169P THE NOCICEPTIN SYSTEM PLAYS A CRUCIAL ROLE IN THE MODULATION OF THE ALLODYNIC-LIKE BEHAVIOUR OF NEUROPATHIC RATS

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Recent studies with models of neuropathic pain indicate that nociceptin (N/OFQ), possibly acting through its specific receptor (OP_4), has anti-nociceptive properties when administered at the spinal level (Yamamoto et al., 1999). Moreover, OP_4 has been described as highly involved in spinal facilitation which occurs in the chronic constriction injury (CCI) rat model (Yamamoto et al., 2000). In the present work, we studied the spinal effect of N/OFQ in this model, examining the molecular changes that occurred in both lumbar enlargement and dorsal root ganglia (DRG) in the development of persistent pain. Male Sprague-Dawley rats (175-200g) were anaesthetised with chloral hydrate and the sciatic nerve was unilaterally damaged by four loose ligatures. Soon after, a chronic intrathecal (ith) catheter was insert in order to administer compounds locally. The mechanical pain threshold of rodents was recorded by von Frey hairs (bending force ranging between 0.1-447g). Data collected were expressed as means ± 95% c.ls for each group of treatment (n=7-8) and were analysed by repeated measures ANOVA. Rats responding to non-noxious stimuli (<3.6g) displayed a severe neuropathy (allodynic-like behaviour) and were selected for pharmacological studies. N/OFQ (0.2-20 nmol/10 µl), injected ith in rats, induced a dose-dependent anti-allodynic activity. At 5 min post administration, a rise in the pain threshold was observed with 2nmol [(23.9g (18.3-31.5) *vs* 2.9g (1.9-4.4) of the saline treated group; $p<0.01$]. This effect was OP_4-mediated as the receptor antagonist, [$Nphe^1$]N/OFQ(1-13)NH_2 (60 and 120nmol; Calo' et al., 2000), given ith in combination with N/OFQ (2 nmol) prevented the activity of the peptide [9.8g (4.3-22.1) and 6.17g (2.2-17.3), respectively *vs* 34.7g (14.5-82.7) of the N/OFQ treated group, at 5 min post injection; $p<0.05$ and $p<0.01$, respectively]. Molecular studies were realised by the semi-quantitative RT-PCR assay using mRNA isolated from quickly dissected and frozen tissues. Data were expressed as relative to control (=1) ± s.e.m. and the Student's *t*-test was used to analyse the results. The two described OP_4 splice variants (Xie et al, 1999) were up-regulated in the ipsilateral spinal tissues of allodynic rats (n=6). The short form increased by 100% ($p<0.01$) while the long form increased by 50% ($p<0.05$) over control values in the spinal cord. In the DRG, the short and the long forms of OP_4 were increased by 60 and 50%, respectively. The up-regulation of both isoforms was time-dependent, starting 3 days after surgery, with the peak effect at day 7 when the allodynic behaviour was apparent, and returned to normal values at 15 days after surgery. No significant changes were found for either the OP_3 receptor or the preproN/OFQ in both sides of the lumbar spinal cord or DRG. Thus, these results demonstrate a crucial role of the N/OFQ-OP_4 system in the development of mechano-allodynia in the CCI rat model. Further studies are necessary to clarify the function of OP_4 receptor reorganization in the spinal plasticity occurring in the persistent pain.

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170P INHIBITION OF C-FIBRE AND MECHANICALLY-EVOKED DORSAL HORN NEURONE ACTIVITY FOLLOWING INTRAPLANTAR FORMALIN INJECTION IN THE ANAESTHETISED RAT

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Intraplantar (i.pl.) formalin injection elicits a biphasic pattern of nocifensive behaviour in conscious rats (Dubuisson & Dennis, 1977) with a temporally related profile of firing in second order dorsal horn (DH) neurones (Dickenson & Sullivan, 1987). The 2nd (late) phase of neuronal activity is dependant upon an initial barrage of C-fibre input and is sustained by a combination of central and peripheral mechanisms (Dickenson & Sullivan, 1987). Here we have examined the viability of somatic inputs to the dorsal horn during the late phase formalin response using in vivo DH recording in the anaesthetised rat.

Male Sprague Dawley rats (300-350g) were anaesthetised (pentobarbitone 30 $mgkg^{-1}h^{-1}$ i.v.) and the spinal cord exposed by laminectomy at vertebral level L1. A recording electrode was inserted into the dorsal spinal cord and wide dynamic range (WDR) neurones were identified which responded to noxious pinch of the hindpaw receptive field (HRF), and to electrical stimulation with response latencies indicative of A- and C-fibre inputs (ie 0-20ms and 90-300ms, respectively). Steady baseline neuronal responses were obtained to electrical and mechanical (application of noxious von Frey filament) stimulation of the HRF before i.pl. injection of formalin (50 µl, 5 %) or control vehicle (saline, 50 µl). Thereafter, electrical stimulation only was continued (trains of 20 stimuli applied every 10 min) for 70 min, during which time ongoing neuronal activity was continuously recorded. Following this, DH responses were again evoked by mechanical stimulation of the HRF. Data were analysed by Student's t-test and a probability (P) value of <0.05 was taken as significant.

