1 and COX-2 in whole cell and microsomal assays (see Mitchell et al., 1995). Moreover, these observations suggest that the low risk of side effects reported for nimesulide and meloxicam is due to their ability to inhibit COX-2.

Mitchell, J.A., Larkin, S. *et al.*, (1995). *Biochem. Pharmacol.*, 50; 1535-1542.

164P THE SODIUM CHANNEL INHIBITOR, 4030W92: EFFECTS ON NEUROGENICALLY-MEDIATED PLASMA PROTEIN EXTRAVASATION AND C-FOS EXPRESSION IN THE TRIGEMINAL NUCLEUS CAUDALIS OF THE GUINEA-PIG

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4030W92 (R (-) 2,4-diamino-5-(2,3-dichlorophenyl)-6-fluoromethyl pyrimidine) antagonises tetrodotoxin-insensitive neuronal sodium channels. The present study investigated whether 4030W92 inhibits two sensory nerve-mediated events: plasma protein extravasation (PPE) and cell activation (expression of Fos-like immunoreactivity (Fos-LI)) in the trigeminal nucleus caudalis (TNC) following trigeminal nerve activation.

In PPE experiments, male Dunkin Hartley guinea-pigs (200-250g) were anaesthetised with pentobarbitone (50mgkg^{-1} i.p.) and the left femoral vein cannulated. A bipolar stimulating electrode was lowered into each trigeminal ganglion and, 5min after [^{125}I]-human serum albumin administration (1.9MBqkg^{-1}, i.v.), the right ganglion was stimulated (1.2mA, 5ms, 5Hz for 5min). 4030W92 (0.1 and 1mgkg^{-1}) or vehicle (saline; 0.5mlkg^{-1}) was administered (i.v.) 15min prior to stimulation. Animals were then perfused (0.9% saline for 3min) transcardially, and the dura, eyelid, tongue and lip dissected out, weighed and counted for radioactivity (expressed as c.p.m. mg^{-1} tissue). Differences between unstimulated and stimulated sides were assessed by ANOVA followed by Dunnett's t-test.

In Fos experiments, male Dunkin Hartley guinea-pigs (340-580g) were anaesthetised (ketamine 40mgkg^{-1} and sodium pentobarbitone 18mgkg^{-1} i.p.) and the left carotid artery and jugular vein cannulated for measurement of blood pressure, and administration of drugs and anaesthetic (30mgkg^{-1}h^{-1} pentobarbitone) respectively. A bipolar stimulating electrode was then lowered into each trigeminal ganglion or the cisterna magna was cannulated to allow intracisternal capsaicin (10nmol in 0.1ml) administration. 4030W92 (10mgkg^{-1}) or vehicle (saline) was administered i.v. (0.5mlkg^{-1}) 2.75h after surgery, and 15mins later either the right ganglion was stimulated (0.6mA, 5ms, 5Hz for 5min) or capsaicin (10nmol in 0.1ml), or its vehicle (ethanol: Tween 80: saline; 0.01:0.01:99.98 v:v), administered. Animals were perfused transcardially with 4% formalin 2h later. Fos-LI in the TNC was localised by the avidin-biotin-peroxidase method (Polley *et al.*, 1997), and the number of stained nuclei counted. Differences in Fos-LI between 4030W92 and vehicle-treated animals were assessed by ANOVA followed by Dunnett's t-test.

Electrical trigeminal ganglion stimulation resulted in PPE in all tissues studied. The PPE ratios, calculated from the c.p.m. mg^{-1} tissue on the stimulated and unstimulated sides, were 1.7±0.1 (dura), 4.1±0.9 (eyelid), 3.1±0.4 (tongue) and 5.3±0.8 (lip) (n=9). Pretreatment of rats with 4030W92 at the lower dose (0.1mgkg^{-1} i.v.) had no effect on PPE in any tissue studied. The higher dose (1.0mgkg^{-1} i.v.) produced significant inhibition of PPE in the dura (by 58.7±9.2%), but although there was a trend towards inhibition in the eyelid, tongue and lip this was not statistically significant (P>0.05).

In vehicle (i.v.)-treated animals, trigeminal ganglion electrical stimulation produced expression of Fos-LI in the TNC (144±8 and 14± 2 stained cells on the stimulated and non-stimulated sides respectively; n=7). 4030W92 (10mgkg^{-1}, i.v.) had no significant effect on Fos-LI (163±29 and 8±1 stained cells on the stimulated and non-stimulated sides respectively; n=3). In vehicle (i.v.)-treated animals, intracisternal capsaicin administration produced a significant Fos-LI (465±53 stained cells compared to 26±8 for its vehicle; n=4; P<0.05). 4030W92 (10mgkg^{-1}, i.v.) had no effect on capsaicin-induced Fos-LI (476±34 stained cells; n=4).

In conclusion, 4030W92 inhibits dural PPE, consistent with sodium channel blockade on peripheral trigeminal nerves. Inability of 4030W92 to inhibit expression of Fos-LI in the TNC may indicate that these channels are absent on central trigeminal nerve terminals.

Polley, J.S., Gaskin, P.J., Perren, M.J. et al. (1997) Regul. Peptides, 68, 23-29.

165P DIFFERENTIAL INHIBITORY EFFECTS OF SALMETEROL AND SALBUTAMOL ON HUMAN EOSINOPHIL RESPONSES *IN VITRO*

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In the treatment of bronchial asthma, salmeterol is known to have a greater anti-inflammatory activity than salbutamol. To determine whether the comparative effects of these drugs on eosinophil function is the basis of such differential anti-inflammatory properties, the effect of the two drugs on interlukin-5 (IL-5) and platelet activating factor (PAF)-induced superoxide anions (O_2^-) release as well as the C5a- and N-formylmethionyl leucyl phenylalanine (FMLP)-induced degranulation of purified human blood eosinophils *in vitro* were studied.

Eosinophils were separated from fresh blood of normal or mildly atopic donors by centrifugation over percoll gradient and further purified (>98% purity) by the immunomagnetic method (Hansel *et al.*, 1991). Purified cells were resuspended in Hank's balanced salt solution at a concentration of 5×10^5 cell/ml and 50 µl aliquots dispensed in microplate wells. Cells were pre-incubated with the drugs for 10 min at 37°C before stimulation, and for 1 h thereafter. The released O_2^- was determined by the superoxide dismutase (SOD) - inhibitable reduction of ferricytochrome c, while the released EPO was measured by the O-phenylenediamine method (Kroegel *et al.*, 1989).

PAF and IL-5 stimulated a concentration-dependent generation of O_2^- in the concentration ranges of 10^{-10} - 10^{-6} M and 0.01 - 100 ng/ml, respectively, and maximal releases of 28.7 ± 5.2, and 36.6 ± 6.6 nmoles reduced ferricytochrome c/10^6 cells/h, respectively, (n = 5-8). Concentrations of the stimuli giving comparable effects (10^{-6} M for PAF and 30 ng/ml for IL-5) were chosen for the study of the inhibitory effects of the drugs.

Salmeterol significantly inhibited IL-5-induced O_2^- release in a concentration-dependent manner with an IC_{50} of 2.2×10^{-6} M (95% CI, 1.6 - 2.7 x 10^{-6} M, n = 6) and a maximal inhibition of around 70%. In contrast, salbutamol had no significant effect even at 10^{-5} M. Both drugs significantly inhibited PAF-induced O_2^- generation, but salmeterol was approximately 20 times more potent than salbutamol [IC_{50} values of 3.2×10^{-7} M (95% CI, 2.1 - 4.3 x 10^{-7} M) and 6.3×10^{-6} M (95% CI, 4.7 - 8.1 x 10^{-6} M), respectively, n = 5-6]. Both drugs failed to block C5a-induced EPO release, whereas for FMLP-induced release, salbutamol, but not salmeterol, produced significant inhibition. The inhibitory actions of salbutamol, but not salmeterol, were reversed by the β_2-adrenoceptors antagonist ICI 118551.

These results confirm that human eosinophils can be directly modulated by β_2-adrenoceptor agonists but that salmeterol and salbutamol have differential effects which are dependent on the stimulus employed. Furthermore, the reported greater *in vivo* anti-inflammatory effect of salmeterol may be a reflection of its superior ability to inhibit eosinophil O_2^- release, rather than degranulation.

Hansel T.T., De Vries I.J.M., Iff T. *et al.*, (1991). An improved immunomagnetic procedure for the isolation of highly purified human eosinophils. J. Immunol. Meth. 145, 105-110.

Kroegel C., Yukawa T., Dent G. *et al.*, (1989). Stimulation of degranulation from eosinophils by platelet activating factor. J. Immunol., 142, 3518-3526.

166P THE EFFECT OF FATTY ACIDS ON NEUTROPHIL ACTIVATION *IN VITRO*: REVERSIBILITY BY PLASMA

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Fatty Acid intervention with fish oils is thought to be useful in the treatment of certain inflammatory disorders in which neutrophil accumulation and their subsequent activation plays a key role. This is thought to be due to the production of less active eicosanoid derivatives or a reduced production of inflammatory cytokines (Blok *et al.*, 1996). Similarly, the n-6 fatty acids γ-linolenic acid (GLA) and dihomo-γ-linolenic acid (DGLA) have also been reported to inhibit neutrophil mediated inflammatory disorders, (Bates, 1995). The aim of this study was to determine the effects of these fatty acids on neutrophil activation *in vitro*.

Neutrophils were isolated by dextran sedimentation followed by centrifugation using a discontinuous percoll gradient, and contaminating red blood cells removed by lysis. Superoxide anion generation (SAG) was assayed by spectrophotometric evaluation of the reduction of ferricytochrome C to ferro-cytochrome C (A550nm). Myeloperoxidase (MPO) release was determined using tetramethylbenzidine (0.1%) and H_2SO_4 (4M) for spectrophotometric evaluation (A450nm). Experiments were carried out in 5% autologous plasma, with fatty acids being added to the neutrophils 10 min before the stimulant.

At 10µM none of the fatty acid potassium salts tested had any effect on neutrophil SAG in response to stimulation with n-formyl-methionyl-leucyl-phenylalanine (fMLP). However at 100µM, GLA and DGLA (n=3), both shifted the fMLP curve to the right giving EC_{50} for fMLP of 15.1 ± 1.1nM for the control curve and 24.6 ± 2.3 and 61.0 ± 12.9nM with GLA and DGLA respectively, without reducing the maximum fMLP response.

Eicosapentaenoic acid (EPA) at 100µM significantly inhibited fMLP stimulated neutrophil SAG, reducing the maximum from 27.4 ± 1.4 to 7.74 ± 2.0 nmolesO_2^-/million cells/10min (n=4), whereas arachidonic acid (AA) itself stimulated neutrophil SAG giving 28.9 ± 1.2 nmolesO_2^-/million cells/10 min (n=4).

The fatty acids were more effective when neutrophil MPO release was used as a marker of neutrophil activation. At 10µM DGLA shifted the fMLP curve to the right, giving EC_{50} for fMLP of 23.7 ± 1.8 for control cells and 30.7 ± 2.7nM with DGLA (p=0.005), reducing the maximal effect of 1µM fMLP to 83.7 ± 1.0% (p=0.01) n=6. Both EPA and AA 10µM inhibited MPO release, n=6, giving EC_{50} for fMLP of 21.7 ± 0.7nM with control cells and 29.9 ± 2.9 (p=0.03) and 32.1 ± 3.3nM (p=0.02) with EPA and AA. At 100µM, DGLA, EPA and AA completely suppressed MPO release by fMLP whereas GLA was less effective (84.3 ± 2.2% of fMLP response, n=6, p=0.05).

In conclusion the fatty acid potassium salts tested can markedly affect both neutrophil SAG and MPO release, with MPO release being more sensitive to these fatty acids. All of the fatty acids tested can inhibit MPO release including AA which stimulates neutrophil SAG. Furthermore, this inhibition is observed in the presence of autologous plasma (5%) as would be the case *in vivo*, where fatty acids are bound to plasma proteins. However, increasing the plasma concentration to 50% abolished the effect of GLA, EPA and AA on neutrophil SAG. Only DGLA (100 µM) was still inhibitory, giving EC_{50} for fMLP of 17.43 ± 2.42 and 35.4 ± 4.2nM (p=0.05), n=4. This is consistent with reports that albumin can reverse the effects of AA (Cohen *et al.*, 1986).

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Blok, W.L. *et al.*, (1996) *J. Nutr.*, 126:1515-1533
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167P POLY-L-LYSINE INDUCED NEUTROPHIL ACCUMULATION IN RABBIT SKIN AND THE INHIBITORY EFFECT OF HEPARIN

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Polycations such as poly-L-arginine produce plasma protein exudation (PPE) in rabbit skin (Needham et al., 1988) and oedema and neutrophil (PMN) infiltration in rat lung (Santana et al., 1993). In rat paw, poly-L-arginine has been reported to cause oedema but only a minor accumulation of PMNs (Antunes et al., 1990). We have previously studied poly-L-lysine (PLL)-induced PPE in rabbit skin and have demonstrated an inhibitory effect of unfractionated heparin (UH) on this parameter (Sasaki et al., 1991). Here, we have investigated the ability of PLL to cause PMN accumulation in rabbit skin and have tested the effect of UH on this response.

Intradermal injections (in 0.1 ml sterile saline) were made in a balanced block design on the shaved flank skin of pentobarbitone anaesthetised, male NZW rabbits (2.3-3.0 kg). Rabbit PMNs, separated from the arterial blood of donor animals by Hespan sedimentation and Ficoll density centrifugation, were labelled in autologous plasma with ^{111}Indium chloride and mercaptopyridine, washed and injected into the anaesthetised recipient animals (10^7 kg^{-1}). ^{111}In-PMN accumulation in excised skin sites was expressed as cells site^{-1}. PPE in the same sites was measured as local accumulation of intravenously injected ^{125}I-albumin and expressed as µl plasma. Mean ± s.e. mean values (n = 4 or 5) were calculated and the significance of differences between means was assessed by analysis of variance followed by a modified t-test which allowed multiple comparisons.

Intradermal injection of PLL (>150kD; 100µg site^{-1}) caused PMN accumulation which was significant ($P<0.01$) at 2h and 3h after injection (8372 ± 566 and 18334 ± 2460) but not at 0.5h or 1h (1079 ± 88 and 2458 ± 414). Accumulation in saline injected sites ranged from 205 ± 21 at 0.5h to 430 ± 80 at 3h. PPE was significant ($P<0.01$) at all four times following injection of PLL. PLL (10-100µg site^{-1})-induced PMN accumulation measured over 3h was dose-related and significant at 30µg site^{-1} (21217 ± 4484; $P<0.01$) and 100µg site^{-1} (35605 ± 5186; $P<0.001$) compared to saline (572 ± 97). PPE was also significant ($P<0.01$-0.001) at these doses. Intradermal injection of UH (5-100 iu site^{-1}), 10 min prior to PLL (100µg site^{-1}), caused dose-related reductions in cell accumulation from control values of 40513 ± 7772 to 8069 ± 1932 ($P<0.01$) with UH 100 iu site^{-1}. This dose of UH also produced a significant ($P<0.01$) reduction in PPE. In contrast, preinjection of the polyanion, poly-L-glutamic acid (PLG; 50-100kD; 5-100µg site^{-1}) had no significant effect on PLL-induced cell accumulation or PPE.

These results demonstrate that, in rabbit skin, PLL can induce a significant PMN accumulation which is slower in onset than PPE but which, like PPE, can be inhibited by local pretreatment with UH but not with the polyanion PLG. The effect of heparin is, therefore, not a simple charge-related effect but remains to be elucidated.

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Santana, A., Hyslop, S., Antunes, E., et al. (1993) *Agents Actions* 39, 104-110.
Sasaki, M., Paul, W., Douglas, G.J. & Page, C.P. (1991) *Br.J.Pharmacol.* 104,444P.

168P CONSTITUTIVE β_2-ADRENOCEPTOR ACTIVITY IN BOVINE TRACHEAL SMOOTH MUSCLE INDUCED BY FENOTEROL TREATMENT

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The β_2-adrenoceptor appears to exist in an equilibrium between an inactive conformation and a constitutively active conformation, which can couple to the G_s-protein in the absence of an agonist. Formation of the active conformation is stimulated by β-adrenoceptor agonist binding and by mutations of the receptor in the third intracellular loop (Samama *et al.*, 1994). Conversely, various β-adrenoceptor antagonists, also referred to as inverse agonists, favor the inactive conformation, and may thus inhibit the constitutive β-adrenoceptor activity. Under physiological conditions, the constitutive receptor activity is low and has only been visualized by overexpression of the native receptor (Chidiac *et al.*, 1994). In the present report, data are presented showing that enhanced constitutive β-adrenoceptor activity may be induced by incubation of bovine tracheal smooth muscle (BTSM) with the β-adrenoceptor agonist fenoterol.

BTSM strips were incubated with fenoterol (0.1-10.0 µM) for various periods of time (5 min, 30 min and 18 h) at 37 °C. After extensive washout during 3 h at 37 °C, isometric contraction was measured in response to increasing concentrations of isotonic KCl (6-40 mM), in the absence and subsequent presence of different antagonists. Antagonists were pre-incubated for 30 min before recording KCl-induced contraction.