I.pl. formalin injection evoked a characteristic biphasic pattern of DH neuronal firing (peak firing rates: Phase 1, 149 ± 43 Hz; Phase 2, 14.4 ± 3.3 Hz; both n=5) lasting >60 min. In contrast, i.pl. saline injection resulted in only a brief (3 min) excitation of DH neurones (peak firing rate 48 ± 22 Hz; n=3). Hindpaw saline injection did not alter DH neuronal responses to mechanical stimuli (response to 10 s stimulus: before, 195 ± 57 spikes; 1 h after, 226 ± 86 spikes, n=3) or to responses evoked by electrical activation of A- or C- fibres (responses after 1h, 91 ± 15 % and 92 ± 13 % of pre-saline responses, respectively, n=3). However, i.pl. formalin injection significantly reduced DH responsivity to mechanical stimulation of the HRF (response to 10 s stimulus: before, 203 ± 40 spikes; 1 h after, 100 ± 33 spikes; $P<0.05$, n=5) and within 10 min had markedly attenuated responses evoked by C-fibre activation, which persisted for the remainder of the experiment (responses after 1 h, 14 ± 6% of pre-formalin values, $P<0.05$, n=5). A-fibre mediated responses were unaffected by i.pl. formalin injection over the entire recording duration.

These data indicate that i.pl. formalin causes a rapid inactivation of the C-fibre mediated component of WDR DH neuronal firing and suggests that the late phase of firing is maintained by an intrinsic central component or continued A-fibre input.

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171P A SINGLE TRANSMEMBRANE LOCATED AMINO ACID INFLUENCES THE ACTION OF GABA AND PENTOBARBITONE ACTING ON AN INVERTEBRATE GABA RECEPTOR (RDL)

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Many intravenous general anaesthetics enhance the actions of GABA acting at the $GABA_A$ receptor and at higher concentrations directly activate this receptor *via* GABA-modulatory and GABA-mimetic effects respectively (Belelli *et al.*, 1999). Certain general anaesthetics also potentiate GABA acting at a homomeric GABA receptor isolated from *Drosophila melanogaster* (RDL), but they do not directly activate the receptor (Belelli *et al.*, 1999). The interaction of general anaesthetics with both GABA- and glycine-gated chloride channels is greatly influenced by the nature of an amino acid within transmembrane region two (TM2). The positive allosteric actions of ethanol on the glycine receptor are negatively correlated with the molecular volume of this TM2 residue (Belelli *et al.*, 1999). Here, we have determined the effects of GABA and pentobarbitone on wild type (WT) and mutant RDL receptors wherein the naturally occurring TM2 amino acid methionine was replaced by the small volume amino acids alanine RDL_{M314A}, or glycine RDL_{M314G}.

Mutations were introduced by standard site-directed mutagenesis protocols. Voltage-clamp (V_h = -60 mV) experiments were performed on *Xenopus laevis* oocytes injected previously (2-12 days) with cRNA (0.25-1.6 $\mu g\ \mu l^{-1}$, 20-50 nl) prepared from WT or mutant *Rdl* cDNAs. Pentobarbitone was pre-applied for 20-60 s prior to co-application with GABA at the appropriate EC_{10} to determine both its GABA-mimetic and GABA-modulatory effects. The pEC_{50} and Emax (expressed as a percentage of the response to a saturating concentration of GABA) for either effect of pentobarbitone is reported as the mean ± s.e.m. Statistical significance was determined by two-tailed t-test ($P<0.01$)

The potency of GABA at RDL_{M314A} (pEC_{50} = 6.05 ± 0.05) and RDL_{M314G} (pEC_{50} = 6.64 ± 0.04) receptors was significantly increased in comparison to the WT receptor (pEC_{50} = 4.82 ± 0.17) (n = 5). At the WT receptor, pentobarbitone caused a concentration-dependent enhancement of the GABA-evoked current (pEC_{50} = 3.36 ± 0.03; E_{max} = 63 ± 4 %; n = 4), but did not directly activate the receptor. Pentobarbitone exhibited a GABA-modulatory action at RDL_{M314A} receptors (pEC_{50} = 4.17 ± 0.06; E_{max} = 40 ± 3 %), and additionally directly activated the receptor (pEC_{50} = 3.53 ± 0.06; E_{max} = 86 ± 12 %) (n = 4). By contrast, at the RDL_{M314G} receptor, pentobarbitone had a large GABA-mimetic effect (pEC_{50} = 4.25 ± 0.05; E_{max} = 123 ± 10 %) but lacked a GABA-modulatory action (n = 4).

Previous work suggests RDL receptors to possess a pentobarbitone modulatory site, but unlike mammalian $GABA_A$ receptors, not a binding site *via* which barbiturates can directly activate the receptor-channel complex. Here, we have shown that both of these actions are critically dependent on the nature of a TM2 located amino acid. Whether these mutations influence barbiturate binding directly, or indirectly by affecting transduction, remains to be determined.