Incubation of BTSM strips with 10 µM fenoterol for 5 min, 30 min and 18 h resulted in a time-dependent reduction of KCl-induced contraction, to 51±1, 43±6 and 38±5% of control, respectively, at 40 mM KCl (n=4-8, $P<0.005$ for all observations). In addition, a decreased sensitivity to KCl was observed, as indicated by an EC_{50} shift from 23.7±1.4 mM to 31.0±0.2, 30.9±0.7 and 31.6±1.0 mM, respectively ($P<0.005$). Incubation of BTSM with 0.1, 1.0 and 10 µM fenoterol during 18 h resulted in a concentration-dependent decrease of the 40 mM KCl response to 70±5, 47±5 and 43±9 % of control, respectively (n=5, $P<0.05$). The reduced 40 mM KCl-induced contractions were reversed in the presence of 1 µM timolol. Moreover, the EC_{50} of KCl-induced contraction in the presence of timolol was significantly reduced after fenoterol incubation (from 26.0±1.3 mM in controls to 17.4±0.7 mM after 18 h incubation with 10 µM fenoterol, $P<0.005$). Various antagonists were tested for their ability to potentiate the decreased KCl contractions after 18 h incubation of BTSM strips with 10 µM fenoterol. Based on the effects on a 40 mM KCl-induced tone, ranging from 178±16 to 268±32% of the fenoterol-incubated control strips, a rank order of inverse efficacy of 1µM timolol= 1µM propranolol=1µM pindolol>1µM alprenolol=100µM sotalol >10µM labetalol=10µM acebutolol was found, which was similar to that found in β_2-adrenoceptor overexpressing cells (Chidiac *et al.*, 1994). Labetalol (10 µM) and pindolol (1 µM), which were both partial inverse agonists at a 25 mM KCl tone, inhibited timolol-induced inverse agonism at this tone in an apparently competitive manner. The pEC_{50} for timolol was reduced by both antagonists, from 7.7±0.2 in the absence of competing antagonist, to 6.2±0.1 ($P<0.0001$) and 5.5±0.1 ($P<0.0001$) in the presence of labetalol and pindolol, respectively (n=7-8). Both the competitive inhibition by labetolol and pindolol of the timolol effect after fenoterol treatment and the enhanced sensitivity of BTSM contraction to KCl in the presence of timolol after this treatment, as described above, indicate that the fenoterol-induced effects cannot be explained by residual β-agonist binding.

In conclusion, treatment of BTSM with the β-agonist fenoterol causes a time- and concentration-dependent development of constitutive β_2-adrenoceptor activity, which can be reversed by various inverse agonists. The fenoterol-induced changes could represent a new regulation mechanism of β-adrenoceptor activity by β-agonists.

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169P POSTJUNCTIONAL RESPONSES TO THE PREJUNCTIONAL REGULATION OF AUTONOMIC NEUROTRANSMITTER RELEASE IN THE GUINEA-PIG TRACHEA

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The airways of many species are innervated by both parasympathetic and sympathetic neural pathways. The physiological effect of the parasympathetic nerves is excitatory, causing airway constriction, whereas the sympathetic innervation is inhibitory, causing dilatation. Recently, we showed by measuring the electrically evoked release of endogenous acetylcholine (ACh) and noradrenaline (NA) from guinea pig tracheal preparations that NA release appeared to be subject to prejunctional α_2-adrenergic auto- and M_2-muscarinic heteroinhibition whereas ACh release was subject to M_2 autoinhibition but only to minor α_2 heteroinhibition (de Haas *et al.*, 1996). In the present study we assessed the postjunctional responses (contraction and relaxation) to this mutual prejunctional regulation in the guinea pig trachea using electrical field stimulation (EFS).

Adult guinea pigs of either sex (Dunkin Hartley, Harlan, UK), weighing 600 - 800 g, were killed by a sharp blow on the head after which the trachea was rapidly removed and placed in gassed (5% CO_2, 95% O_2) Krebs-Henseleit buffer solution (37 °C). After removal of the epithelium, proximal open-ring preparations were mounted for isotonic recording of EFS (16 Hz, 150 mA, 0.8 ms, 4s every 80s) -induced cholinergic contraction followed by adrenergic + iNANC relaxation. Drugs were applied after precontraction of the preparations to 40% of maximal contraction using histamine. Responses were measured 30 minutes after each drug application. Data were expressed as percentages of basal responses ± s.e.m. and analysed by one way ANOVA and Student's *t*-test.

Both atropine (1 μM) and the selective M_3-muscarinic receptor antagonist UH-AH 371 (0.3 μM) completely blocked cholinergic twitch responses ($p<0.005$; n=5 and n=14, respectively), without altering relaxations. The NO synthesis inhibitor L-NAME (100 μM) reduced relaxations to 69±4% of control ($p<0.005$; n=11). Coincubation of L-NAME and the non-selective β-adrenoceptor antagonist timolol (1 μM) almost completely blocked relaxation to 9±4% ($p<0.005$; n=7);. twitch responses increased to 154±5% ($p<0.005$; n=11) and with L-NAME + timolol to 177±7% ($p<0.05$; n=7), both increments probably being the result of functional antagonism. The selective M_2-muscarinic receptor antagonist AQ-RA 741 (0.03 μM) increased twitches to 145 ± 8% ($p<0.005$; n=6) without affecting relaxations. No effect of AQ-RA 741 was found on adrenergic relaxations elicited in the presence of UH-AH 371 and L-NAME (n=8) either. The selective α_2-adrenoceptor antagonist yohimbine (1 μM) increased twitches to 108 ± 3% ($p<0.05$; n=15) while increasing relaxations to 153 ± 12% ($p<0.005$; n=15). The increase of relaxations could be attributed completely to increased adrenergic but not iNANC activity since yohimbine application after timolol did not increase the relaxation phase. Remarkably, administration of yohimbine after AQ-RA 741 increased relaxations to 235±58% ($p<0.05$) without significantly affecting the AQ-RA 741 potentiated cholinergic twitch.

Our results indicate that under the present experimental conditions prejunctional *auto*regulation plays an important role in the functional responses of the guinea pig trachea to endogenously released ACh and NA. In addition, autoregulation of the noradrenergic relaxatory response by α_2-adrenoceptors is negatively controlled by muscarinic M_2-heteroreceptor activation.

De Haas, J.R.A. *et al.* (1996) *Am. J. Respir. Crit. Care Med.*, **153(4)**, A848

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170P INVOLVEMENT OF THE NK_1-RECEPTOR IN THE DEVELOPMENT OF ALLERGEN-INDUCED AIRWAY HYPERREACTIVITY AND AIRWAY INFLAMMATION IN CONSCIOUS, UNRESTRAINED GUINEA-PIGS

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Airways are innervated by excitatory nonadrenergic noncholinergic nerves containing various transmitter peptides, including substance P (SP) and neurokinin A (NKA). Upon activation, SP and NKA are being released, leading to a series of responses collectively referred to as 'neurogenic inflammation', which may be involved in allergic asthma (Barnes *et al.*, 1991). These responses are partially mediated by the activation of NK_1 receptors, which may cause microvascular leakage, mucus secretion and recruitment and activation of inflammatory cells in the airways. Using a conscious and unrestrained guinea pig model of allergic asthma (Santing *et al.*, 1994), we investigated the involvement of NK_1 receptors in allergen-induced early (EAR) and late (LAR) asthmatic reactions, airway hyperreactivity (AHR) after these reactions and infiltration of inflammatory cells in the airways.

On two different occasions, separated by one week interval, ovalbumin (OA)-sensitized guinea pigs (SPF Dunkin Hartley, 400-500 g, of either sex) inhaled either saline (3 min) or the selective NK_1 receptor antagonist SR140333 (Emonds-Alt *et al.*, 1993; 100 nM, 3 min) at 30 min before, as well as at 5.5 h after OA provocation. Saline and SR140333 inhalations were alternated randomly. To assess the effects of these treatments on the EAR and LAR, pleural pressure (P_{pl}) changes were measured continuously during 24 h after OA provocation, using an on-line computer system (Santing *et al.*, 1994). Airway reactivity to inhaled histamine was assessed 24 h before and at 5 h (after EAR) and 23 h (after LAR) after OA provocation, by determining the provocation concentration causing 100% increase in P_{pl} (PC_{100}). Bronchoalveolar lavage (BAL) was performed at 25 h after the second allergen provocation, as well as in a control group, which received saline instead of OA.

SR140333 had no significant effect on both the EAR (3082±397 %x5 min) and LAR (10941±2365 %x5 min) compared to control (2799±654 %x5 min and 9362±2629 %x5 min, respectively, NS, n=3-6). However, SR140333 significantly reduced the OA-induced AHR to histamine, both after the EAR (1.77±0.13-fold increase in histamine reactivity vs. 2.50±0.25-fold increase in the controls, $P<0.01$) and LAR (1.15±0.12-fold increase vs. 1.98±0.34-fold increase, respectively, $P<0.05$, n=7). Moreover, SR140333 had an inhibitory effect on the allergen-induced infiltration of pro-inflammatory cells. Thus, compared to a control challenge with saline, OA challenge caused a significant increase in the numbers of BAL eosinophils (from $3.33\pm0.69\times10^6$ to $28.12\pm5.21\times10^6$, $P<0.001$), neutrophils (from $0.10\pm0.04\times10^6$ to $1.46\pm0.33\times10^6$, $P<0.005$) and lymphocytes (from $0.34\pm0.10\times10^6$ to $1.50\pm0.32\times10^6$, $P<0.01$) in the animals which were treated with saline instead of SR140333, while the numbers of infiltrated cells were significantly reduced in the SR140333-treated (second week) animals ($14.41\pm1.62\times10^6$ ($P<0.05$), $0.50\pm0.21\times10^6$ ($P<0.05$), and $0.33\pm0.05\times10^6$ ($P<0.005$), respectively) (n=6-7).

From these results we conclude that the NK_1 receptor is involved in the development of allergen-induced AHR to histamine, and that NK_1 receptor-mediated infiltration of inflammatory cells in the airways could contribute to this AHR.

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171P A MODEL OF ESTABLISHED AIRWAY INFLAMMATION IN BROWN NORWAY RATS: EFFECT OF DELAYED TREATMENT WITH FLUTICASONE PROPIONATE

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Single ovalbumin (OA) challenge in sensitised Brown Norway rats leads to airway eosinophilia and an increase in activated T lymphocytes, which are characteristics of human bronchial asthma (Underwood et al., 1997). Prophylactic treatment with fluticasone propionate inhibits airway inflammation in this model (Underwood et al., 1997). However, an effective anti-inflammatory drug needs to resolve established airway inflammation. The aim of this study was to develop a model of established airway inflammation and to investigate the effect of fluticasone propionate on existing airway inflammation.

Male Brown Norway rats (250-300 g) were sensitised i.p. with OA (100 μg) and $Al(OH)_3$ (100 mg) in saline on days 0, 12 and 21. From day 28 onwards, rats were challenged with aerosolised OA (10 g/l, 30 min) on 5 consecutive days. Treated groups were administered vehicle (0.5 % methylcellulose/0.2 % polysorbate 80 in saline) or fluticasone propionate (300 μgkg^{-1}) intratracheally 1 hour before the 3rd, 4th and 5th OA challenge. 24 hours after the final OA challenge, rats were euthanised, bronchoalveolar lavage (BAL) was performed, lungs were removed and flushed with RPMI/10% foetal calf serum. Airway inflammation was measured by differential cell counts of BAL and lung tissue samples obtained by incubation with collagenase. T lymphocyte subsets were quantified by flow cytometry (Underwood et al.,1997). Results are expressed as mean ± s.e.mean., n = 10 in each group. Statistical analysis was carried out using Kruskal Wallis multiple comparison test, $P < 0.05$ was accepted as significant.

Rats challenged for two days exhibited a significant increase in eosinophil and lymphocyte numbers in both lung tissue and airway lumen (table 1). Total T lymphocyte numbers ($CD2^+$), T helper ($CD4^+$) and activated lymphocytes ($CD25^+$) were enhanced. Therefore, airway inflammation was observed prior to fluticasone administration. Five ovalbumin challenges produced a further rise in inflammatory cell numbers in comparison to animals challenged only twice (table 1). Delayed administration of fluticasone propionate led to a significant reduction in airway eosinophilia and T lymphocytes (table 1).

Multiple ovalbumin challenge in Brown Norway rats resulted in sustained airway inflammation Fluticasone propionate, administered after the onset of airway inflammation, inhibited the increase in eosinophil and lymphocyte cell numbers. This study demonstrates that multiple ovalbumin challenge in Brown Norway rats is a useful model to investigate the efficacy of anti-inflammatory drugs in established airway inflammation.

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Table 1 - Effect of fluticasone propionate on airway inflammatory cell numbers following multiple ovalbumin challenge. (*$P < 0.05$ in comparison to vehicle treated group)

Treatment	No. of challenges	BAL (10^3 cells/ml)	Lung Tissue (10^3 cells/mg)			
		Eosinophils	Eosinophils	$CD2^+$	$CD4^+$	$CD25^+$
Unchallenged	0	6.1 ± 2.1	1.8 ± 0.6	2.3 ± 0.6	0.7 ± 0.2	0.1 ± 0.02
Challenged	2	74.0 ± 15.8	3.0 ± 0.3	3.8 ± 0.4	1.9 ± 0.2	0.4 ± 0.1
Challenged/ Vehicle	5	278.9 ± 67.3	5.7 ± 0.5	6.7 ± 0.6	3.4 ± 0.3	1.0 ± 0.1
Challenged/ Fluticasone	5	139.5 ± 23.6*	2.3 ± 0.2*	2.0 ± 0.3*	0.5 ± 0.1*	0.1 ± 0.01*

172P DIFFERENTIAL INHIBITION BY TIXOCORTOL PIVALATE OF THE RELEASE OF INTERLEUKIN-5 RATHER THAN INTERFERON-γ FROM HUMAN BRONCHOALVEOLAR LAVAGE CELLS IN *IN VITRO*

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Previous work, in guinea-pigs, has shown that some steroids selectively inhibit interleukin-5 (IL-5) - dependent inflammation (Whelan *et al.*, 1995; Whelan, 1996). Tixocortol pivalate (Tix) is a glucocorticoid of similar potency to hydrocortisone used in the treatment of rhinitis, which is devoid of the systemic side-effects associated with other glucocorticoids (Davies, *et.al.*, 1981). In this presentation, we present evidence indicating that Tix and beclomethasone dipropionate (BDP) inhibited IL-5 release from human bronchoalveolar leukocytes in culture but that Tix was less potent as an inhibitor of interferon γ (IFNγ) release.

Bronchoalveolar lavage fluid (BAL) was recovered from patients undergoing bronchoscopy for diagnostic purposes. Cells in the BAL fluid were washed twice by centrifugation in RPMI 1840 medium containing penicillin (50 $U.ml^{-1}$) and streptomycin (50 $U.ml^{-1}$). Aliquots of the resulting suspension (1 x 10^6 cells. ml^{-1}) were incubated for 1h at 37°C with Tix, BDP or vehicle after which PHA (10μg.ml^{-1}) and PMA (10ng.ml^{-1}) were added to all cultures to activate the cells. Forty eight h after the addition of PHA/ PMA, culture supernatants were harvested and assayed for IL-5 and IFNγ by ELISA. An experimental design was used where patients served as their own control in that aliquots of cells cultured with glucocorticoid were compared with an aliquot of cells from the same patient cultured with vehicle. Inhibition was measured as the percent reduction in cytokine release relative to vehicle treated cells and are presented as mean ± s.e.mean of the number of subjects shown in parentheses.

The BAL fluid recovered from patients contained approximately 80% macrophages, 20% lymphocytes, with the balance consisting of mast cells and epithelial cells. After 48h culture with PHA/PMA supernatants contained 2.22±0.34 ng.ml^{-1} IL-5 (n=19) and 0.24±0.03 ng.ml^{-1} IFNγ (n=17).

Tix (10^{-5} and $3x10^{-5}$M) significantly ($p<0.05$ paired t test) inhibited IL-5 release from human BAL cells (46.39±14.62%,n=7 and 45.94±12.22%, n=5 respectively). A lower concentration of Tix (10^{-6}M) did not significantly ($p>0.05$) inhibit IL-5 release (-5.87±38.44%, n=5%). Tix ($3x10^{-5}$M) significantly ($p<0.05$) inhibited IFNγ release (22.32±10.17%,n=5) whereas the lower concentrations of Tix (10^{-5} and 10^{-6}M) had no significant ($p>0.05$) inhibitory effect (10.76±12.11%,n=7 and 18.96±15.72%, n=6 respectively). BDP (10^{-7}M) significantly ($p<0.05$) inhibited IL-5 and IFNγ release from human BAL cells (97.68±1.44%, n=6 and 96.35±1.80%, n=6 respectively). A lower concentration of BDP (10^{-8}M) did not significantly ($P>0.05$) inhibit IL-5 or IFNγ release (9.18±29.76%, n=5 and -15.94± 33.30%, n=5 respectively).

The data show that Tix caused a concentration-related inhibition of IL-5 release but only the highest concentration tested inhibited IFNγ release. In contrast, all concentrations of BDP tested inhibited IL-5 and IFNγ release equally. These data show that Tix appears to be a selective inhibitor of IL-5 release while BDP is not. Further experiments are required to establish the reason for the apparent selectivity of Tix in these experiments.

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173P INTERLEUKIN-1 TYPE-1 RECEPTOR EXPRESSION IN THE RAT HYPOTHALAMUS

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Interleukin (IL), acts directly on the central nervous system to induce fever. Functional studies on experimental animals show that intracerebroventricular (icv) injection of IL-1β induces an increase in body temperature by interacting with thermoregulatory sites in the hypothalamus (Rothwell and Hopkins, 1995). Previous radioligand binding studies using radioiodinated IL-1α and IL-β in the rat failed to detect the main IL-1 receptor (IL-1RI) in this brain region (Takao *et al.*, 1992; Marquette *et al.*, 1995). In the present study a sensitive quantitative RT-PCR technique was used to investigate the expression and regulation of this receptor in hypothalamic tissue taken from rats after an icv injection of a pyrogenic dose of IL-1β or saline.

RNA was prepared from hypothalamic tissue taken from adult, male, Sprague Dawley rats (200-300g) two hours after injection with IL-1β (5ng/rat, icv; n=6) or saline (2μl/rat, icv; n=6) via stereotaxically pre-implanted icv guide cannulae or from normal uncannulated control conscious animals (n=5). RNA was also prepared from the liver of each animal for use as a positive control.
RNA samples were diluted to a standard concentration, spiked with an external standard cRNA specific for rat IL-1RI and reverse transcribed, cDNA products were amplified by PCR using the same pair of specific primers. Optical densities of the PCR products were determined and the results expressed relative to the external standard. Results are presented as mean±sem and statistical analysis was performed using ANOVA followed by the Tukey-Kramer Multiple Comparisons Test.

In the hypothalamus, IL-1RI expression in control (untreated) rats could not be detected. However, in rats injected icv with saline the level of IL-1RI expression was 6.8±1.8 pg/μg total RNA. Administration of IL-1β did not alter this expression (11.9±1.8 pg/μg total RNA). In contrast, IL-1RI expression was detected (55.0±8.5 pg/μg total RNA) in liver of untreated control animals and was not affected by icv injection of saline (62.6±6.9 pg/μg total RNA). However, IL-1RI expression was significantly increased by icv injection of IL-1β (277.7±41.2 pg/μg total RNA; $p<0.001$).

In contrast to previous data, these results demonstrate IL-1RI mRNA expression in the hypothalamus. This expression may be influenced by local damage resulting from implantation of the guide cannulae or insertion of the injection needle into the brain. Upregulation of IL-1RI mRNA in the liver by central injection of IL-1β supports the proposal that brain cytokines can modify events in the periphery.

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174P TUMOUR NECROSIS FACTOR-α (TNF- α) ENHANCES GUINEA-PIG AIRWAYS SMOOTH MUSCLE CONTRACTILITY VIA A CALCIUM SENSITIZATION PATHWAY

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A major characteristic of asthma is a hyper-responsiveness of the bronchial smooth muscle. This hyper-responsiveness is associated with an activation of inflammatory cells, such as neutrophils, which release the cytokine, TNF-α. *In vivo* exposure of sheep airways to aerosolized TNF-α produces an increase in bronchial smooth muscle contractility to agonists (Wheller *et al.*, 1990). The mechanism of this TNF-α-induced hyper-responsiveness is not yet known. The aims of this study were to investigate *in vitro* the effects of TNF-α in intact and permeabilized guinea pig bronchial smooth muscle.

Smooth muscle strips 200 μm wide were cut from guinea pig bronchi, attached to a senstive force transducer to measure tension and mounted in a "bubble chamber" adapted for rapid solution changes. Strips were placed in HEPES buffered Krebs at 28_C containing 3 μM indomethacin. Initially, strips were stimulated every hour with 154 mM K^+ and 10^{-4} M carbachol until stabilized contractile responses were obtained. Strips were stimulated with either 10^{-6} M or 10^{-4} M carbachol, relaxed and incubated in HEPES buffered Krebs containing 1 μg/ml human recombinant TNF-α for one hour. Following this incubation, bronchial strips were again challenged with the same concentration of carbachol. For permeabilized preparations, 200 μm wide strips were mounted in a bubble chamber and permeabilized with *staph. aureas* α-toxin. Immediately following permeabilization, a maximal calcium-activated response was obtained with pCa 4.5 solution. All pCa solutions are buffered with 10 mM EGTA to 'clamp' the intracellular Ca^{2+} concentration. Strips were submaximally contracted with pCa 6.3 buffer , relaxed in Ca^{2+} free buffer containing 1 mM EGTA (G1) and incubated for 45 minutes in G1 with 1 μg/ml TNF-α. Controls had no TNF-α added. Following this incubation, strips were contracted in pCa 6.3 and the tension measured. This protocol was also used to investigate the effects of a 45 minute incubation with 0.4 units/ml purified sphingomylinase which releases ceramide, a mediator of some TNF-α effects. All results are mean ± sem.

In intact strips of bronchial smooth muscle, contractions to carbachol were significantly increased after a one hour incubation in TNF-α (10^{-6} M carbachol: TNF treated 197 ± 16% of carbachol-induced contraction before TNF incubation vs control 82 ± 11%, n=4; 10^{-4} M carbachol: TNF treated 138 ± 8% vs control 94 ± 5%, n=5, $p<0.05$). In permeabilized strips of smooth muscle the pCa 6.3 induced contraction was 12 ± 1% of maximum Ca^{2+}-activated contraction in pCa 4.5(n=15). After 45 minutes, TNF-α incubation in G1 produced a small but significant rise in the baseline tension (TNF 10 ± 1% of pCa 4.5; control -2 ± 3%, n=6, $p<0.005$). There was a fivefold increase in the pCa 6.3 response following TNF-α incubation compared to controls (TNF treated 370 ± 49% of initial pCa 6.3 vs control 74 ± 10%, n=6, $p<0.05$) which was not additive with carbachol-induced sensitization. However, strips treated with sphingomyelinase for 45 minutes in G1 showed no significant change in the pCa 6.3 contractile response (67 ± 7% of initial pCa 6.3).

In conclusion, recombinant TNF-α caused a potentiation of agonist-induced bronchial smooth muscle contraction *in vitro* which may contribute to the bronchial hyperresponsiveness in asthma. This study has revealed that the mechanism for this potentiation is via a TNF-α-activated calcium sensitization but does not appear to involve a ceramide activated pathway.

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175P PHARMACOLOGICAL DIFFERENCES BETWEEN THE BINDING OF [^{125}I]IL8 AND UNLABELLED IL8 TO THE HUMAN RECOMBINANT CXCR1 AND CXCR2 RECEPTORS

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Interleukin-8 (IL8) is a pro-inflammatory chemokine involved in the recruitment of neutrophils to sites of inflammation. This effect is mediated by CXC chemokine receptors 1 and 2 (CXCR1 and 2). (Oppenheim *et al.*, 1991). Conventional saturation binding experiments require the use of large amounts of radioligand, which presents substantial difficulties for radio-iodinated compounds, such as [^{125}I]IL8. To circumvent this problem, binding parameters of radio-iodinated ligands are frequently determined by either (i) 'spiking' the radioligand with the non-iodinated ligand and peforming saturation binding, or (ii) using homologous competition experiments. However, a key assumption in using either experimental design is that both radioligand and homologous cold ligand have the same K_D value for the receptor (Hulme & Birdsall, 1992). We report data which suggests that this is not the case for IL8 and [^{125}I]IL8 binding to recombinant CXCR1 and 2 receptors.

Membranes from CHO cells stably expressing CXCR1 or CXCR2 were prepared. Bound radioligand was measured using a scintillation proximity assay (SPA). Briefly, membranes (10 and 100 µg protein for CXCR1 and CXCR2 respectively) were incubated with 0.5 mg SPA bead and increasing concentrations of [^{125}I]IL8 (0.5 pM - 1nM, saturation binding), or 50 pM [^{125}I]IL8 in the presence of increasing concentrations of cold IL8 (0.4 pM - 0.1 µM; competition binding) for eight hours. For spiking experiments, membranes were incubated with [^{125}I]IL8 (2.5 pM - 5 nM) after the specific activity had been reduced 10-fold by adding a 9-fold molar excess of cold IL8. Non-specific binding was determined using a saturating concentration of IL8. IL8 was radioiodinated by Amersham Life Science using sodium [^{125}I]iodide and lactoperoxidase. B_{max} and pK_D values were estimated by direct non-linear regression and expressed as mean ± s.e.mean of 3 experiments. Data was analysed statistically using one-way analysis of variance followed by a Tukey-Kramer test for significance.

IL8 competed for [^{125}I]IL8 at the CXCR1 receptor with a pK_i value which was 6.9 fold lower ($P<0.05$) than the pK_D determined from unspiked saturation experiments (Table 1). Spiked saturation experiments yielded a pK_D value not significantly different from the pKi ($P>0.05$), but 6.2 fold lower ($P<0.05$) than the pK_D determined from unspiked saturation experiments. Data obtained for CXCR2 showed a similar trend, with a pK_D derived from spiked saturation experiments which was significantly lower ($P<0.05$) than the pK_D determined from unspiked saturation. B_{max} estimates obtained from competition and spiked saturation binding at CXCR1 and CXCR2 were approximately five and two fold higher ($P<0.05$), respectively, than the B_{max} values obtained from unspiked saturation experiments.

Table 1

	CXCR1		CXCR2	
	pK_D/pK_i	B_{max}[1]	pK_D/pK_i	B_{max}[1]
Control Saturation	9.64 ± 0.03	20 ± 2	9.41 ± 0.03	1.1 ± 0.1
Spiked Saturation	8.85 ± 0.02*	97 ± 3*	9.04 ± 0.09*	2.4 ± 0.1*
Competition	8.80 ± 0.05*	93 ± 8*	9.22 ± 0.03	1.7 ± 0.2*

* $P<0.05$ vs pK_D or B_{max} (control saturation). [1] pmoles/mg protein

These results demonstrate that [^{125}I]IL8 and cold IL8 are not pharmacologically identical and the use of cold IL8 to determine K_D and B_{max} values yields erroneous results. Therefore, results derived from such experimental paradigms should be treated with caution.

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176P SPERMINE SUPPRESSES L-ARGININE TRANSPORT ACTIVITY IN RAT ALVEOLAR MACROPHAGES (AMΦ) WITHOUT DIRECTLY INHIBITING TRANSPORT

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Certain polyamines, such as spermine have been shown to inhibit nitrite production in immuno-stimulated macrophages and it was suggested that polyamines may suppress the induction of iNOS (e.g. Szabó *et al.*, 1994). NO synthesis by iNOS largely depends on the supply of the substrate L-arginine (L-Arg) which is taken up by the cells via specific cationic amino acid transport systems (see Closs, 1996; Hirschmann *et al.*, 1997). In the present study it was tested whether the polyamine spermine might affect L-Arg transport in rat AMΦ, cells in which iNOS plays a particular role (e.g. Hey *et al.*, 1995).

Rat AMΦ ($0.5*10^6$ cells per well) were cultured for 20 h in DMEM-F12 medium containing 5 % FCS (see Hey *et al.*, 1995) in the absence or presence of 1 µg ml^{-1} lipopolysaccharides (LPS) and/or spermine (1 - 100 µM). Thereafter, 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). Data are expressed either in absolute terms (d.p.m.) or as % of the uptake observed in controls of the respective cell preparation, means± s.e.mean of n experiments are given.

^{3}H-L-Arg accumulated in rat AMΦ, which had been cultured under control conditions, amounted to 4 874±324 d.p.m. $(0.5*10^6$ cells$)^{-1}$ (n=21). When spermine (up to 100 µM) was present only during the acute uptake period, it had no significant effect on ^{3}H-L-Arg uptake. However, when spermine (10 and 30 µM) was present during the 20 h period of culture, the subsequently studied ^{3}H-L-Arg uptake was reduced by 86±2 and 95±1 %, respectively, whereas at lower concentrations (1 and 3 µM), spermine had no significant effects (n=3-9). It should be noted that in rat AMΦ cultured under control conditions iNOS is already expressed, but iNOS activity is largely enhanced after culture in the presence of LPS (Hey *et al.*, 1995; Hammermann *et al.*, 1997). On the other hand, after culture in the presence of LPS, ^{3}H-L-Arg uptake was not significantly affected and spermine also inhibited ^{3}H-L-Arg uptake in LPS-stimulated cells. Spermine (10, 30 and 100 µM) reduced ^{3}H-L-Arg uptake by 92±2, 96±1 and 98±0.2 %, respectively, whereas 3 µM spermine had no significant effects (each n=3).

In conclusion, in rat AMΦ spermine does not interfere directly with the L-Arg transport, but following prolonged exposure can cause reduction of the L-Arg transport activity in these cells. Suppression of cationic amino acid transporter activity may be one mechanism by which polyamines can exert inhibitory effects on NO synthesis in macrophages.

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177P INHIBITION OF L-ARGININE TRANSPORT IN RAT AND GUINEA-PIG ALVEOLAR MACROPHAGES (AMϕ) BY POLY-CATIONIC PEPTIDES

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L-Arginine (L-Arg) serves as substrate of NO synthase and arginase, enzymes which play a particular role in AMΦ (Hey *et al.*, 1995). The cellular uptake of L-Arg is carried out by specific cationic amino acid transporters (CATs). Eosinophils can release cationic proteins such as major basic protein (MBP) or eosinophilc cationic protein (ECP) which appear to be involved in the development of airway hyperresponsiveness (Gleich *et al.*, 1993). Biological effects of MBP can be mimicked by poly-L-arginine (p-L-Arg) or poly-L-lysine (p-L-Lys) (e.g. Coyle *et al.*, 1993). Here, it was tested whether cationic peptides may affect transport of L-Arg in AMΦ.

Rat or guinea-pig AMΦ ($3*10^6$ cells per well) were cultured for 20 h in DMEM-F12 medium containing 5 % FCS (see Hey *et al.*, 1995). Thereafter, 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).

Under control conditions, rat and guinea-pig AMΦ accumulated 20 239±1 340 (n=57) and 75 720±12 530 (n=22) d.p.m. ^{3}H-L-Arg per $3*10^6$ cells, respectively (means±s.e.mean of n experiments). ^{3}H-L-Arg uptake was inhibited by p-L-Arg (5 000-15 000), p-L-Lys (4 000-15 000), protamine or MBP as summarized in Table 1.

Table 1. Concentration-dependent effects of cationic peptides in the absence or presence of 100 μg/ml heparin on ^{3}H-L-Arg uptake in rat and guinea-pig AMΦ expressed as % of respective controls.

	10	30	100	300 μg/ml
Rat AMΦ:				
p-L-Arg	74±22	52±14*	35±9**	16±4**
p-L-Arg+heparin			130±1+	127±4+
p-L-Lys	122±9	106±8	62±5*	58±3**
p-L-Lys+heparin			143±15+	130±8+
Protamine	102±5	91±4	78±5*	51±4**
MBP		90±3	77±2*	67±5*
Guinea-pig AMΦ:				
p-L-Arg		63±9*	42±6**	33±6**
p-L-Arg+heparin			137±4+	96±1+

means±s.e.mean, n=3-8; *P<0.05, **P<0.01 vs respective controls; +P<0.01 vs respective value in the absence of heparin.

In conclusion, L-arginine transport in AMΦ can be inhibited by cationic peptides, an effect which may contribute to the biological effects of eosinophils-derived cationic peptides.

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178P TEMPORAL AND SPATIAL EXPRESSION OF THE INDUCIBLE ISOFORMS OF CYCLOOXYGENASE, NITRIC OXIDE SYNTHASE AND HEME OXYGENASE IN TISSUES FROM RATS INFUSED WITH LPS IN THE CONSCIOUS STATE

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We have, in previous studies, analysed the regional and cardiac haemodynamic effects of a continuous infusion of LPS in conscious rats, and provided evidence for involvement of complex interactive mechanisms (Gardiner *et al.*, 1995). In the present work, using this model of endotoxaemia, we have investigated tissue changes in the inducible isoforms of cyclooxygenase (COX-2), nitric oxide synthase (iNOS) and heme oxygenase (HO-1) by Western blotting and immunocytochemical techniques (Tomlinson *et al.*, 1994; Willis *et al.*, 1996).

Male, Long Evans rats (350-450 g) were anaesthetised (sodium methohexitone, 40-60 mg kg^{-1}, supplemented as required) and had catheters implanted in the right jugular vein. The following day, an infusion of saline (n = 12) or LPS (*E.coli* serotype 0127: B8, Sigma, 150 μg kg^{-1} h^{-1}) (n = 12) was begun, and at 6 h or 24 h, 6 animals from each group were anaesthetised (sodium pentobarbitone, 60 mg kg^{-1} i.v.) and decapitated, and tissues (heart, kidney, lung, liver, spleen, mesentery, aorta) were frozen in isopentane pre-cooled in liquid N_2, and processed for the expression and localisation of COX-2, iNOS and HO-1 protein by Western blotting and immunocytochemistry.

In all tissues from saline-treated control animals, at both 6 and 24h, all three inducible enzymes were detected by Western blotting, with no change between the time points. Constitutive expression of iNOS has been reported by others (e.g., Park *et al.*, 1996; Hoffman *et al.*, 1997). In animals given LPS for 6h, densitometry of Western blots showed a 6-fold increase iNOS protein in lung and 4-fold in spleen; COX-2 expression was unchanged in all tissues. After 24 h infusion of LPS, iNOS protein had returned to control values in all tissues. In contrast, all tissues showed at least a 2-fold increase in HO-1 protein (except spleen, which has high endogenous HO-1 levels), with a 4-fold increase in lung. As at 6h, no detectable change in COX-2 protein expression was observed.