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172P ALTERATIONS IN $GABA_B$1B AND $GABA_B$ 1C RECEPTOR GENE EXPRESSION IN THE BASAL GANGLIA AND THALAMUS OF RATS BEARING A NIGROSTRIATAL TRACT LESION

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Loss of striatal dopamine innervation due to the degeneration of substantia nigra pars compacta (SNc) in Parkinson's Disease (PD) produces downstream changes in associated pathways within the basal ganglia (BG) and thalamus. We have previously shown in rats, that such changes include increased $GABA_B$1A receptor splice variant gene expression in the subthalamic nucleus, entopeduncular nucleus (EP) and SN pars reticulata (SNr) and reductions in $GABA_B$1A and $GABA_B$2 gene expression in the SNc (Johnston *et al.*, 2000).

The aim of this study was to examine whether similar changes in expression of genes encoding two additional $GABA_B$ receptor splice variants (1B and 1C) accompanied 6-hydroxydopamine (6-OHDA)-induced nigrostriatal tract lesioning in the rat.

Male Sprague Dawley rats (250-270g) were killed by halothane overdose, 3 weeks after a unilateral 6-OHDA (12.5 µg in 2.5 µl), or sham (2.5 µl 0.1% ascorbate in H_2O), lesion of the median forebrain bundle. Brains were removed, snap frozen and sectioned coronally (15 µm). For in situ hybridisation, sections were fixed, dehydrated and defatted. ^{35}S-labelled oligonucleotide probes complementary to mRNA encoding the $GABA_B$1B and $GABA_B$1C subtypes were diluted in hybridisation buffer to a specific activity of $3x10^6$ cpm ml^{-1}. Sections were hybridised overnight at 37°C then washed in a series of standard saline citrate solutions (1 x SSC: 0.15M NaCl, 0.015M sodium citrate in H_2O) to a maximum stringency of 60°C and 0.1x SSC. Sections, together with ^{14}C standards, were exposed to film for 20 days. Levels of $GABA_B$ receptor mRNA were semi-quantified by densitometric image analysis. Differences in mRNA levels between lesioned and intact hemispheres were determined using a paired t-test ($P < 0.05$).

Subsequent to a 6-OHDA lesion, expression of both subtypes was significantly reduced in the SNc ($GABA_B$1B ~ 40%; $GABA_B$1C ~ 75%). In addition, $GABA_B$1B gene expression was significantly reduced in the SNr (~40%) and EP (~32%) (Table 1.). No significant changes in mRNA levels were noted in the sham animals (data not shown).

	$GABA_B$ Receptor mRNA levels (OD – nCi/g)			
	1B		1C	
Region	Lesioned	Intact	Lesioned	Intact
SNc	7.7 ± 2.0*	30.7 ± 1.7	5.8 ± 0.5*	15.5 ± 0.6
SNr	5.5 ± 0.9*	9.2 ± 0.9	3.4 ± 0.2	5.0 ± 0.2
EP	9.1 ± 1.1*	13.4 ± 0.9	5.6 ± 0.5	5.6 ± 0.1

Table 1. $GABA_B$1B and $GABA_B$1C receptor mRNA levels in rats with a 6-OHDA lesion. Values are mean ± s.e.mean. (n=6). * $P<0.05$; lesioned vs. intact hemisphere. OD = optical density.

The concurrent decrease in $GABA_B$1B and 1C expression within the SNc suggests that, as previously noted for $GABA_B$1A and $GABA_B$2, both these subtypes are present on nigrostriatal tract neurones. The relevance of a decreased gene expression of the 1B variant in the SNr and EP following 6-OHDA lesioning remains to be seen. However, these changes are opposite to those previously observed for $GABA_B$1A (Johnston *et al.*, 2000), indicating that expression of the various splice variants resulting from the parent $GABA_B$1 gene are subject to differential regulation under conditions of nigrostriatal tract lesion.

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173P THE EVOKED RELEASE OF GABA AND TAURINE FROM THE STRIATO-PALLIDAL (INDIRECT) PATHWAY: A DUAL PROBE MICRODIALYSIS STUDY IN THE FREELY-MOVING RAT

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Cortical information is processed in the striatum and then transmitted to the output nuclei of the basal ganglia, the entopeduncular nucleus (EP) and to the substantia nigra pars reticulata (SNr), by either the direct or the indirect pathways. The direct pathway comprises a direct projection from the striatum to the EP or SNr, the indirect pathways consist of a striatal projection to the globus pallidus (GP) and thence to the output nuclei or to the output nuclei via the subthalamic nucleus (STN). The projection neurones from STR, GP, SNr and EP are GABAergic, whereas those of the STN use glutamate. Thus, activation of the direct pathway leads to inhibition of the EP and SNr, whereas activation of the indirect pathway causes disinhibition (Smith & Bolam, 1990).

Previous dual-probe microdialysis studies have shown intra-STR application of the glutamate receptor agonist, kainate (KA), to release endogenous GABA and taurine from STR and, distally, from SNr in a DNQX- (6,7-dinitroquinoxaline-2,3-dione) and terodotoxin (TTX)-sensitive manner (Bianchi et al., 1998). The aim of the present study was to compare those results with release of endogenous GABA and taurine from the indirect striato-pallidal pathway.