In general, where Western blotting indicated an increase in protein, this was mirrored by an increase of immunolabelling, especially in influxing inflammatory cells. However, cells were observed to be immunopositive for iNOS at 6h and for COX-2 at 6 and 24h, where Western blotting indicated no change. This probably reflects differences in sensitivities of the techniques. Combined results from these two experimental approaches suggests a less pronounced pattern of COX-2 expression than for the other two inducible enzymes. iNOS was induced in most tissues at 6h, with a return to control values by 24h. In contrast, there was a marked induction of HO-1 after 24h LPS infusion, indicative of a stress response feasibly mediated by nitric oxide, since previous findings indicate expression of COX-2 and iNOS is associated with pro-inflammatory events, whilst that of HO-1, a stress protein, coincides with inflammatory resolution (Tomlinson *et al.*, 1994; Willis *et al.*, 1996). The temporal pattern of changes is consistent with our earlier studies showing maximum iNOS activity at 6h, but maximum vasodilatation 24h after onset of LPS infusion (Gardiner *et al.*, 1995). The present findings indicate that HO-1 may contribute to the delayed vasodilatation.

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179P APOPTOSIS INDUCED BY MONOCULAR DEPRIVATION IN THE LATERAL GENICULATE NUCELUS (LGN) OF NEW-BORN RATS IS PREVENTED BY N$^{\omega}$-NITRO-L-ARGININE-METHYL-ESTER (L-NAME)

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During early post-natal life, the mammalian visual system is particularly susceptible to plastic adaptation in response to altered experience. Brief occlusion of vision in one eye during this period causes the initially binocular visual cortex to respond almost exclusively to stimulation of the unoccluded eye. So that vision in the deprived eye is irreversibly impaired. Anatomically, monocular deprivation (MD) causes cell shrinkage in the LGN and a reduction of geniculo-cortical axon arbors connected with the deprived eye (Rauschecker, 1991). Here we report that MD induces apoptosis in the LGN of new-born rats and this is prevented in a stereoselective fashion by L-NAME, an inhibitor of nitric oxide synthase (NOS). The right eyelid of Long Evans new-born rats (post-natal day=15; n=6 per group) were sutured for 2, 7 and 14 days. Age-matched, non-deprived rats (n=6 per group) were used as controls. Serial brain coronal sections (15μm) were processed for *in situ* detection of DNA fragmentation according to the TUNEL technique (Gavrieli *et al.*, 1992). Morphological characteristics and cell counting of adjacent brain sections were assessed under light microscopy using haematoxylin and eosin (H&E) staining as previously described (Nucci *et al.*, 1997). DNA fragmentation (TUNEL + cells) was observed in areas of sections (n=6 per brain) corresponding to (Pellegrino *et al.*, 1981) the LGN of rats deprived for 2 days (8.5±0.3), 7 days (8.0±0.3) and 14 days (8.0±1.1). Nuclear chromatin marginalization and condensation, typical features of apoptosis, were observed in H&E stained sections. No TUNEL + cells were observed in the LGN of controls. In adjacent sections (n=6 per brain) stained with H&E, the total number of cells was counted (100x magnification) in 176μm^2 to include approximately the whole LGN area and the resulting means±s.e.m. from MD and controls evaluated statistically for differences. The results are given in Table 1.

Table 1: L-NAME prevents cell loss caused by monocular deprivation (MD) in the LGN of new-born rats

Days	N° of rats	Control	MD	MD+L-NAME
2	6	62.4±0.7	54.9±0.4*	
7	6	63.3±0.9	40.1±1.0*	66.1±0.6
14	6	62.4±2.1	28.2±0.3*	

*p<0.05 vs age-matched controls (Student's "t" test)

As shown in table 1, treatment with L-NAME (3mg kg^{-1}, given i.p. twice daily for 7 days, n=6) reduced 7 days MD-induced cell loss in the LGN and the number of TUNEL + cells (0.2±0.1) did not differ from control (p>0.05). In all instance, D-NAME (same doses as for L-NAME) resulted ineffective.

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Partial financial support from the CNR (Rome).

180P EVIDENCE THAT INDOMETHACIN PREVENTS APOPTOSIS INDUCED BY HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) COAT PROTEIN gp120 IN THE NEOCORTEX OF THE RAT

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Recently, we have shown that intracerebroventricular (i.c.v.) injection of gp120 causes apoptosis in the brain neocortex of rat (Bagetta *et al.*, 1996a,b) and suggested that this may be implicated in neuronal loss often described at *post-mortem* in the brain of patients suffering from AIDS-associated neurological syndrome (see Bagetta *et al.*, 1997). Here we report that gp120 enhances cyclo-oxygenase type 2 immunoreactivity (COX2-I) and increases PGE_2 in the rat brain and this may be involved in the mechanisms of gp120-induced apoptosis because the latter is prevented by indomethacin, an inhibitor of COX activity. HIV-1 gp120 IIIB (100ng) or bovine serum albumin (BSA; 100ng) were administered (i.c.v.) daily for 7 days to individual rats with a 5μl Hamilton syringe (1μl volume) as previously described (Bagetta *et al.*, 1996a). In the antagonism study, gp120 injection was preceded (1h beforehand) by indomethacin (3.0 and 6.0 mgkg^{-1} i.p.). Apoptosis was assessed in coronal tissue slices (n=6 per brain) from the brain of rats sacrificed 24h after the last injection of gp120 by using the *in situ* terminal transferase (TUNEL) method, haematoxylin and eosin staining and transmission electron microscopy (TEM) (see Bagetta *et al.*, 1996a,b). Cryóstat brain sections (30μm) were incubated with a polyclonal rabbit anti-mouse antibody (Cayman Co.; diluted 1:1500) to detect COX2-1.Whole brain tissue content of PGE_2 was assayed by ELISA using a commercial kit (Cayman Co.). Quantification of apoptotic (TUNEL+ cells [1161μm^2]$^{-1}$) cells and COX2-I cells ([4753μm^2]$^{-1}$) in the brain neocortex was performed according to Bagetta *et al.* (1996a, 1997). In the neocortex of rats, gp120 causes *in situ* DNA fragmentation, nuclear chromatin marginalisation and condensation. Apoptotic cell counts are given in Table1.

Table 1. Indomethacin reduces the number (mean±s.e.m.) of TUNEL+ cells yielded by 7 days i.c.v. injection of gp120 in the untreated (U) and treated (T) side of the rat neocortex.

Treatment	N° of rats	U	T
gp120 (100 ng, i.c.v.)	6	1,8±0,4	3,6±0,4
gp120+Indo (3.0 mgkg^{-1})	6	2,1±0,4	2,5±0,4
gp120+Indo (6.0 mgkg^{-1})	6	0,2±0,1*	0,5±0,1*

*P<0.01 vs gp120 (ANOVA & Tukey-Kramer test).

The viral protein also increased COX2-I cells (gp120=113.8±9.4 vs BSA=81.8±1.4; P<0.01, Student's "t" test) in the same region of the rat (n=12) brain. The latter effect coincided with a 50% increase in brain (n=6 per group) PGE_2 content (gp120=1474±424 vs BSA=992±281, ng PGE_2/g tissue) and this may be involved in gp120-induced apoptosis because the latter was prevented by indomethacin (Table 1).

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181P THE USE OF A SCINTILLATION PROXIMITY ASSAY TO DETERMINE THE INTERACTIONS BETWEEN β-AMYLOID AGGREGATION INHIBITORS AND β -AMYLOID PEPTIDE

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We have described the inhibition of beta-amyloid aggregate formation by a novel series of benzofurans. Although the compounds prevent the formation of toxic aggregates of the peptide, the immunoassay and neurotoxicity data do not provide information on whether the inhibitors actually bind to the peptide. In this communication, we describe the use of a scintillation proximity assay (SPA) to study the interaction between peptide and the aggregation inhibitor SKF-74652 (Howlett *et al.*, this meeting).

SKF-74652 was radiolabelled to 48.7 Ci/mmol by tritium exchange and was purified on a silica gel Sep-Pak column (Waters). Beta-amyloid 1-40 peptide (Batch ZM605 - Bachem) was incubated in the presence of [^{3}H]-SKF-74652, a mouse monoclonal raised to the 1-16 sequence of beta amyloid, and anti-mouse SPA reagent (Amersham). Association of [^{3}H]-ligand and beta-amyloid was demonstrated by the formation of a complex between peptide, monoclonal and SPA reagent bringing the radioactive moiety and SPA reagent into close proximity.

Competition for [^{3}H]-SKF-74652 binding (10nM) by cold SKF-74652 was observed between 0.2 and 20 μM of unlabelled compound, with a maximum of 56 +/- 2.4% (mean +/- s.d.; n=4) inhibition at 20μM. Another aggregation inhibitor of the same chemical series inhibited [^{3}H]-SKF-74652 binding suggesting competition for the same site (SKF-64346 (Howlett *et al.*, this meeting): 60 +/- 8 % inhibition at 20 μM; n=4) whilst an analogue which was inactive in aggregation assays (SKF-73033) was also ineffective in preventing binding (IC_{50}>1mM).

Other known inhibitors of beta-amyloid aggregation were examined in the [^{3}H]-SKF-74652 SPA. Of these, daunomycin inhibited the binding (IC_{50} 26 +/- 12μM; n=4) whilst benzoquinone was ineffective (IC_{50} > 1mM).

Although competition was observed between tritiated and unlabelled SKF-74652, in a typical experiment the total binding of SKF-74652 increased exponentially from 0.16 pmole/nmole beta 1-40 at 31 nM to 0.77 nmole/nmole beta 1-40 at 203 μM free SKF-74652 (this was repeated 3 times). SPA signal was not detected in the absence of beta 1-40 peptide. Attempts to demonstrate association between peptide and inhibitor by nuclear magnetic resonance spectroscopy indicated that self-aggregation of SKF-74652 (and SKF-64346) occurred at high concentrations (200μM) of benzofuran. Thus, it would appear that the increases in binding are associated with concentration-dependent co-aggregation of SKF-74652 (incorporating tritiated ligand) and subsequent binding to beta-amyloid peptide.

These data, together with the anti-aggregation results (Howlett *et al.*, this meeting), suggest that inhibition of formation of toxic aggregates of beta-amyloid by SKF-74652 is associated with production of non-toxic beta-amyloid/SKF-74652 complexes.

Howlett, D.R. *et al.*, (1997) This meeting

182P PROGESTERONE INHIBITS NICOTINE-EVOKED RELAXATION OF THE GUINEA-PIG ISOLATED BASILAR ARTERY

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O'Shaughnessy & Connor (1994) have described an *in vitro* method for stimulating trigeminal terminals of the guinea-pig basilar artery (GPBA) with nicotine. A nicotine-evoked relaxation was substance P-mediated and blocked by sumatriptan or capsaicin. Progesterone has been shown to inhibit plasma protein extravasation in the dura mater following trigeminal stimulation in an *in vivo* model in the rat (Limmroth *et al.*, 1996) and is reported to block neuronal nicotinic receptor responses in mouse-brain synaptasomes (Bullock *et al.*, 1997). We have investigated the effects of progesterone in an *in vitro* model using the GPBA.

Male, Hartley guinea-pigs (250-400g) were killed by stunning and exsanguination. Ring segments (1.5-2 mm in length) of the GPBA were mounted on tungsten wires (0.1mm diameter) in 10ml tissue baths under a tension (isometric recording) of 0.4g. Tissues were maintained in Krebs' solution at 37°C, gassed with 5% CO_2 in O_2 for 90 min before a concentration-response curve to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10nM-10μM) was obtained. Substance P (3nM) was added to confirm an intact endothelium. Tissues were then washed and equilibrated with guanethidine (3μM) and atropine (3μM). After 1h tissues were re-contracted with $PGF_{2\alpha}$ (1 or 3μM) and the nicotine (0.1mM) relaxation was determined. Tissues were washed before progesterone (0.1 or 1μM) or vehicle (ethanol, final concentrations 0.1 and 0.01μl/ml respectively) was incubated with the tissues for 60min. Tissues were then re-contracted with $PGF_{2\alpha}$ and the response to nicotine obtained. One concentration of progesterone was tested in a tissue. The ratio of the response to nicotine in the presence of progesterone or vehicle (S2) with the initial response to nicotine (S1) was calculated in each tissue. Mean values ± s.e. mean are quoted.

The maximum increase in tension with $PGF_{2\alpha}$ in control tissues was 0.52±0.05g and the relaxation evoked by the first exposure to nicotine was 0.15±0.04g (n=10). Only 50% of tissues responded to nicotine. A significant reduction in the nicotine response was seen in the presence of progesterone (1μM). The nicotine S2:S1 ratios were 0.28±0.03 and 1.06±0.16 in the presence of progesterone (1μM) and ethanol vehicle respectively (p=0.008, paired t test, n=5). Progesterone (0.1μM) did not significantly reduce nicotine-evoked relaxations, S2:S1 ratios being 0.48±0.07 and 0.74±0.19 in the presence of progesterone (0.1μM) and ethanol vehicle respectively (p=0.180, paired t test, n=6). Responses to $PGF_{2\alpha}$ (1 or 3μM) were not significantly different in progesterone (0.1 or 1μM) and vehicle treated groups (S2:S1 ratios 1.06±0.08, 0.97±0.03, 0.98±0.02 and 0.99±0.06 for progesterone 1μM, 0.1μM and their respective ethanol vehicle controls, n=5).

The results show that nicotine-evoked relaxation of the GPBA is blocked following 1h equilibration with a high concentration of progesterone (1μM). The mechanism of action of progesterone is unknown but high concentrations of progesterone have been reported to block the ion channel of the neuronal nicotinic receptor (Bullock *et al.*, 1996; Ke & Lucas, 1996). A $GABA_A$ receptor mechanism is also possible (Limmroth *et al.*, 1996).

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183P EFFECTS OF A BRIEF SWIM STRESS ON PLASMA CORTICOSTERONE AND BRAIN MONAMINE LEVELS IN MICE WITH GENETIC MANIPULATION OF α_{2C}-ADRENOCEPTOR EXPRESSION

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Recently strains of mice have been genetically manipulated to either over-express (by three-fold) the α2C-receptor (α2Cxs) or have receptor inactivation, i.e. so-called knock-out mice (α2Cko) (Link *et al.* 1996; Sallinen *et al.* 1997). It was shown that though both strains of mice respond to non-subtype selective α2-agonists with characteristic changes in the levels of brain monoamine metabolites, there were some subtle differences (e.g. in levels of homovanillic acid, HVA) between mutant mice and controls (Sallinen *et al.* 1997). However, the central effects of non-subtype selective agonists are predominantly mediated via α_{2A}-receptor subtype. Therefore this experiment was designed to investigate the effects of minor stress on these mice strains, i.e there was no drug intervention.

The characteristics of the α2Cko and α2Cxs mice, including the method of production have been reported elsewhere (Sallinen *et al.* 1997). It should be noted that the strains of mice used to generate the transgenics differ. The α2Cko mice are predominantly C57BL/6J with a contribution of DBA/2J and 129/Sv strains whereas the α2Cxs are derived from FVB/N strain. This means that they are not mutually comparable i.e. comparisons are only valid between transgenics and their own wild type (wt) controls. Each group consisted of 9 or 10 male mice, average weight 31 ± 0.4 g s.e. mean. The mice were left to swim for 2.5 min in a 2 l beaker of water at 25°C. After the short swim, they were placed back in the home cage and 20 min later they were decapitated, blood drawn for assay of plasma corticosterone (by RIA) and brain monoamines and metabolites by HPLC and electrochemical detection. Statistical analysis was 2 factor ANOVA (stress; strain) followed by Scheffe's test.

Plasma corticosterone levels were elevated by the swim stress but the extent of the increase was somewhat blunted in transgenics, especially in the α2Cxs strain (table 1). Of all the brain monoamines/metabolites measured, only HVA showed both strain and stress related significant differences but neither it nor any other index revealed strain x stress interactions.

Table 1: Concentrations (± s.e. mean) of corticosterone in plasma and HVA in mouse brain 20 min after a 2.5 min swim

α2Cko mice	Corticosterone (ng/ml)		HVA (nmol/g)	
	wt	α2Cko	wt	α2Cko
Control	19.0±3.3	15.5±3.7	1.13±.04	1.04±.03
Swim	99.7±10.8#	80.0±5.8#	1.36±.04#	1.26±.04#
α2Cxs mice				
Control	32.6±7.8	38.6±16.8	0.65±.02	0.82±.07*
Swim	170.0±8.8#	138.5±11.1*#	0.88±.04#	0.89±-06

* Significant difference; transgenic vs wt ($P<0.05$)
\# Significant difference; swim vs control ($P<0.05$ at least)

In conclusion, manipulation of α2C-adrenoceptor gene expression either by knock-out or over-expression does not eliminate physiological or neurochemical responses to minor stress. However, in mice with α2C-receptor over-expression, there did seem to be a blunting of the responses. This may mean that these mice are constitutively stressed and the stress-relieving properties of α2-agonists are mediated, at least partly, via α2C-adrenoceptors.

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184P THE ROLE OF 5-HT_{1b} RECEPTORS IN THE MODULATION OF PLASMA ACTH LEVELS

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Measurement of neuroendocrine responses to a 5-HT drug challenge provides a valid means of assessing brain 5-HT function in both animals and humans (Cowen, 1993). In the present study the role of 5-HT_{1B} receptors in the modulation of plasma ACTH levels was evaluated using a repeat sampling, cannulation method.

Male Sprague Dawley rats (250-300g) were anaesthetised with Sublimaze (0.9ml/100g i.p.) and Domitor (0.4ml/100g i.m.) and the jugular vein and carotid artery cannulated. Animals were allowed to recover overnight before blood collection (0.5ml) via the arterial cannula commenced. Following collection of baseline samples, drugs or vehicle were administered and 4 further samples were collected at 30 min intervals. ACTH levels in blood samples were detected using a Diagnostic Systems Laborotories, Inc, RIA kit. Data was analysed using ANOVA followed by least significance difference t-test, using SAS-RA software. Significance was taken at the $p<0.05$ level.

Using this cannulation technique it was feasible to monitor drug induced changes in plasma ACTH levels over a 3-4h time period. The mixed 5-$HT_{1A/1B/1D}$ receptor agonist SKF-99101 (20 mg/kg i.p., Hatcher et al., 1995) caused a significant ($p<0.05$) increase in plasma ACTH levels 30 mins post administration when compared to vehicle controls. Levels reached 464.8 ±101.9 pg/ml (n=4) compared to vehicle control levels of 97.5 ± 36.7 pg/ml (n=4).

The selective 5-HT_{1B} receptor antagonist, SB-224289 (Roberts *et al.*, 1997) at doses of 2, 4 and 8 mg/kg p.o., had no significant effect on plasma ACTH levels *per se*, though a small transient increase in ACTH levels was observed at the 4mg/kg dose. At a dose of 2 mg/kg, SB-224289 had no significant effect on the SKF-99101 response. In contrast, at a dose of 4 mg/kg and 8 mg/kg SB-224289 significantly ($p<0.05$) attenuated the SKF-99101 induced increase in plasma ACTH levels.

The 5-HT_{1A} receptor antagonist, WAY-100635 (1 mg/kg i.p.), elicited a transient, but non-significant increase in plasma ACTH levels when compared to vehicle controls, 1h post treatment. Pretreatment with WAY-100635 (1 mg/kg i.p.) partially attenuated the effects of SKF-99101, however, this effect did not achieve significance.

The present study has validated the cannulation technique as a robust method for monitoring changes in plasma ACTH levels over time. Lack of effect of WAY-100635 on the SKF-99101 response and the attenuation of this response by the selective 5-HT_{1B} receptor antagonist, SB-224289, suggests that 5-HT_{1B} receptors modulate changes in plasma ACTH levels. In addition, these data provide a pharmacodynamic model for measuring 5-HT_{1B} receptor function *in vivo*, which may be transferable to man.

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185P PROLIFERATIVE EFFECTS OF CCK PEPTIDES AND PROCESSING INTERMEDIATES IN RAT GH_3 ANTERIOR PITUITARY CELLS

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Despite extensive hybridization screening in multiple species and tissues under both high and low stringency conditions, molecular studies identify only two CCK receptor family members, CCK-A and CCK-B (Wank *et al.*, 1994). Suggestions of further heterogeneity have arisen from a variety of studies in the literature. The glycine-extended precursor of bioactive amidated gastrin G-Gly, was recently reported to cause potent proliferative effects via a putative novel site on AR4-2J cells with maximal effects at 0.1nM, four orders of magnitude below its CCK-B receptor affinity, which were not blocked by the antagonist L-365,260 (Todisco *et al.*, 1995). Novel gastrin receptors were also proposed to mediate mitogenic effects of gastrin and G-Gly in CCK receptor-lacking Swiss 3T3 cells (Singh *et al.*, 1995). Growth-promoting effects of CCK have been documented in a variety of tissues and cells of peripheral origin, including gastric, pancreatic and lung cancer cell lines, but there is less information regarding proliferative effects in neuronal or pituitary tissue. We therefore examined effects of CCK and G-Gly on mitogenesis in GH_3 cells, previously shown to express a single population of functionally coupled CCK-B receptors (Smith *et al.*, 1994).

Cells were seeded at 5-8x10^4/ml in poly-L-lysine-coated 24-well plates and allowed to attach for 24h in serum-containing medium (1:1 DMEM/Hams F10 + 15% horse serum). A change to serum-free conditions was achieved over a 24h period with two changes of medium. Peptides were then added in fresh serum-free medium for a further 72h and 1μCi/well [^{3}H]thymidine was present for the final 24h. Antagonists were added 1h prior to agonists.

CCK peptides elicited small but significant dose dependent increases in growth over 3 day periods in the absence of serum as determined by [^{3}H]thymidine incorporation. CCK-8s, gastrin(1-17) and G(1-17)-Gly all produced similar maximal increases over control growth of 39±10% (P=0.002, n=20), 40±11% (P=0.007, n=9) and 44±14% (P=0.019, n=7) respectively. Significance of difference from control was determined by unpaired two-tail t-Test. Interleukin-2, a potent mitogen for GH_3 cells, was routinely included as a control (252±33% increase in cell proliferation). CCK-8s responses were maximal at 100nM with an EC_{50} of 0.12nM. The CCK curve was shifted to the right in a parallel manner in the presence of 1μM of the competitive CCK-B receptor antagonist L-365,260 with IC_{50} of 25nM, in good agreement with the IC_{50} for inhibition of GH_3 cell [^{125}I]BHCCK binding of 18nM (14;24, n=7) and an apparent pA_2 for inhibtion of proliferation of 8.32. The CCK-A receptor antagonist devazepide (10nM) was ineffective against stimulation by 10nM CCK-8s (35±10% increase over control, n=4). 0.1% DMSO solvent controls were without effect. Gastrin also potently stimulated cell proliferation with maximal effects at 100nM which were also blocked by 1μM L-365,260. G-Gly promoted cell growth with similar efficacy but markedly lower potency. Maximum proliferative effects were observed at 1μM G-Gly (44±14%, n=7) with no effects observable below 100nM. The affinity for displacement of [^{125}I]BHCCK binding from GH_3 cells by G-Gly was 1.09μM (1.01;1.17, n=6) and 5.53μM (3.71;5.99, n=4) in guinea-pig cortex. In the presence of 1μM L-365,260 the response to 1μM G-Gly was reduced from 44±14% to 6±7% (n=4) increase over control. These results are consistent with mediation of G-Gly effects via normal CCK-B receptors. L-365,260 alone reduced control responses from 100±1% to 89±4% (P=0.018, n=14) and may indicate that GH_3 cells produce an endogenous CCK peptide which influences growth. These results suggest that GH_3 cells do not contain any novel G-Gly-responsive site and further reinforce the concept of a role for CCK-B receptors in control of cell proliferation.

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186P OXYTOCIN AND VASOPRESSIN (V_{1a}) RECEPTOR ANTAGONISTS IN HUMAN MYOMETRIUM AT TERM: COMPLEXITIES IN FUNCTIONAL PHARMACOLOGY

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Oxytocin (OT) is a potent uterotonin whose role in normal human parturition remains controversial. Both OT and vasopressin V_{1a} receptors can be activated by OT to elicit myometrial smooth muscle contraction. Here we have used L-371,257, a non-peptide OT receptor antagonist (Williams, *et al.*, 1995) and atosiban, a cyclic peptide OT/V_{1a} receptor antagonist (Fuchs *et al.*, 1989) to probe myometrial responses to OT. Binding affinities (pK_i) at cloned human OT and V_{1a} receptors (Allen *et al.*, in press) are: L-371,257 8.1 & 6.2; Atosiban 7.4 & 8.5, respectively.

Briefly, myometrial biopsies were obtained from the lower uterine segment of women undergoing Caesarean section at term, with full written consent. Following suspension in siliconised organ baths containing Krebs solution at 36°C for isometric recording, tissue strips (10x2x2mm) were allowed to equilibrate for a 90-minute period prior to addition of antagonist or vehicle. Spontaneous phasic contractions were observed in 81% of drug-naïve tissue strips; quiescent and spontaneously active strips could be obtained from directly adjacent smooth muscle. Sixty minutes after addition of antagonist a single cumulative concentration-effect (E/[A]) curve to OT was obtained. Contact time for each concentration of OT was 30 min. Curve parameters were estimated by fitting a four-parameter logistic function to activity integral data. Data was analysed by one-way ANOVA followed by Dunnets test. Values quoted as mean ± s.e.mean.

Atosiban and L-371,257 (10nM-1μM) reduced spontaneous activity in a concentration-dependent manner but did not antagonise responses to exogenously applied OT in a simple competitive manner (Table 1).

Table 1. OT E/[A] curve parameters in the absence and presence of antagonist (n=4-8). Asymptote describes curve height in integral units (g.s); CR denotes concentration ratio. * Denotes P<0.05 vs. control.

	pEC_{50}	slope	asymptote	LogCR
Control	7.56±0.26	2.03±0.61	990±180	-
10nM atosiban	7.26±0.28	0.75±0.17	1990±824	-0.04±0.68
30nM atosiban	7.39±0.29	0.92±0.10	1313±546	0.16±0.37
100nM atosiban	7.61±0.50	0.63±0.15	2946±996	-0.04±0.56
300nM atosiban	7.40±0.31	0.49±0.09	2355±743	0.27±0.35
1μM atosiban	6.15±0.54*	0.78±0.14	2011±701	>1.6*
Control	7.92±0.11	1.65±0.55	1334±340	-
10nM L-371,257	7.85±0.18	1.74±0.81	719±177	0.1±0.13
30nM L-371,257	7.81±0.27	2.44±0.91	1258±206	0.16±0.23
100nM L-371,257	7.67±0.07	1.81±0.93	1370±295	0.31±0.17*
300nM L-371,257	7.49±0.20	1.58±0.70	2041±410	0.49±0.23*
1μM L-371,257	7.18±0.20*	1.58±0.56	2012±564	0.80±0.19*

Overall concentration-ratios were smaller than expectations based on the relative binding affinities of these antagonists. Degradation of OT by oxytocinase enzymes or supression of basal activity can generate resistance to antagonism. The oxytocinase inhibitor bestatin (100μM; Naruki *et al*, 1996) did not affect OT E/[A] curve parameters (log CR = -0.03±0.41; n=7). Furthermore, computerised simulations suggest that suppression of OT/V_{1a} receptor-mediated basal activity cannot account for these results. These complexities in the myometrial OT/V_{1a} system *in vitro* are the subject of ongoing investigations.

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187P THE THIAZOLIDINEDIONE, MCC-555, REDUCES OB EXPRESSION IN MATURE ADIPOCYTES, BUT NOES NOT ALTER PLASMA LEPTIN CONCENTRATIONS

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Thiazolidinediones enhance insulin action in glucose intolerant and diabetic rats (Whitcomb and Saltiel, 1995) thereby leading to increased glucose entry into adipose tissue and an increase in fat mass. The adipose derived hormone, leptin, a product of the *ob* gene, provides feedback information to the brain on the fat mass. Plasma leptin concentrations are proportional to fat mass in normal and obese animals (Campfield et al., 1996). We have investigated the effect of the novel thiazolidinedione, MCC-555 (Upton et al., 1997) on plasma leptin concentrations and *ob* mRNA density in white adipose tissue (WAT) of obese Zucker and diabetic ZDF rats, to investigate whether leptin signals are altered in response to increasing fat mass.

Groups of male obese (300g) and lean (200g) Zucker rats and diabetic ZDF (200g) and lean (200g) rats were orally dosed with MCC-555 (10mg/kg/day) for 21 days. Rats were killed by CO_2 inhalation and blood removal by cardiac puncture into cold heparinised tubes. Gonadal WAT mass was removed, weighed and snap frozen in liquid N_2. Plasma leptin concentrations were measured by radioimmunoassay. *Ob* mRNA concentrations were measured by Northern blotting and expressed as the ratio of *ob* mRNA/18S RNA. Ratios were normalised to those measured in WAT from lean rats. Chronic treatment of MCC-555 for 21 days resulted in a significant increase in gonadal WAT pad mass in both obese Zucker rats (MCC-555 treated obese = 7.6 ± 0.3 g versus controls 6.4 ± 0.3 g, P <0.05, Student's t-test, mean ± s.e.m.) and diabetic ZDF rats (MCC-555 treated 7.0 ± 0.2 g versus controls 5.9 ± 0.2 g P<0.01). Northern blotting demonstrated a significant reduction in *ob* mRNA density in MCC-555 treated obese Zucker rats and diabetic ZDF rats, as compared to controls (ratio *ob* mRNA/18S RNA density; Zucker rats, 2.5 ± 0.2 versus 3.4 ± 0.4 *; diabetic ZDF rats 1.5 ± 0.2 versus 2.5 ± 0.4 *P<0.05, Student's t-tests). However, plasma leptin concentrations were not significantly different between MCC-555 treated Zucker rats and controls (11.7 ± 1.4 versus 9.8 ± 1.1) or between MCC-555 treated diabetic ZDF rats and controls (5.8 ± 0.5 versus 5.7 ± 0.5) Microscopic examination of hematoxylin/eosin stained wax embedded sections of WAT tissue, fixed with formyl saline. revealed a higher cell density in tissue from MCC-555 treated Zucker and diabetic ZDF rats, as compared to controls. This is consistent with data demonstrating that thiazolidinediones can promote preadipocyte differentiation (Whitcomb and Satiel, 1995). In conclusion, we demonstrate that MCC-555 increases WAT mass and cell number and produces an opposing reduction in *ob* mRNA synthesis thereby cancelling out any change in plasma leptin concentrations.

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188P THE HYPERPHAGIA IN MODERATE STREPTOZOTOCIN-INDUCED DIABETES IS NOT ACCOMPANIED BY CHANGES IN HYPOTHALAMIC NPY ACTIVITY

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The potent orexigenic neurotransmitter, neuropeptide Y (NPY) which is found in rat hypothalamus may mediate the hyperphagic response observed in streptozotocin-diabetic rats (Frankish et al., 1993). Previous studies have demonstrated increased hypothalamic NPY synthesis and release in rats with extreme streptozocin-induced diabetes which is associated with up to 90 % depletions in plasma insulin concentrations (Frankish et al., 1993; 1995). As NPY is also activated during severe weight loss and following large reductions in both leptin and insulin, we wished to study whether the hypothalamic NPY system is regulated by moderate reductions in leptin and insulin in diabetes. Male Wistar rats (200g) were injected with streptozocin (55 mg/kg) or sterile saline (controls) via the tail vein under light halothane anaesthesia. Between 14 and 21 days after the establishment of the diabetes, daily food and water intake were measured. Rats were then killed by CO_2 inhalation, blood collected into cold heparinised tubes via cardiac puncture and the brains removed. In some rats, hypothalamic blocks were disected free and snap frozen in liquid N_2 for later measurements of NPY mRNA using Northern blotting. The brains from other rats were frozen on dry ice for measurements of NPY receptor densities using 30 pM [^{125}I]PYY and quantitiative receptor autoradiography as described previously (Widdowson, 1997). The remaining rats received chronic intracerebroventricular cannulas as described previously for injection of the NPY antagonist, 1229U91 (Kanatani et al., 1996). Injection of the streptozocin was accompained by an increase in plasma glucose (control = 4.5 ±0.2 mmol.l^{-1}, diabetic =26.3 ±1.8 mmol.l^{-1}, n =25, mean ± s.e.m., P<0.01; Student's t-test), a reduction in plasma insulin (controls = 20.5 ±0.9 μU.ml^{-1}; diabetic = 11.5 ±0.8 μU.ml^{-1}, n = 25, P<0.01) and leptin concentrations (controls = 2.6 ±0.3 ng.ml^{-1}; diabetic = 0.6 ±0.1 ng.ml^{-1}) and an increase in daily food (controls =33.8 ±0.7g, diabetic = 47.2 ±0.8g, n = 9* P<0.01) and water intake (controls = 41 ±1 ml, diabetic = 179 ±6 ml* P<0.01). However, Northern blots failed to detect changes in NPY mRNA density nor was there evidence for a down-regulation in NPY receptors in hypothalamic regions (specific binding to the hypothalamic lateral periformical hypothalamus, control = 6.6 ±0.7 fmol.mg tissue^{-1}, diabetic = 6.2 ±0.5 fmol.mg tissue^{-1}, n=9). Finally, intracerebroventricular injections of the NPY antagonist, 1229U91 (30 μg twice daily), which reduces feeding following overnight starvation, did not attenuate the hyperphagia and polydypsia. In conclusion, although moderate streptozocin-induced diabetes resulted in a reduction of both plasma insulin and leptin concentrations, these changes were not of sufficient magnitude to produce an increase in hypothalamic NPY activity. Thus the hyperphagia observed in moderate diabetes cannot be attributed to increased hypothalamic NPYergic activity.

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189P CHARACTERISATION OF THE 5-HT RECEPTORS MEDIATING THE CONTRACTILE EFFECT OF 5-HT IN THE PROXIMAL REGION OF THE RAT SMALL INTESTINE

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In a systematic study we have shown that different 5-HT receptors are involved in mediating the contractile effect of 5-HT in different regions of the *Suncus murinus* intestine (Javid *et al*, 1996, 1997). We have extended these experiments to intestinal tissues taken from the rat and in this study report on tissues taken from the proximal region.

Segments (1-1.5 cm long) taken from the intestine (2 cm distal to the pyloric sphincter) of adult Hooded Lister rats (210-320 g) of either sex were mounted in 10 ml organ baths containing Krebs' solution (37°C, 95% O_2, 5% CO_2). The tissues were allowed to equilibrate for 60 min and washed every 20 min. The resting tension was maintained at 0.5 g and recorded isometrically. Non-cumulative concentration-response curves to 5-HT (1nM-30μM) were established with a 1 min contact time and 20 min intervals. The procedure was repeated in the presence of antagonists (1 h equilibration time): methysergide (methy, 1μM), ritanserin (rit, 0.1μM), atropine (atr, 1μM), ondansetron (ond, 1μM), SB204070 (SB, 1nM), a combination of methysergide (1μM) plus ondansetron (1μM), SB204070 (1nM) or atropine (1μM). The control responses to 5-HT alone and in the presence of antagonist/s were established using a randomised experimental design. Tension changes were expressed as the mean±s.e.mean of n=9 and analysed using one-tail Dunnett's t-test.