Male Wistar rats (250 g) were anaesthetised with chloral hydrate (i.p. 400 mg/kg b.w.) and microdialysis probes stereotaxically implanted, one into the right STR and one into the ipsilateral GP, as previously described by Bianchi et al. (1998; 1999). After 24 h recovery, the probes were perfused with artificial CSF (flow-rate, 2 µl/min). After 90 min stabilisation, 20 min samples were collected and amino acids determined by derivatisation followed by HPLC with fluorimetric detection. Areas under the concentration-time curves (AUCs) were calculated and statistically analysed, as described previously (Bianchi et al., 1998), before and after 20 min STR exposure to 100 µM KA alone or in the presence of 100 µM DNQX or 10 µM TTX. All values are means ± s.e.m., n = 4-7, with MANOVA $P<0.05$ being regarded as significant.

The intrastriatal application of KA significantly enhanced the local GABA and taurine release, from 26 ± 6 to 105 ± 22 and from 909 ± 118 to 6897 ± 1064, basal to maximal, respectively (nM, mean ± s.e.m., n=7). As previously shown, the local release of GABA, but not taurine, was abolished in the presence of DNQX and TTX. The distal (pallidal) release of GABA and taurine induced by intrastriatal KA application was significantly increased (from 23 ± 2 to 42 ± 5 and from 588 ± 59 to 935 ± 203 nM, respectively) and this was abolished by the presence of striatal DNQX and TTX.

These data indicate that GABA and taurine release in the GP is dependent on non-NMDA receptor activation in the STR and on the propagation of action potentials along the striato-pallidal pathway. Thus, GABA may be released from striatal axon collaterals and pallidal terminals of the indirect pathway. Furthermore, taurine, known to be present in the direct pathway, may also be involved in the striato-pallidal system.

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174P ACh AND GABA RELEASE FROM THE CORTEX AND HIPPOCAMPUS OF THE RAT DURING NOVELTY AND HABITUATION.

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The involvement of the forebrain cholinergic system in arousal, learning and memory has been well established. Other neurotransmitter systems such as the GABAergic and glutamatergic systems may be involved in the mechanisms of memory through their modulatory activity on the cortical and hippocampal cholinergic pathways. We studied the activity of cortical and hippocampal cholinergic, GABAergic and glutamatergic systems during novelty and habituation in the rat by means of the microdialysis technique coupled to HPLC. Male adult Wistar rats (250 g) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and transversal microdialysis membranes were inserted in the fronto-parietal cortex or dorsal hippocampus (Giovannini et al., 1998). The day after surgery, the membrane was perfused with Ringer solution containing 7 µM physostigmine (flow rate 4 µl/min) and the system was allowed to stabilize for 1 h. Samples were then collected at 10 min intervals. After collecting 5 samples to detect basal release, the animals were gently transferred to a novel environment (an arena of 70 x 60 x 30 cm) and allowed to explore it twice consecutively for 30 min each, spaced by 60 min (Arena I and II). Between and after explorations the animals were returned to their home cage. Samples were collected throughout the experiment and the release of ACh, GABA and Glu was measured. During Arena I and II motor activity was measured at 10 min intervals. During the two explorations both cortical (Arena I: $p<0.001$ vs basal, n= 6; Arena II: $p<0.05$ vs basal, n= 7) and hippocampal (Arena I: $p<0.001$ vs basal, n= 5; Arena II: $p<0.05$ vs basal, n= 4) ACh release increased above basal, slowly going back to basal levels when the animal was returned to the home cage. Area under the curve (AUC) showed that during Arena I the increase was higher and longer-lasting in the dorsal hippocampus than in the cortex ($F_{3,28} = 12.57$; $p<0.0001$). In either structure ACh release was higher during Arena I (+63%, n=6 and +38%, n=6 respectively, $p<0.05$) than Arena II (+200%, n=5, and +59%, n=5, respectively, $p<0.01$) and was TTX-dependent. Motor activity increased mainly during exploration of Arena I, with a maximum during the first 10 min (327.4±39.9 counts; n=9; $p<0.001$ vs Arena II), and was significantly correlated to cortical ($r^2 = 0.2243$; $p<0.01$) but not to hippocampal ACh release ($r^2 = 0.1169$; n.s.). Cortical GABA release increased during exploration of Arena II only (+159%; $p<0.01$ vs controls), but was not correlated to motor activity ($r^2 = 0.068$; n.s.). Hippocampal GABA release increased in 2 out of 7 rats only both during Arena I and Arena II. Cortical and hippocampal glutamate release did not vary significantly during the two exploration of the Arena. Our data indicate that the cortical and hippocampal cholinergic pathways are activated during exploration of a novel environment, probably due to increased arousal and attention. However, during the second exploration of the arena, habituation takes place, as demonstrated by the lower and shorter release of the neurotransmitter. The cortical GABAergic system which activates during the second exploration of the arena may act as an inhibitory brake on the cholinergic system during habituation.

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175P RECOMBINANT EXPRESSION OF $GABA_B$ RECEPTORS IN NG108-15 CELLS USING POLYETHYLENIMINE (PEI)

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The metabotropic $GABA_B$ receptor is linked to a variety of effector mechanisms. Previously, we have demonstrated that recombinantly expressed $GABA_B$ receptor subunits, $GABA_{B1}$ and $GABA_{B2}$, functionally couple to voltage-dependent calcium channels (VDCCs) in NG108-15 cells (Easter & Spruce, 2000). However, less than 50% of the cells transfected by electroporation responded to the specific $GABA_B$ agonist, baclofen. Here, we report a much higher transfection efficiency using polyethylenimine (PEI), enabling better assessment of the functional role of individual $GABA_B$ subunits.

NG108-15 cells were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 5% foetal calf serum (FCS). On day 0, undifferentiated cells were plated in 35 mm petri dishes. Cells were transfected with 0.8 mls of a mixture of plasmid cDNA (5 μg), PEI (9 μg / μl RNase free water, pH 7.0), and serum-free medium (Boussif et al., 1995). Various combinations of plasmid cDNAs encoding $GABA_{B1a}$ (a $GABA_{B1}$ splice variant), $GABA_{B2}$ and GFP (a fluorescent marker for transfected cells) were used. The cells were incubated in the transfection medium for 2-4 hrs, before replacement with differentiation medium (DMEM, 1% FCS, 50 μM IBMX, 10 μM PGE_1). Whole-cell calcium current was recorded from fluorescent cells on days 3-7 (holding potential −90 mV). During repeated voltage steps to +20 mV every 30 s, the effect of 50 μM (-) baclofen on peak I_{Ca} was measured over 2 min and normalised to baseline recordings. Responding cells were defined as those where current was reduced by >4 %. All data are reported as mean ± SEM. A Students t-test was used to compare the percentage change in I_{Ca} during baclofen application between $GABA_B$ and control (GFP) transfections.

The properties of I_{Ca} were not altered by transfection: peak I_{Ca}, 1.0 ± 0.3 nA (n = 4; $GABA_{B1a/B2}$) and 1.0 ± 0.2 nA (n = 7; untransfected). For double $GABA_B$ receptor subunit transfections, baclofen inhibited I_{Ca} in 30/31 cells transfected with $GABA_{B1a/B2}$ but only 2/12 cells responded from the control (GFP) transfection group. The reduction in normalised peak current was significantly greater in $GABA_B$-transfected cells (31 ± 3 %; n = 31; $p < 0.001$) compared to control (GFP) transfections (7 ± 3 %; n = 12). For single subunit transfections, using $GABA_{B2}$, 6/10 cells responded and I_{Ca} was reduced overall by 12 ± 4 % (n = 10; $p < 0.05$), compared to the control group where no cell responded (1 ± 2 %; n = 13). In contrast, using $GABA_{B1a}$, baclofen had no effect in 9 cells.

The results demonstrate that PEI can be used to successfully transfect NG108-15 cells with $GABA_B$ plasmid cDNA. Robust coupling to VDCCs is seen in most cells using both $GABA_{B1a}$ and $GABA_{B2}$ and in the majority using $GABA_{B2}$. Since the functional receptor is assumed to be a heterodimer, perhaps $GABA_{B2}$ can associate with endogenously expressed $GABA_{B1}$.

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176P CALCIUM CHANNEL SUBTYPES MODULATED BY RECOMBINANTLY EXPRESSED $GABA_B$ RECEPTORS IN NG108-15 CELLS

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Presynaptic $GABA_B$ receptors modulate transmitter release via inhibition of voltage-dependent calcium channels (VDCC). Neuronal studies have revealed that $GABA_B$ receptors couple, to varying degrees, to multiple VDCC subtypes (Kerr & Ong, 1995). Recombinant expression of the cloned $GABA_B$ subunits, $GABA_{B1}$ and $GABA_{B2}$, in a cell-line expressing multiple VDCC subtypes has provided preliminary evidence for the involvement of different channel subtypes (Easter & Spruce, 2000). We pursued this line of investigation using selective VDCC antagonists.

NG108-15 cells were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 5% foetal calf serum (FCS). On day 0, undifferentiated cells were plated and transfected with plasmid cDNAs encoding $GABA_{B1a}$, $GABA_{B2}$ and GFP using polyethylenimine (PEI) solution (9 μg PEI/ μl in RNase free water, pH 7.0). Cells were incubated in transfection medium for 2-4 hrs, before replacement with differentiation medium (DMEM, 1% FCS, 50 μM IBMX, 10 μM PGE_1). Whole-cell calcium current was recorded from fluorescent cells on days 3-7 (holding potential −70 mV). The effect of 50 μM (-) baclofen on peak I_{Ca} was measured from voltage steps to +20 mV elicited every 30 s. Current was normalised to that prior to drug application, to allow comparisons of percentage inhibition for different conditions (using student's t-test). Drugs were perfused on to the cell via a pipette fed by different solution reservoirs. The voltage dependence of inhibition by baclofen was studied using a prepulse protocol. All data are reported as mean ± SEM.