5-HT (1nM-30μM) produced a concentration-dependent contraction. Methysergide, a 5-$HT_{1/2}$ receptor antagonist and ritanserin, a 5-HT_2 receptor antagonist, significantly ($P<0.05$) shifted the concentration-response curve to 5-HT to the right with a significant ($P<0.05$) reduction at 30 μM of 5-HT.

Ondansetron, a selective 5-HT_3 receptor antagonist and SB204070, a selective 5-HT_4 receptor antagonist, (Wardle *et al*, 1994), significantly ($P<0.05$) reduced the response to 5-HT at concentrations >30nM. Atropine also significantly ($P<0.05$) reduced the response to 5-HT at concentrations >30nM. A combination of methysergide plus atropine or ondansetron or SB204070 significantly ($P<0.05$) shifted the concentration-response curve to 5-HT to the right with a significant ($P<0.05$) reduction in the maximum response (Figures 1.a-c).

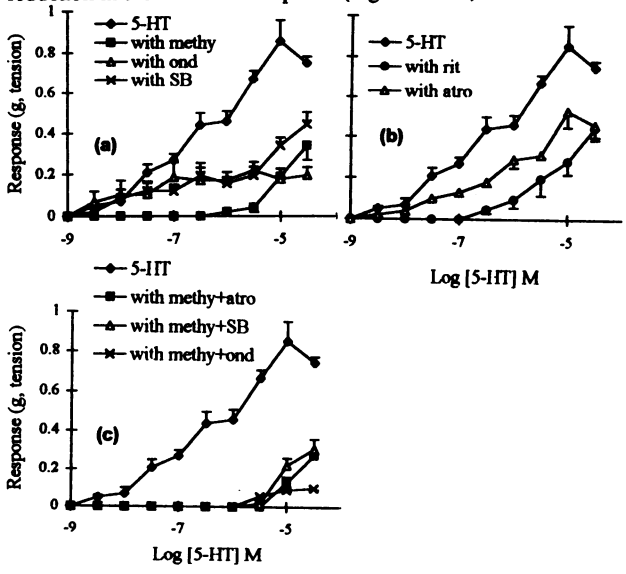

Figure 1. The effect of 5-HT receptor antagonists on the contractile response to 5-HT in the rat proximal intestine.

The data suggest that in the proximal region of the rat small intestine the contractile response to 5-HT is mediated via 5-HT_2, 5-HT_3 and 5-HT_4 receptors, unlike the proximal region of *Suncus murinus* intestine where contractions to 5-HT are mediated via 5-HT_2 receptors.

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190P INHIBITION OF PERISTALSIS WITH PDE4 BUT NOT PDE3 INHIBITORS IN THE GUINEA-PIG ISOLATED ILEUM

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An increasing number of isoenzyme-selective inhibitors of PDE3 and PDE4 families of phosphodiesterase enzymes have been developed due to their potential clinical use in heart failure and asthma respectively. However, there is a paucity of information regarding their gastrointestinal effects. In the present study we investigate the effect of these inhibitors in an animal model of intestinal motility using peristalsis in the guinea-pig isolated ileum.

Up to four segments of ileum (taken 5-30 cm from the ileo-caecal junction) were obtained from guinea-pigs (Dunkin Hartley) of either sex (500-1000g) and cannulated at the oral and aboral ends and secured horizontally in a bath containing Krebs-Henseleit solution kept at 37°C and oxygenated with 95% O_2 and 5% CO_2. Peristalsis was measured and recorded using the methodology previously described (Costall *et al.*, 1993). Cumulative concentration response curves to PDE inhibitors (added serosally) were constructed approximately 30 min after the mounting of the tissues as follows: peristalsis was elicited (by closing the lower outlet) and once a regular peristalsis was achieved vehicle or an increasing concentration of the PDE inhibitor was applied at 3 min intervals. If peristalsis was not abolished with the last concentration tested, a 100 μM concentration of rolipram was added to abolish peristalsis. The concentration response curves were expressed as a percentage change in peristaltic threshold from the threshold before the addition of the PDE inhibitor or the vehicle taking the maximum pressure generated inside the tissue when peristalsis was abolished as 100%. The comparison of the potency of PDE inhibitors to inhibit peristalsis were made at 50% inhibition. The drugs were dissolved in either water or DMSO with use of appropriate vehicle controls.

The peristalsis remained stable with no significant change in the peristaltic threshold in the tissues used as the vehicle controls. The PDE4 inhibitors rolipram (30-100 μM), Ro 20-1724 (10-300 μM) (Beavo & Reifsnyder, 1990) and RP 73401 (30-100 μM) (Ashton *et al.*, 1994) inhibited peristalsis with pEC_{50} values of 4.40±0.06 (n=6), 4.27±0.04 (n=4) and 4.64±0.10 (n=4) respectively. Peristalsis was abolished in all the tissues at the maximum concentration tested with these compounds. Unlike PDE4 inhibitors, no significant changes in the threshold for peristalsis were observed with the PDE3 inhibitors milrinone (1-100μM) and SKF 94120 (1-100 μM) (Beavo & Reifsnyder, 1990) (n=4 each). Moreover, milrinone and SKF 94120 did not alter the ability of rolipram (100 μM) to abolish peristalsis.

The results suggest that unlike PDE3 inhibitors tested, the PDE4 inhibitors rolipram, Ro 20-1724 and RP 73401 can inhibit peristalsis in the guinea-pig ileum. Such an effect of the compounds on peristalsis might be relevant to the gastrointestinal motility related effects of these compounds in the clinic. However, further studies are required to understand whether the inhibition of peristalsis by the above PDE4 inhibitors are due to an inhibition of phosphodiesterase4 enzymes or other mechanisms.

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191P EFFECT OF DARIFENACIN AND OTHER MUSCARINIC ANTAGONISTS ON GASTRIC ACID SECRETION IN CONSCIOUS DOGS

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Darifenacin is a competitive muscarinic receptor antagonist. It has been shown *in vitro* to be selective for the M_3 receptor over both M_1 and M_2 subtypes (Wallis *et al*, 1995). In the conscious dog it has minimal anticholinergic effects on the heart or pupil at doses which inhibit small bowel digestive motility (Sawyer *et al*, 1996). Gastric acid secretion is known to involve a cholinergic pathway sensitive to M_1 selective antagonists (Hirschowitz *et al*, 1983). Experiments were conducted with standard muscarinic antagonists to confirm the role of M_1 receptors in the control of gastric acid secretion in a Heidenhain pouch model. In addition, the potency of darifenacin was assessed to define its M_3 over M_1 selectivity *in vivo*.

Male beagle dogs (n=4), with Heidenhain pouches were used in these experiments. They were deprived of food for 18 hours and gastric acid secretion stimulated by a 3 hour intravenous infusion of pentagastrin ($2\mu gkg^{-1}h^{-1}$). Test substances were administered orally by gavage 30 minutes into the infusion. Secretions were collected every 15 minutes and titrated against 0.01M sodium hydroxide to determine their acid concentration.

In vehicle treated animals gastric acid secretion was 106 ±18 mEq H+ between 0.5 and 3 hours of pentagastrin infusion. Darifenacin ($0.3\text{-}3mgkg^{-1}$) had no effect on gastric acid secretion at doses up to $1mgkg^{-1}$. Only the highest dose tested, $3mgkg^{-1}$, produced a significant ($p<0.01$) effect, an inhibition of (31%). By comparison pirenzepine (M_1 selective) ($0.01\text{-}0.3mgkg^{-1}$) produced a dose-related inhibition of gastric acid secretion which was significant ($p<0.01$) at all doses, with an ED_{50} of $0.037mgkg^{-1}$ (95% confidence limits 0.010, 0.150 $mgkg^{-1}$). Methoctramine (M_2 selective) ($0.1\text{-}1mgkg^{-1}$) did not significantly inhibit gastric acid secretion. However, a significant ($p<0.01$) tachycardia of 62 ± 7 beats per minute at $1mgkg^{-1}$ indicated antagonism at cardiac M_2 receptors. Atropine (non-selective) ($0.001\text{-}0.1mgkg^{-1}$) also produced significant ($p<0.01$) inhibition of gastric acid secretion at all doses tested, the ED_{50} being $0.007mgkg^{-1}$ (95% confidence limits 0.001, 0.049 $mgkg^{-1}$).

In conclusion experiments with standard antimuscarinics have confirmed a role for M_1 receptors in the control of gastric acid secretion in the conscious dog. Furthermore they indicate that darifenacin has weak activity on canine gastric M_1 receptors when compared to pirenzepine and atropine, and that darifenacin is over 30 times more potent as an inhibitor of small bowel digestive motility (ED_{50}, 0.1 $mgkg^{-1}$, Sawyer et al, 1996) than gastric acid secretion. This confirms the *in vitro* selectivity of darifenacin and suggests that it will have minimal M_1 antagonist activity at doses which inhibit small bowel digestive motility.

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192P EFFECT OF CISAPRIDE AND METOCLOPRAMIDE ON POSTOPERATIVE ILEUS IN RATS

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Postoperative ileus is a common complication after abdominal surgery. We previously showed the involvement of adrenergic and nitrergic nerves in the pathogenesis of postoperative ileus using a rat model (De Winter *et al.*, 1997). In the present study, we first evaluated the effect of two well established prokinetic agents namely cisapride and metoclopramide. Then we compared their effect with that of granisetron, a 5-HT3 receptor antagonist and that of a new prokinetic agent R 093877, a selective 5-HT4 receptor agonist (Briejer *et al.*, 1997). Male Wistar rats (150-230 g) underwent an abdominal operation under ether anaesthesia. After one hour of recuperation the rats received an intragastric injection of 0.1 ml Evans blue. Twenty minutes later the rats were killed and the migration of Evans blue was measured from the pylorus to the most distal point of migration of Evans blue. Ether anaesthesia and skin incision (SI) had no effect on the transit (De Winter *et al.*, 1997). The laparotomy (LAP) significantly inhibited the transit. This inhibition was even more pronounced when the small intestine and caecum were manipulated (L+M) (table 1). I.v. injection of cisapride 1 mg/kg or metoclopramide 30 mg/kg, just prior to the operations, significantly enhanced the transit after SI (table 1). Cisapride also significantly increased the transit after LAP and L+M, whereas metoclopramide further inhibited the transit after these operations (table 1). I.v. injection of granisetron, a 5-HT3 receptor antagonist (10 and 50 µg/kg) had no significant effect on the transit after the three operations (n=9-10).

Treatment	SI	LAP	L+M
Control	57.8 ± 2.1 cm	34.6 ± 2.4 cm	19.4 ± 2.4 cm
Cisapride	71.4 ± 2.6 cm*	51.6 ± 2.9 cm*	28.9 ± 3.1 cm*
Metoclop	71.3 ± 3.5 cm*	20.8 ± 2.4 cm*#	7.6 ± 1.6 cm*#

*Table 1: Effect of cisapride and metoclopramide on the gastrointestinal transit after abdominal operations; *, P≤0.05, vs. control rats; #, P≤0.05, vs. rats treated with cisapride; one way analysis of variance plus Bonferroni, n=9-10.*

I.v. injection of the new selective 5-HT4 receptor agonist R 093877 (1 and 5 mg/kg) had no significant effect on the transit after SI and L+M. However, R 093877 (1 mg/kg) significantly increased the transit after LAP from 37.4 ± 3.1 cm to 49.9 ± 2.8 cm (n=9). Finally, i.v. treatment of the rats with the combination of granisetron 50 µg/kg and R 093877 1 mg/kg had no effect on the transit after SI but significantly increased the transit after LAP and L+M (table 2), thus mimicking the effect of cisapride.

Treatment	SI	LAP	L+M
Control	63.1 ± 3.3 cm	37.5 ± 2.8 cm	17.4 ± 2.2 cm
Gran. + R	62.1 ± 2.9 cm	45.5 ± 1.6 cm*	24.6 ± 1.8 cm*

*Table 2: Effect of the combination of granisetron and R 093877 on the gastrointestinal transit after abdominal operations; *, P≤0.05, vs. control rats; unpaired Student's t-test, n=9-10.*

In summary, although cisapride and metoclopramide increase the transit after skin incision, cisapride accelerates the transit after abdominal surgery whereas metoclopramide enhances the postoperative ileus in rats . As the effect of cisapride is mimicked by the combination of a 5-HT3 receptor antagonist plus a 5-HT4 receptor agonist, cisapride most likely acts via both receptors to increase the transit after abdominal surgery.

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193P PROTECTIVE EFFECT OF POLYANIONS ON AMINOGLYCOSIDE ANTIBIOTIC-INDUCED INHIBITION OF MTT ACTIVITY IN A HUMAN RENAL CELL LINE

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We have previously demonstrated (Kumar et al., 1995) that the human renal cell line, G401 is sensitive to the aminoglycoside antibiotics. It has been suggested (Hulke et al., 1993), that when co-administered, polyanions may bind to the polycationic antibiotics to form a complex which alters the effect that the antibiotic alone has on enzyme activities. Polyanions may thus be nephroprotective in this case. In this study we have investigated the effect of co-administration of polyanions with aminoglycoside antibiotics on mitochondrial enzyme activity in the G401 using the MTT assay (Mossman, 1983).

G401 cells (passage 81-85) were seeded in 96 well plates and grown to confluence in McCoy's 5α medium supplemented with 10% Foetal Clone at 37°C in 95% O_2/ 5% CO_2. Once confluent, medium was replaced with fresh media containing i) 5mM gentamicin, neomycin or streptomycin alone ii) increasing concentrations of polyaspartic acid or polyasparagine alone (0-50mM) and iii) 5mM of either antibiotic with either polyanion for a further 24h after which time the MTT assay was performed. A control was set up containing media alone. Values are shown as absorbance readings at 595nm λ.

Table 1 clearly shows that MTT activity was significantly inhibited compared to the control, for each antibiotic (AB) alone whereas polyaspartic acid (PAA) alone appears to have no significant effect. When co-administered, a dose-dependent effect was seen and MTT activity was restored back to the control value in each case. The same trend was seen with polyasparagine (data not shown). Our results suggest that the G401 cell line is susceptible to toxic insult by these antibiotics and that this toxicity may be reduced by co-administration with a polyanion. These data are comparable to those shown by Ramsammy et al., (1989) who used rat primary cultures of the proximal tubule.

	MTT Activity				PAA EC_{50} (mM)
	Control	AB alone	PAA alone	AB +PAA	
Gent.	1.059 ± 0.006	*0.486 ± 0.009	1.053 ± 0.017	1.130 ± 0.009	4.00 ± 0.002
Neo.	1.116 ± 0.013	*0.494 ± 0.004	0.941 ± 0.007	1.151 ± 0.012	7.00 ± 0.005
Strep.	0.982 ± 0.037	*0.554 ± 0.013	0.925 ± 0.017	0.982 ± 0.014	7.00 ± 0.003

Table 1. The effect of 5mM gentamicin (gent.), neomycin (neo.) or streptomycin (strep.) alone and/or polyaspartic acid, 50mM on MTT activity. Data are expressed as mean ± s.e.m (n=30). ANOVA and Student Newman Keuls test were used for statistical analysis. *p<0.05 compared to the control.

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194P EFFECTS OF A SOYBEAN OIL-EMULSIFIED PREPARATION OF ANANDAMIDE AT CANNABINOID RECEPTORS IN ISOLATED PREPARATIONS FROM THE GUINEA-PIG

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The use of anandamide (AEA, an endogenous ligand for cannabinoid receptors) is problematic in assays of biological activity because of the action of endogenous amidases (Pertwee *et al.*, 1995), and the need for solubilisation using detergents or organic solvents. In this report, we have compared the effects of an emulsified preparation of AEA in soybean oil (emulsified AEA - droplet size 250 nm), with an ethanol-dissolved preparation (ethanolic AEA).

Binding to cannabinoid receptors of the guinea-pig (male, Dunkin-Hartley, 300-800 g) cerebellum was examined using [^{3}H]-CP55940 (0.5 nM) and 10 μM HU-210 to define non-specific binding (Rinaldi-Carmona *et al.*, 1994). Competition curves for AEA were constructed in quadruplicate assay over the range 10^{-9} M to 10^{-4} M, at half-log intervals. Inhibition of the electrically-evoked contraction of guinea-pig isolated ileum (Pertwee *et al.*, 1995) was assessed by cumulative addition of AEA over the range 10^{-7} M to 10^{-4} M, at half-log intervals. AEA solutions were diluted in buffer. At least 3 preparations were used throughout. Statistical significance was evaluated by use of ANOVA with post-hoc Student-Newman-Keul's test.

Vehicle controls, equivalent to the highest concencentrations of AEA examined in the binding assay, showed no significant effect on binding of [^{3}H]-CP55940. Ethanolic AEA competed for [^{3}H]-CP55940 binding with a pIC_{50} value of 5.50 ± 0.08, which was significantly enhanced (P<0.01) in the presence of the non-specific amidase inhibitor phenylmethylsulphonyl fluoride (PMSF, 50 μM) to 7.41 ± 0.03. In comparison, emulsified AEA evoked a concentration-dependent competition for [^{3}H]-CP55940 with a pIC_{50} value of 5.95 ± 0.12, which was statistically different from the ethanolic AEA (P<0.05). In the presence of PMSF, the potency of emulsifed AEA was enhanced (pIC_{50} 6.98 ± 0.19, P<0.001), although to a lesser extent than the ethanolic AEA (P<0.05).