In cells transfected with $GABA_{B1a}$ and $GABA_{B2}$, baclofen inhibited I_{Ca} by 31 ± 3 % (n = 31). We investigated the effect of VDCC antagonists on baclofen's action. Nifedipine (10 μM; dissolved in DMSO) inhibited I_{Ca} by 29 ± 5 % (n = 4). Upon subsequent application of baclofen, the inhibition of I_{Ca} was increased to 55 ± 5 % (n = 4). Since the inhibition attributed to baclofen was less than that in the absence of nifedipine, this suggests that L-type channels may also be modulated. Indeed, ω-CTX-GVIA (1 μM) inhibited I_{Ca} by 32 ± 3 % (n = 4) and, in the presence of baclofen, the inhibition increased by 7 ± 2 % (n = 4). Further, the combination of nifedipine and ω-CTX-GVIA totally occluded the effect of baclofen (n = 3). The voltage dependence of baclofen's action was also investigated. In the presence of nifedipine, the normalised peak I_{Ca} was inhibited by 42 ± 4 %, but, following a depolarising prepulse to +120 mV, this was reduced to 20 ± 6 % (n = 4; $p < 0.05$, paired t-test).

The results indicate that recombinantly expressed $GABA_B$ receptors formed from $GABA_{B1a}$ and $GABA_{B2}$ subunits couple to both N- and L-type VDCCs. As demonstrated for native receptors, the coupling to N-type channels is strongly voltage-dependent suggesting that it is due to βγ subunits binding directly to the channels.

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177P DIFFERENTIAL EFFECT OF $GABA_B$ RECEPTOR ANTAGONISTS ON ELECTRICALLY-EVOKED RELEASE OF [^{3}H]-GABA IN NIGRAL AND CEREBROCORTICAL SLICES OF RAT BRAIN

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We have previously shown that $GABA_B$ receptor activation by (-) baclofen suppresses the electrically evoked release of accumulated [^{3}H]-GABA from slices of rat substantia nigra (SN), as well as cerebral cortex (CC), in a concentration dependent manner (Meza-Toledo and Bowery 2000). However, whilst we were able to demonstrate the presence of autoreceptors in CC slices we failed to obtain any evidence for their existence in SN slices even though (-) baclofen inhibited [^{3}H]-GABA release. The present study describes the comparative effects of three recognised $GABA_B$ receptor antagonists, CGP35348, CGP56999 and CGP55845 (Froestl and Mickel 1997) on the evoked release in these slices and indicates that whilst they all act as antagonists in CC slices, two of them appear to act like $GABA_B$ receptor agonists in the SN slices.

SN and CC slices (250μM x 250μM) were prepared from male Wistar rats and incubated in [^{3}H]-GABA (45nM for 20 min at 35°C). The slices were then rinsed in an artificial CSF solution containing tiagabine (10μM), aminooxyacetic acid (50μM) and β-alanine (100μM), to prevent uptake and metabolism of GABA, and 150μl aliquots of slice suspension were superfused (0.4 ml.min^{-1}, 35°C, Brandel 2000) for 35 min before collection of consecutive 4 min samples. The slices were stimulated (40mA, 1Hz for 4 min) 48 min (S1) and again 88 min (S2) after the start of superfusion. $GABA_B$ receptor ligands were added to the superfusion solution 30 min before S2. Results were then expressed as S2:S1 ratios where the values of S1 and S2 represented amounts released above basal.

The S2:S1 ratios obtained in control CC and SN slices were 0.84± 0.11 (n=19) and 1.06±0.02 (n=10) respectively. The addition of (-) baclofen (10μM) prior to S2 produced corresponding S2:S1 ratios of 0.62±0.14 (n=9) and 0.61 ± 0.05 (n=13). In CC slices the addition of CGP35348 (10 and 100μM) before S2 increased the S2:S1 ratio to 1.1±0.03 and 1.86±0.08 respectively. Similarly CGP55845 (10μM) and CGP56999 (1μM) increased the ratio to 4.9±1.7 and 3.3 ±0.4 respectively. Each of the antagonists reversed the inhibitory action of (-)baclofen (10μM). The enhancement produced by the antagonists would support the concept of presynaptic autoreceptor modulation in CC slices. By comparison, in SN slices the addition of $GABA_B$ antagonists alone failed to produce any increase in the S2:S1 ratio. In the presence of CGP55845 (10μM) the ratio (0.83 ± 0.17 n=6) was not significantly different from control. By contrast, both CGP56999 (10μM) and CGP35348 (100μM) decreased the evoked release to produce S2:S1 ratios of 0.26±0.07 (n=3) and 0.37 ±0.12 (n=3) respectively. CGP55845 (10μM) reversed the agonist action of (-)baclofen whereas CGP56999 and CGP35348 did not. Preliminary studies indicate that the apparent agonist effects of CGP56999 and CGP35348 could also be blocked by CGP55845 in the SN.

In conclusion, these data support the existence of $GABA_B$ autoreceptors in rat CC and also support the absence of such receptors in rat SN. However, we have also observed that two recognized $GABA_B$ antagonists may be acting as partial agonists in the SN slices whereas they act as pure antagonists in the CC slices.

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178P EXPRESSION OF $GABA_{B(1a)}$, $GABA_{B(1b)}$ AND $GABA_{B2}$ SUBUNIT PROTEINS IN THE CORTEX AND THALAMUS OF RAT

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$GABA_B$ receptors are metabotropic G-protein-coupled receptors associated with slow synaptic transmission. Characterisation of the structure of these receptors has revealed that they exist as heterodimers (Marshall et al. 1999). $GABA_B$ receptors have been implicated in a variety of neuropathological conditions including absence seizures, spasticity and depression.

In the present study we have focused on the somato-sensory system. Using a conventional immunocytochemical (ICC) technique with antibodies developed to specifically recognise $GABA_{B(1a)}$, $GABA_{B(1b)}$ and $GABA_{B2}$ subunits in rat brain, we have compared their distribution patterns in cortex and thalamus. $GABA_{B(1a)}$, $GABA_{B(1b)}$ and $GABA_{B2}$ were detected throughout the cortex and thalamus of rats. $GABA_{B(1a)}$ isoform was randomly distributed in the cortical layers localised mainly in the neuronal somata but also in the neuropil with lower immunoreactivity. $GABA_{B2}$ immunostaining was present mostly in the cell bodies and fainter in the neuropil of the cortex where afferent thalamic GABAergic axons project to the pyramidal cortex. $GABA_{B(1b)}$ immunoreactivity was evident predominantly in the apical dendrites and some pyramidal cell bodies of cortical neurones. The expression patterns of $GABA_{B(1a)}$, $GABA_{B(1b)}$ and $GABA_{B2}$ were also similar throughout the thalamus: $GABA_{B(1b)}$ and $GABA_{B2}$ showed a cognate pattern in the ventrobasal nucleus, by contrast the $GABA_{B(1a)}$ and $GABA_{B2}$ immunosignals were matching in the reticular nucleus. These data suggest that in the thalamo-cortical pathway the $GABA_{B(1b)}$ subunit might be the presynaptic isoform modulating neurotransmitter release.

The fact that $GABA_{B(1b)}$ was present not only in the neuropil, but also in cell bodies may represent the subunit in the presynaptic boutons of synapses (Porkalkali et al.2000). Whereas $GABA_{B(1a)}$ would appear the postsynaptic splice variant present on cell bodies in the cortex and reticular thalamic nucleus. This differs from previous observations in the brain regions so that whilst in the cortex, ventrobasal and reticular thalamic nuclei $GABA_{B(1a)}$ and $GABA_{B(1b)}$ represent respectively the post- and presynaptic forms, in other brain areas such as cerebellum the situation appears be the opposite (Billinton et al.1999; Bishoff et al. 1999).

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179P COMPARATIVE BINDING OF [^{3}H]-CGP62349 AND [^{3}H]-GABA IN THE SPINAL LAMINA II OF CHRONICALLY GABA$_B$ AGONIST TREATED NEUROPATHIC RATS

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Systemic administration of the GABA$_B$ receptor agonist baclofen, produces an antinociceptive response in neuropathic rats compared to naïve and sham animals (Smith et al, 1994). This effect is mediated, at least in part, by an action within the spinal cord where GABA$_B$ receptor activation suppresses the release of transmitters from primary afferent fibres (Malcangio and Bowery,1996). In an attempt to determine whether the increase in sensitivity to baclofen was due to a change in the number of receptors we have examined the binding profile of [^{3}H]-CGP62349, a high affinity GABA$_B$ receptor antagonist (Bittiger et al,1996), as well as that of [^{3}H]-GABA in 12μm sections of L4/L5 spinal cord obtained from naïve, sham operated and neuropathic rats (1-4 weeks after surgery). The data obtained indicated that while there was no change in the binding parameters of [^{3}H]-CGP62349 in all groups, the affinity but not the B_{max} of [^{3}H]-GABA was increased bilaterally up to 6 fold in sham operated and neuropathic animals. There was no difference between sham-operated and neuropathic groups.

We have now extended our study to examine the binding profiles of both radioligands in the L4/L5 spinal cord sections of neuropathic rats chronically treated once a day with (±)baclofen(5mg.kg^{-1}) or CGP44532(0.3mg.kg^{-1}), a novel GABA$_B$ receptor agonist (Froestl and Mickle, 1997) for 7,14 and 21 days. (±)Baclofen and CGP44532 completely reversed the mechanical hyperalgesia in the hind paw pressure test up to 14 days but their analgesic activity was lost thereafter. The contralateral hind paw threshold was of similar value to that of naïve animals. Binding experiments were performed according to Castro-Lopes et al. (1995). Fresh frozen L4/L5 spinal cords were sectioned at 12μm and mounted onto slides. Sections were thawed and preincubated in Tris HCl buffer (50mM pH 7.4) plus $CaCl_2$(2.5mM) for 90 min and air-dried. Binding solutions, containing [^{3}H]-GABA (25-200nM) or [^{3}H]-CGP62349 (0.03-10nM), were applied over the sections and left for 20 and 60 min at 21°C, respectively. Non-specific binding was determined by including (-) baclofen(100μM) and CGP54626A(10μM) in the solutions containing [^{3}H]-GABA and [^{3}H]-CGP62349, respectively. Sections were washed ([^{3}H]-GABA=2x3s; [^{3}H]-CGP62349=2x1min), dried, apposed to dry K5-photographic emulsion dipped coverslips and exposed for 14 days. After developing and staining silver grains were counted using an MCID-M4 imager. Results were analysed using ANOVA($p<0.01$,n=3).