In the proximal end of the guinea-pig isolated ileum, emulsified AEA caused a concentration-dependent inhibition of electrically evoked contractions (e.g. $3x10^{-6}$ M 74 ± 6 % & 10^{-5} M 58 ± 8 % control), which was not significantly different from ethanolic AEA (e.g. $3x10^{-6}$ M 78 ± 10 % & 10^{-5} M 53 ± 6 % inhibition). The presence of 50 μM PMSF failed to enhance significantly the inhibitory effect of 10 μM emulsified AEA (emulsion 105 ± 2 %; PMSF 96 ± 10; AEA 58 ± 7; AEA+ PMSF 38 ± 8).

The increased potency of the emulsified AEA compared to ethanolic AEA in the binding assay may represent increased stability against long-term exposure (i.e. 30 min) to amidase activity. The indistinguishable activities of the emulsified and ethanolic AEA in the isolated ileum suggests that both preparations show comparable bioavailabilities, at least, in this preparation. These results suggest the emulsified preparation may be advantageous for investigating the effects of AEA, when the presence of ethanol is undesirable, e.g. *in vivo*.

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195P THE EFFECTS OF REDUCED pH ON A_{2B} ADENOSINE RECEPTOR-EVOKED CYCLIC AMP GENERATION IN THE GUINEA-PIG CEREBRAL CORTEX

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It has been hypothesized that the levels of adenosine required to activate A_{2B} adenosine receptors would only be reached during periods of stress, such as hypoxia/ischaemia (Fredholm *et al.*, 1994), at a time when tissues would be expected to be acidotic. We have therefore investigated the effects of reduced pH on the stimulation of cyclic AMP (cAMP) responses by adenosine and the stable adenosine analogue, 5'-N-ethylcarboxamidoadenosine, NECA.

Generation of [^{3}H]-cAMP in [^{3}H]-adenine labelled slices of cerebral cortex from male, Dunkin-Hartley guinea-pigs (300-800 g) was assessed essentially as previously described (Alexander *et al.*, 1996). The pH of the Krebs-Henseleit (checked by use of a pH meter) was lowered by reducing the $NaHCO_3$ concentration from 25 to 15 mM prior to gassing with O_2:CO_2 (95:5). Slices were prepared at pH 7.4, and then dispensed into medium at pH 7.1 or 7.4 prior to agonist exposure. Agonist-evoked responses were initially expressed as a percentage conversion from the total [^{3}H]-adenine nucleotides with basal levels subtracted, from experiments carried out on at least 4 separate occasions. Concentration-response curves were constructed to NECA (10^{-7} M to 10^{-4} M) and adenosine (10^{-7} M to $10^{-3.5}$ M). Statistical significance was evaluated by use of paired t-test.

Basal accumulations of [^{3}H]-cAMP in guinea-pig cerebral cortex slices at 15 minutes (0.74 ± 0.13 % conversion, mean ± s.e.mean) were not significantly changed by reducing the ambient pH (0.59 ± 0.06 %). cAMP responses in the presence of 100 µM NECA were increased at pH 7.1 (control 1.79 ± 0.39 %, pH 7.1 2.45 ± 0.42 %, P=0.022). Analysis of concentration-response curves showed no significant alteration in the potency of the NECA-evoked cAMP response (control pEC_{50} 5.86 ± 0.07, pH 7.1 5.94 ± 0.04). Adenosine-evoked cAMP responses were also enhanced at lower pH (maximal responses: control 1.29 ± 0.26 %, pH 7.1 2.11 ± 0.37, P=0.027), without significant effect on the potency (pEC_{50} control 4.59 ± 0.21; pH 7.1 4.53 ± 0.17).

The cAMP response to 100 µM NECA, but not 1 mM adenosine, at pH 7.4 was maintained for up to 120 minutes (Smith & Alexander, 1997). At pH 7.1, however, the response to NECA was not maintained. For example, at 120 minutes, NECA-evoked cAMP levels were 49 ± 12 % of the pH 7.4 NECA response at 15 minutes. The adenosine-evoked response at 120 minutes, pH 7.1, was not significantly different from basal (12 ± 3 % of the 15 minute NECA response at pH 7.4).

Thus, at pH 7.1, cAMP responses to NECA and adenosine are enhanced, although these responses are not as well-maintained, compared to responses at pH 7.4. Whether the site of modulation by reduced pH is the receptor, or some other component of the signalling system, requires further analysis.

JKS is a BBSRC Glaxo Wellcome CASE Student.

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196P A CHANNEL MUTANT OF $GABA_A$ RECEPTORS REVEALS CHANGES IN ALLOSTERIC MODULATION

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We have previously noted that the binding affinity (pKi) for GABA agonists were generally two to three orders of magnitude higher than the pEC_{50}'s measured electrophysiologically (Ebert *et al.*, 1997). Mutation of a conserved leucine in the putative second membrane-spanning domain of several ligand gated ion channels results in increased agonist sensitivity (Chang *et al.*, 1996). We have mutated this conserved leucine to serine in human $GABA_A$ β2 and investigated the actions of a number of $GABA_A$ agonists, antagonists and modulators on human α1β2ΔL259Sγ2s compared to wild type α1β2γ2s $GABA_A$ receptors, expressed in *Xenopus* oocytes.

Significantly smaller currents to a maximum concentration of GABA were observed in α1β2ΔL259Sγ2s receptors (88 ± 6nA n=56) compared to α1β2γ2s receptors (2016 ± 340nA n=14). The mutant receptors also displayed a greater leak current (between 200-400nA) compared to the wild type (between 30-100nA). As expected, this mutation decreased the GABA EC_{50} (110 fold) resulting in mean EC_{50} values of 182 ± 61nM on α1β2ΔL259Sγ2s compared to 20 ± 3µM on α1β2γ2s. The partial agonists THIP and P4S (Ebert *et al.*, 1997) also displayed a decrease in EC_{50} (90 and 70 fold respectively).

The current-voltage relationship revealed a reversal potential of -26.3 ± 3.9mV for α1β2ΔL259Sγ2s which was not significantly different from -21.3 ± 1.2 mV for α1β2γ2s.

The competitive antagonists, bicuculline and SR95531 (Ebert *et al.*, 1997) behaved as inverse agonists on α1β2ΔL259Sγ2s producing outward currents in the absence of GABA. The pIC_{50}'s of 5.5 ± 0.09 (n=4) for bicuculline and 6.8 ± 0.05 (n=4) for SR95531 correlate well with the pKi's for competitive antagonism of GABA (Ebert *et al.*, 1997). In addition to producing outward currents the two antagonists competitively shifted GABA concentration-response curves.

The effects of a range of allosteric modulators (flunitrazepam, CL-218,872, loreclezole, pentobarbital and 5α-pregnan-3α-ol-20-one) were examined on a submaximal GABA concentration (EC_{20}). None of these modulators potentiated the EC_{20} response, however they all directly activated the receptor in the absence of GABA. Pentobarbital (100µM) produced the largest direct activation (79 ± 6% of maximum GABA) while loreclezole (10µM) produced the smallest (12 ± 1% of maximum GABA). The benzodiazepine (BDZ) inverse agonist DMCM, produced outward currents in the absence of GABA. Direct effects of BDZ's were blocked by the BDZ antagonist, flumazenil (1µM). The direct activation by pentobarbital of α1β2ΔL259Sγ2s receptors also showed a 10-fold decrease in EC_{50} compared to wild type.

To conclude, the above mutation resulted in receptors which exhibit a degree of spontaneous activity, and are more sensitive to GABA agonists. These receptors can be activated but not modulated by BDZ's and other agents. Bicuculline and SR95531 can also act as allosteric channel modulators through the GABA binding site.

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197P EFFECTS OF INCREASED MEMBRANE CHOLESTEROL ON $GABA_A$ CURRENTS IN ACUTELY DISSOCIATED HIPPOCAMPAL NEURONES

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The functions of various membrane proteins are influenced by cholesterol in the membrane interacting directly with the protein (Yeagle, 1991). An initial study on the $GABA_A$ receptor in neuronal membrane fragments has shown that enhancement of flunitrazepam binding by other classes of GABA-potentiating drugs is affected by cholesterol enrichment of the membranes (Bennett & Simmonds, 1996). We now report the effects of cholesterol enrichment of whole neurones on their electrophysiological responses to GABA.

Brain slices containing hippocampus from 10-16 days old male Wistar rats were incubated in 0.03% pronase for 20 min at 31°C followed by 0.03% protease type X for 20 min in a physiological salt solution (PSS) containing (mM): NaCl 140, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11, HEPES 10; adjusted to pH 7.4 with Tris-base. The hippocampal tissue was separated and neurones were dissociated by trituration with glass pipettes. The tissue remaining suspended after standing for 10 min was centrifuged at 175×g for 4 min to form a very loose pellet which was then layered onto a 5% solution of bovine serum albumin (BSA) in PSS for further centrifugation to separate a loose pellet of neurones from suspended cell debris. Enrichment of neurones with cholesterol was achieved by incubation at 31°C for 60 min in oxygen-saturated PSS containing 1% BSA and cholesterol + phosphatidylcholine liposomes (Bennett & Simmonds, 1996) at a final cholesterol concentration of 0.15-0.20 $mg.ml^{-1}$. Unenriched neurones were subjected to the same processes but without liposomes. The enrichment procedure increased neuronal cholesterol by 9-40% above the value of 0.47±0.02 μmoles.mg $protein^{-1}$ (mean ± s.e.mean, n=17) in unenriched neurones. The neurones remaining viable (trypan blue exclusion) at this stage were about 70-80% of enriched and 80-90% of unenriched neurones.

Electrophysiological recordings were made from dissociated single neurones which adhered to the bottom of the recording chamber and appeared bright and 3-dimensional under phase contrast. Whole cell membrane currents were recorded with patch pipettes at 20°C and the membrane clamped at -20 mV. The patch pipette solution contained (mM): CsCl 140, $CaCl_2$ 1, $MgCl_2$ 2, Na_2ATP 2, EGTA 11, HEPES 10; adjusted to pH 7.2 with Tris-base. The recording chamber was continuously perfused with PSS. GABA dissolved in PSS was applied close to the recorded neurone by the "U-tube" adaptation of the method of Wakamori *et al.* (1993) for periods of 5-15 s at intervals of 1-2 min. Full log. concentration - response curves were constructed over the range 0.1-300 μM GABA. Results from the analysis of the curves are shown below.

	Unenriched (n = 16)	9 - 12% enrichment (n = 9)	20 - 40% enrichment (n = 9)
Maximum (nA)	2.72 ± 0.12	1.97 ± 0.17*	1.50 ± 0.04#
log. EC_{50}	-5.297±0.082	-5.276±0.153	-5.196±0.047
EC_{50} (μM)	5.05	5.30	6.37

* different from Unenriched (P<0.01)
\# different from Unenriched (P<0.001) and 9-12% enrichment (P<0.05)

Enrichment of hippocampal neurones with cholesterol by up to 40% caused no significant change in the EC_{50} for GABA but there was a correlated reduction in the maximal response, suggesting a loss of functional $GABA_A$ receptors.

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198P DIRECT MODULATION OF MURINE RECOMBINANT $GABA_A$ RECEPTORS BY PROTEIN TYROSINE KINASE INHIBITORS

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Protein tyrosine phosphorylation has a potentially important role in the modification of neuronal function in the central nervous system demonstrated by the regulation of neuronal and recombinant $GABA_A$ receptors by protein tyrosine kinases (PTK; Moss *et al.*, 1995; Valenzuela *et al.*, 1995). $GABA_A$ receptors are hetero-oligomers composed of subunits selected from α, β, γ, δ and ε subunit classes. Consensus sequences for PTK phosphorylation have been identified in the postulated large intracellular loop between transmembrane domains 3 and 4 of $GABA_A$ receptor β and γ2 subunits. These consensus sequences are phosphorylated at 2 sites in the β1 (Y370 and Y372) and γ2L (Y365 and Y367) subunits, but only phosphorylation of the tyrosines in the γ2L subunit enhanced the GABA-activated current (Moss *et al.*, 1995). This study addressed whether the sites of PTK phosphorylation in the γ2S subunit confer similar regulation on $GABA_A$ receptor function. Tyrosines 365 and 367 in the γ2S subunit were mutated to non-phosphorylated phenylalanine (F) residues. *Xenopus* oocytes were injected with murine $GABA_A$ receptor cDNAs encoding for wild-type α1β1γ2S or mutant α1β1γ2S(Y365,367F) subunits and studied using a two-electrode voltage clamp technique.

Application of the tyrosine kinase inhibitor, genistein (100μM), which acts at the adenosine triphosphate binding site on PTK, to α1β1γ2S wild-type (w.t.) receptors, produced a depression of the GABA concentration-response curve. The maximum normalised (max.) response was reduced from 1.90±0.02 (control) to 0.69±0.04 (+genistein) and the EC_{50} was reduced from 38.45±1.54 to 18.33±3.01 (mean ± s.e.mean, P<0.05, t test; n = 3-17 oocytes). In comparison, 100μM daidzein, the inactive analogue of genistein, did not affect the max. response (1.90±0.02 (control) and 1.80 ± 0.05 (+daidzein) n = 3-17), nor the GABA EC_{50} 38.5±1.5μM (control) and 33.8±2.2μM (+daidzein) (n = 3-17) in accordance with regulating $GABA_A$ receptor function by tyrosine kinases. Unexpectedly, application of 100μM genistein to the tyrosine mutant receptor, α1β1γ2S(Y365,367F), produced a greater depression of the GABA concentration-response curve (max. response 1.93±0.06 reduced to 0.26±0.01; n=3-9). Furthermore, 100μM daidzein non-competitively depressed the GABA-induced conductance (max. response reduced to 1.31±0.06; n=3), although the EC_{50} was unaffected (40.25±5.2 (control) and 36.2±7.5μM (+daidzein)). Interestingly, the inhibition by genistein increased with the GABA concentration (0 and 64.4% inhibition for 5μM and 1mM GABA, n =3-7), which is representative of uncompetitive antagonism.

Comparative experiments with alternative PTK inhibitors, the tyrphostins, which target the substrate binding site on PTK, also indicated a direct interaction with $GABA_A$ receptors. Tyrphostin A25 (200μM) produced a non-competitive depression of the GABA concentration response curve in w.t. receptors (max. responses 2.04±0.1 and 0.48±0.04, EC_{50}s 45.6±3.9 and 23.9±6.3μM , P<0.05, for control and in the presence of tyrphostin A25 respectively, n = 3-17). A similar inhibition was also observed for GABA concentration-response curves obtained for the tyrosine mutant receptor (max. responses 2.00±0.07 and 0.34±0.01, EC_{50}s 44.1±6.2 and 31.7±4.1μM (n=3-18) in control and in tyrphostin A25). Similarly, the inactive tyrphostin A1 non-competitively inhibited the GABA-activated response of both the w.t and mutant receptor (73.2% and 64.7% inhibition of the maximum responses to GABA, respectively, n = 3).

These data suggest that 2 classes of inhibitors of tyrosine kinases can directly interact with a binding site(s) on the external surface of the $GABA_A$ receptor resulting in different types of inhibition. This study also demonstrates the non-specificity of these agents with respect to phosphorylation studies when applied externally to $GABA_A$ receptors.

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199P USE OF A 96 WELL FORMAT ^{36}Cl FLUX ASSAY TO MEASURE $GABA_A$ ION CHANNEL FUNCTION IN STABLY EXPRESSED RECOMBINANT RECEPTORS: CONSTITUTIVE CHANNEL OPENING IN THE ABSENCE OF AGONIST REVEALED

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$GABA_A$ receptors are multisubunit oligomers with integral chloride channels belonging to the ligand-gated ion channel receptor family. $GABA_A$ ion channel opening can be modulated by compounds acting at various allosteric sites on the receptor eg. benzodiazepines, barbiturates and steroids. This study describes the development and preliminary characterisation of a 96 well assay to measure human recombinant $GABA_A$ receptor function.

Ltk$^-$ cells stably expressing human recombinant $GABA_A$ subunit combinations were grown in Dulbecco's Modified Eagle Medium with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100µg/ml streptomycin and 1mg/ml geneticin. Cells were subsequently seeded into 96 well plates at densities of 1 to $4x10^4$ cells/ml in a volume of 200µl/well and grown in the presence of 10% serum for 7 days. Cells were induced one day prior to use with 1µl/ml dexamethasone. Cell density was found to be critical for optimal signals and number of days of induction important for observing benzodiazepine modulation. Optimised assay conditions included use of chloride-free assay buffer by substitution with acetate salts, performing the assay at 4°C, inclusion of the chloride transport inhibitors DIDS (10µM) and furosemide (0.1mM), and using a short assay time of 30 secs. At extended assay times the specific signal was progressively reduced with increasing basal accumulation and desensitization of the GABA response. Cells were initially washed with HEPES/Krebs buffer at room temperature (pH 7.4 using 1M Tris) using a Dynatech 96 plate washer. Benzodiazepines were added in a 30 sec preincubation step, particularly important for detection of compounds with slower on-rates such as the full agonist abecarnil. A Robbins Hydra 96 then simultaneously added 40µl of ^{36}Cl ligand solution ± GABA in chloride-free assay buffer at 4°C, to each well for 30 secs. Solutions were then aspirated and cells washed with 100µM picrotoxin-containing stop buffer at 4°C. Influx was determined by scintillation counting on a Packard TopCount.

The pharmacology of ^{36}Cl influx was examined in this 96 well format, generating concentration response curves to GABA and muscimol in cells expressing α subunits in combination with β3γ2. Both agonists elicited similar maximum increases in ^{36}Cl influx, although muscimol exhibited higher potency. EC_{50} values (µM) for GABA and muscimol respectively were : α1 3.9±0.5 (n=13) and 0.54±0.06 (n=17); α2 1.6±0.4 (n=12) and 0.27±0.04 (n=14); α3 10.3±0.7 (n=12) and 2.0±0.2 (n=17); α5 1.6±0.3 (n=17) and 0.36±0.03 (n=16). Agonist responses were blocked by the competitive $GABA_A$ antagonist bicuculline (100µM) and the chloride channel blocker picrotoxin (100µM). Benzodiazepine modulation of the EC_{20} response to GABA was demonstrated at each of these α subtypes. At α3, for example, maximal GABA stimulation was from 81±3 (basal) up to 666±24 cpm ($P<0.001$), and modulation of EC_{20} GABA by the full agonist chlordiazepoxide from 161±7 to 443±15 cpm ($P<0.001$). Interestingly, benzodiazepine agonists were observed to enhance basal influx in the absence of exogenous GABA and further, basal ^{36}Cl influx could be reduced by picrotoxin. This could infer activation by an endogenous substance or spontaneous ion channel activation. Conditioned ^{36}Cl solution, pre-exposed to cells for a 10 min period, was applied to fresh, washed cells for 40 secs but no stimulatory effects were observed. Additionally, increasing prewashing of cells prior to the start of the assay did not reduce effects of 1µM flunitrazepam on basal influx. These observations are supported by electrophysiological measurements which show spontaneous channel activity in the absence of GABA, with conductance states being consistent with mediation via a $GABA_A$ receptor chloride channel.

In summary, a 96 well format ^{36}Cl flux assay has been developed to measure functional activity at recombinant $GABA_A$ receptors.

200P SIMULTANEOUS MULTI-CHANNEL RECORDINGS REVEAL SYNCHRONOUS EPILEPTIFORM-LIKE BURSTING IN CULTURED RAT HIPPOCAMPAL NEURONES INDUCED BY BICUCULLINE

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Reduction in GABA-mediated inhibition is thought to be important in enhancing and amplifying excitatory post-synaptic potentials leading to seizure generation, since drugs that block $GABA_A$ receptors induce synchronous burst discharges in the hippocampus (Scharfman, 1994). To date, electrophysiological studies of epileptic bursting have largely concentrated on recordings from one or two single-units or field potentials. We are using a system which has been developed to record extracellular discharge activity simultaneously from up to 64 microelectrodes (Gross & Schwalm, 1994). The system was utilised to study bicuculline-induced epileptiform discharges within a monolayer network of dissociated hippocampal neurones and the actions of the anti-epileptic drugs sodium valproate and flurazepam.

Primary dissociated hippocampal neurones from E18 rat fetuses were cultured directly onto Multiple Micro-Electrode Plates (MMEPs), which comprised a planar 64-channel microelectrode array incorporated onto a glass substrate. Extracellular discharge activity was captured and processed using a computer-controlled *Multi-channel neuronal acquisition processor* (Plexon Inc, Texas, USA). Data were analysed off-line using *SpikeWorks* (Plexon Inc) and multiple spike train analysis software (*Stranger*, Biographics, North Carolina, USA). Statistical differences between groups of data were analysed using a Student's paired t-test.

The hippocampal cultures formed monolayer networks with characteristic morphology and exhibited spontaneous extracellular discharge activity. Recordings were made from 6-20 cells per MMEP which expressed firing rates of 0.003-20.45 Hz (3.32±0.48 Hz, mean±s.e.mean, n=66). GABA produced a concentration-dependent decrease in firing rate (EC_{50}=9.14±0.04 µM, n=9) which was blocked by prior application of bicuculline (10 µM). GABA-evoked suppression of firing rate (5 µM: 37.6±5.9%, n=18) was significantly potentiated by flurazepam in all cells tested (20 µM: 89.8%±4.3%; $P<0.001$). Addition of bicuculline (10 µM) induced epileptiform-like bursting which, within 10 minutes, became highly synchronised between all cells recorded (n=13). After bicuculline, the burst rate (a burst was defined as a minimum of 3 spikes occurring within a 0.01 s period) was significantly increased from 1.10±0.68 bursts min^{-1} (basal) to 43.6±7.03 bursts min^{-1} ($P<0.001$). Synchronised epileptiform-like bursting within the network was abolished by the addition of sodium valproate (10 µM), with the burst rate returning to basal levels.

Using this multi-channel recording system we have demonstrated bicuculline-induced synchronised epileptiform-like activity in a hippocampal network which is abolished by sodium valproate. The system should prove a useful model for electrophysiological studies to the genesis of epileptic activity and the mode of action of anti-convulsant drugs.

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201P EFFECTS OF BROMOCRIPTINE AND HALOPERIDOL ON PREPULSE INHIBITION OF THE EYE-BLINK STARTLE RESPONSE AND N1/P2 AUDITORY EVOKED POTENTIAL IN MAN

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The acoustic startle response can be suppressed by presentation of a brief low-intensity auditory stimulus 30–500 ms before the 'startle-eliciting' stimulus ('prepulse inhibition', PPI). Evidence from experiments with animals indicates that D_2-dopamine receptors are involved in regulating PPI (Swerdlow *et al.*, 1992). Recently we reported that single oral doses of a D_2 receptor agonist bromocriptine attenuated PPI of the electromyographic (EMG) component of the startle eyeblink response in humans, and that this attenuation was reversed by administration of the D_2-receptor blocking neuroleptic haloperidol (Abduljawad *et al.*, 1997). Here we report the effects of bromocriptine and haloperidol on PPI of the EMG response and the N1/P2 complex of the auditory evoked potential (Mauguiere, 1995).

11 males (18–30 years) participated in 4 sessions in which they received oral doses of placebo (PLAC), bromocriptine 1.25 mg (BROM), haloperidol 3 mg (HAL), and bromocriptine 1.25 mg + haloperidol 3 mg (BROM+HAL), according to a balanced double-blind design. EMG recording via electrodes placed over the orbicularis oculi muscle, and electroencephalographic recording via a vertex electrode, was carried out 120 min after HAL and 90 min after BROM. Subjects received 60 trials, separated by variable intervals (mean 25 s, range 15–35 s); the acoustic stimuli (1 kHz) were: (i) 40 ms, 115 dB ('pulse alone' [PA] trials); (ii) 40 ms, 85 dB ('prepulse-alone' trials); (iii) 40 ms, 85 dB, followed after 120 ms by 40 ms, 115 dB ('prepulse/pulse', [PP] trials) (20 trials of each type, in random sequence). Mean amplitudes [A] of the EMG response and the N1/P2 complex were derived from the PA and PP trials, and in the each case, percent PPI was calculated as $100.[A_{PA}-A_{PP}]/A_{PA}$ (Swerdlow *et al.*, 1992). Results were analyzed by ANOVA, followed by comparisons with PLAC using Dunnett's test.

Under the PLAC condition, both the EMG response and the N1/P2 complex showed >50% PPI (Table 1). The treatments had no significant effect on the amplitude of either the EMG response ($F<1$) or the N1/P2 complex ($F<1$). PPI of the EMG response was significantly reduced by BROM ($F_{3,30}=3.8$, $P<0.02$; PLAC *vs* BROM, $t=2.9$, $k=4$, $P<0.01$); the degree of inhibition seen in the BROM+HAL and PLAC conditions did not differ significantly ($t=1.1$, $k=4$, $P>0.2$). There was no significant effect of the treatments on PPI of the N1/P2 complex ($F<1$).

Table 1: Amplitudes of eyeblink EMG response and N1/P2 complex evoked by 115 dB stimuli, and percent inhibition (%PPI) induced by 85 dB prepulses, under all treatment conditions (mean ± s.e.mean)

	eyeblink EMG		N1/P2 complex	
Treatment	amplitude, mV	%PPI	amplitude, μV	%PPI
PLAC	0.54±0.19	53.9±11.3	50.4±4.4	70.9±5.1
BROM	0.53±0.19	32.4±11.3*	47.7±4.7	63.1±9.6
HALO	0.58±0.14	55.7±10.1	59.3±17.1	59.1±12.1
HALO+BROM	0.82±0.44	45.6±13.4	45.6±7.4	74.3±5.3

* Significantly different from PLAC ($P<0.01$): see text

The suppression of PPI of the eyeblink EMG response by BROM, and the reversal of this suppression by HAL, are consistent with our previous results (Abduljawad *et al.*, 1997). The failure to find an equivalent effect in the case of the N1/P2 complex suggests that different mechanisms may be involved in PPI of the eyeblink and the N1/P2 component of the auditory evoked potential, and that D_2-dopamine receptors may not be involved in the latter case.

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202P MODULATION OF [^{3}H]-FLUNITRAZEPAM BINDING BY NEUROSTEROIDS AND CHOLESTEROL

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Neurosteroids are known to enhance the binding of [^{3}H]-flunitrazepam (FNZ) to neuronal membranes (Harrison *et al.*, 1987), presumably through allosteric interactions on the $GABA_A$ receptor. We have[e] shown recently that this effect is influenced by the cholesterol content of the membranes (Bennett & Simmonds, 1996). Some aspects of these phenomena are now explored further.

Details of the methods have been described before (Bennett & Simmonds, 1996). Briefly, membranes were prepared from the cerebral cortex of male Wistar rats (200-250 g) to yield a synaptosomal fraction which was lysed and thoroughly washed to remove endogenous GABA. Enrichment of membranes with cholesterol was achieved by incubation with liposomes containing equal amounts of cholesterol and phosphatidylcholine for 3h at 37°C. This increased the membrane cholesterol to 185-240% of the levels in membranes incubated without liposomes (unenriched). Binding of [^{3}H]-FNZ to the membranes was performed with 1 nM final concentration of ligand at 4°C for 60 min. Neurosteroids were dissolved in acetone before dilution in the incubation medium. A nominal final concentration of 0.5% acetone was included in all samples. The neurosteroids were added to the membranes and incubated at 37°C for 10 min before addition of the [^{3}H]-FNZ. Non-specific binding was determined with 10 μM FNZ and was less than 4%. Specific binding data are presented.

Control binding of [^{3}H]-FNZ was significantly reduced ($P<0.001$) in cholesterol enriched membranes to 535±26 fmol.mg protein^{-1} compared with 730±34 fmol.mg protein^{-1} in unenriched membranes (mean ± s.e.mean, n=22). This may represent a loss of binding sites since the binding affinity of FNZ was previously shown to be unchanged (Bennett & Simmonds, 1996).

Pregnanolone (5β-pregnan-3α-ol-20-one) 0.1 - 30 μM caused a concentration-dependent enhancement of [^{3}H]-FNZ binding to a maximum determined by hyperbolic curve fitting (GraphPad Prism) of 148.1±2.5% control for enriched membranes and 143.9±2.3% control for unenriched membranes (difference not significant, P=0.22). The EC_{50} for pregnanolone was significantly lower (P=0.009) at 1.01±0.22 μM in enriched membranes compared with 2.30±0.43 μM in unenriched membranes. These data were derived from 11 experiments in 2 of which the effect of enrichment was in the opposite direction, similar to the result published previously (Bennett & Simmonds, 1996).

Experiments with 3 μM allopregnanolone (5α-pregnan-3α-ol-20-one) and 3 μM alphaxalone (5α-pregnan-3α-ol-11,20-dione) also showed significantly greater enhancements in [^{3}H]-FNZ binding in enriched membranes: 123.2±1.1 and 117.2±1.8% control (n=4) for allopregnanolone in enriched and unenriched membranes, respectively; 118.3±1.9 and 109.9±1.4% control (n=3) for alphaxalone compared with 134.2±3.1 and 122.5±2.1% control (n=11) for 3 μM pregnanolone. In both enriched and unenriched membranes, a combination of 10 μM pregnanolone + 10 μM alphaxalone enhanced [^{3}H]-FNZ binding significantly less ($P<0.01$) than did pregnanolone alone and not significantly more ($P>0.05$) than did alphaxalone alone, suggesting that alphaxalone is a partial agonist compared with the fuller agonist action of pregnanolone in this effect.

Possible interpretations are that cholesterol enrichment enhanced the sensitivity of the FNZ binding site to modulation by neurosteroids or that the 27% of FNZ binding sites which disappeared with enrichment had a lower sensitivity to neurosteroids than those that remained.

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203P PERIPHERAL TYPE BENZODIAZEPINE BINDING SITES IN HUNTINGTON'S DISEASE

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Huntington's disease (HD) is a hereditary disorder associated with the development of dyskinesias and dementia, characterised by profound atrophy of the striatum and to a lesser extent in other areas including the cerebral cortex. A relative gliosis has been described in HD although it is unclear whether microglial proliferation occurs. One marker for such inflammatory gliosis is an elevation of peripheral type benzodiazepine binding sites (PTBBS) (Bourdiol *et al.*, 1991); these have been shown to be increased following excitotoxic neuronal damage (Benavides *et al.*, 1987). The present study investigates PK11195 binding to PTBBS in HD brain tissue.

Post mortem tissue was taken from 10 HD patients and 10 control subjects of comparable age. Temporal cortex (BA 21), frontal cortex (BA 10) and putamen were examined. Tissue was homogenised in Tris-HCl buffer (50nM, pH 7.4), centrifuged and rehomogenised. For saturation binding assays samples contained tissue at a 1:160 dilution in a final volume of 250 µl with 6 concentrations of [^{3}H]PK11195 ranging from 0.5 to 10 nM. Non-specific binding was determined in the presence of 1 µM unlabeled PK11195. The reaction mixture was incubated for 120 mins at 4°C and the reaction stopped by rapid vacuum filtration and subsequent washing with buffer. Radioactivity on the filters was determined by liquid scintillation counting.

PK11195 binding revealed a change in the B_{max} of PTBBS for two of the examined brain areas. A highly significant increase of 69% was observed for the putamen. A moderate, but significant increase in the B_{max} was observed for the frontal cortex (+25%). No significant changes in the B_{max} were seen for the temporal cortex. The receptor affinity did not changed in any brain area.

Table 1. Density of peripheral type benzodiazepine binding sites in brain regions of control subjects and HD patients.

	B_{max} mean ± SD (fmol/mg tissue)		
	control subjects	HD patients	ANOVA
BA 10	39.5 ± 6.9	49.6 ± 11.8	p=0.033
BA 21	46.9 ± 14.1	52.5 ± 12.4	p=0.353
Putamen	46.6 ± 8.4	78.9 ± 30.6	p=0.007

The observed changes in the number of PTBBS in the putamen and frontal cortex correlate with other neurotransmitter changes indicative of neuronal atrophy in these areas of the HD brain (Pearson & Reynolds, 1994). An increase in the B_{max} of PTBBS in degenerated brain areas may be mediated by mechanisms involving activation of microglia and astrocytes (Bourdiol *et al.*, 1991). The finding of a changed B_{max} rather than an altered activity agrees with earlier findings for astrocytosis *in vitro* (Canat *et al.*, 1993). These results closely resemble those for other diseases where inflammatory processes may be involved, e.g. Alzheimer's disease (Rogers *et al.*, 1996). Our findings therefore indicate the possible involvement of inflammation on the neurodegenerative process of HD.

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204P NMDA RECEPTORS IN THE STRIATUM IN SCHIZOPHRENIA

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There is substantial evidence implicating a dysfunction of glutamatergic systems in schizophrenia. Although several studies have reported changes in pre- and/or post-synaptic glutamatergic markers in cortex or hippocampus there have also been abnormalities identified in the striatum in the disease. Thus a deficit of neuronal glutamate uptake sites has recently been observed in striatal and accumbens tissues in schizophrenia (Aparicio-Legarza *et al.* 1997), while dizocilpine binding to NMDA receptors in putamen is reportedly increased (Kornhuber *et al.* 1989).

We have undertaken saturable radioligand binding of [^{3}H]L-689,560 to the glycine site of the NMDA glutamate receptor complex to determine the density of this receptor in putamen, caudate and accumbens nuclei taken post mortem from schizophrenic patients and age-matched controls. The method employed has been previously reported (Reynolds *et al.* 1994). In addition, the possible contribution of antipsychotic drug treatment was determined following 21 days administration i.p. of haloperidol (1.5 mg/kg/day), clozapine (25mg/kg/day) or vehicle.

The results (table 1) demonstrate a significant increase in the NMDA receptor density in schizophrenia in the putamen, but not in the other two regions. No effect of haloperidol on the binding of [^{3}H]L-689,560 to rat striatum was observed (table 2), however, in those receiving clozapine, a decrease in mean values was apparent which failed to reach significance.

Table 1. [^{3}H]L-689,560 binding in human post mortem brain tissue.

	N. Accumbens	Caudate	Putamen
Schizophrenics	44.2±15.9 (n=13)	32.6±10.0 (n=8)	50.6±9.7* (n=12)
Controls	45.0±11.5 (n=11)	28.5±4.5 (n=5)	39.2±10.5 (n=12)

Data are Bmax values of saturable [^{3}H]L-689,560 binding expressed as pmol/g tissue. Values are means±SD. *p=0.012

Table 2. [^{3}H] L-689,560 binding density in drug-treated and non-treated rat striatum

Haloperidol-treated (n=8)	106.9±21.2
Clozapine-treated (n=8)	93.2±15.2*
Controls (n=8)	107.3±13.2

Data are Bmax values of saturable [^{3}H] L-689,560 binding expressed as pmol/g tissue. Values are means±SD. *p=0.067

These results provide further evidence for dysfunction of glutamatergic systems in the striatum in schizophrenia.

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