No change was observed in the binding capacities(B_{max} values) of either [^{3}H]-GABA or [^{3}H]-CGP62349 in lamina II of L4/L5 spinal cord sections from chronically GABA$_B$ receptor agonist treated neuropathic rats compared to the saline treated group. However, the affinity of [^{3}H]-GABA, but not [^{3}H]-CGP62349, was increased bilaterally by up to 5.7 fold in all groups of neuropathic rats when compared to naïve animals.

We conclude that neither the changes in sensitivity to GABA$_B$ receptor agonists in neuropathic animals nor the decrease in the antinociceptive response to GABA$_B$ agonists following their chronic administration is reflected in the binding profiles of the GABA$_B$ receptor ligands in the L4/L5 spinal cord sections.

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180P CIRCADIAN ACTIVITY IN MICE DURING CHRONIC ORAL NICOTINE ADMINISTRATION

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Nicotine is critical in the maintenance of tobacco use. Similarly to other drugs of abuse nicotine elicits stimulatory effects on mesolimbic dopamine transmission. This effect is associated with behavioural correlates including locomotor stimulation. We have earlier shown that nicotine can be administered in the drinking water to mice in doses resulting in plasma nicotine concentrations similar to those reported in smokers (Pekonen *et al.*, 1993). Further, we found that the locomotor activity and the striatal dopamine (DA) metabolism were increased in the forenoon in mice still drinking nicotine. Also, the circadian rhythm in striatal DA was affected (Pietilä *et al.*, 1995; Gäddnäs *et al.*, 2000). Most experiments with nicotine have been carried out during the light phase of the day. In the present experiment we wanted to study the effects of chronic oral nicotine administration on activity during the active (dark) phase and inactive (light) phase and determine if chronic nicotine treatment alters the circadian rhythm of activity in mice. Also, we measured circadian brain concentrations of nicotine during chronic administration.

Male NMRI mice (body weight 20-25 g) were kept in a 12:12-h light/dark cycle (lights on from 06:00 to 18:00) and were given nicotine in their drinking water as the sole source of fluid. The concentration of nicotine was gradually increased from 50 to 500 μg ml^{-1} as described earlier (Pekonen *et al.*, 1993). Brain concentrations of nicotine were estimated by GC-MS at 05:00, 11:00, 15:00 and 21:00 on the 50th day of nicotine administration. The gross motor activity was measured by using infrared sensors in groups of three mice continuously over 24 h on the 50th day of nicotine administration. Activity data were calculated over 1.5-h intervals and analyzed by two-way ANOVA followed by Student's t-test for multiple comparisons.

The brain concentration of nicotine was at its highest (329 ± 53 ng g^{-1}, mean±s.e.mean, n=11) at the end of the dark period at 05:00 and at its lowest (243 ± 35 ng g^{-1}, n=12) in the light period at 15:00. On the 50th day of nicotine administration the mice were more active than control mice drinking tap water. Significant differences between nicotine-treated and control mice were found in four consecutive 1.5-h intervals between 07:30 and 13:30 (P<0.05) and between midnight and 03:00 (P<0.01). Total activity scores were in the light period 24358 ± 2758 (mean±s.e.mean, n=12) and 40036 ± 3624 (n=13; P<0.01) and in the dark period 33896 ± 2470 (n=14) and 42641 ± 2535 (n=14; P<0.05), in control and nicotine-treated mice respectively. However, the circadian rhythmicity of activity was not altered by chronic nicotine.

In summary, we found that the activity of mice was increased during chronic oral nicotine administration. The effects of nicotine varied depending on the baseline activity of mice and tended to be most prominent during the light period.

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181P THE EFFECT OF THE IMIDAZOLINE$_2$ (I_2) SITE SELECTIVE LIGAND BU224 IN RAT EXPOSED TO A FORCED SWIM TEST PARADIGM

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Ligands selective for the imidazoline$_2$ binding site (I_2BS) elevate extracellular levels of monoamines in the rat brain (Hudson *et al.*, 1999). The selective I_2BS ligand 2-(2-benzofuranyl)-2-imidazoline (2-BFI) has previously been shown to reduce immobility of rats in the Porsolt forced swim test (FST) (Nutt *et al.*, 1995). Taken together, these findings suggest that I_2BS selective ligands may have potential as antidepressant drugs. The present study has investigated the effect of another highly selective I_2 site ligand 2-(4,5-dihydroimidazol-2-yl)quinoline (BU224) on the behaviour of rats in a FST paradigm.

Male Sprague Dawley rats (300-350 g) were injected i.p. with saline or BU224 (10 mg kg^{-1}) 24, 18 and 1 hr prior to a single 15 min exposure to the FST. The length of time the rats spent immobile, struggling and swimming calmly during the first 5 min of the test was measured. Defecation was also measured by counting the number of faecal boli excreted during the 15 min FST exposure.

Administration of BU224 to rats significantly reduced immobility in the FST by 28% compared with saline treated animals exposed to the FST (Figure 1). There was a significant elevation (33%) in mild swimming behaviour of BU224 treated rats compared with saline treated controls (Figure 1). The length of time spent struggling did not differ significantly between the BU224 treated rats (47 ± 6 secs) and the saline treated controls (39 ± 3 secs). Defecation during FST exposure was not significantly affected following BU224 treatment (4.6 ± 0.1 faecal boli/15 min) compared with saline treated controls (4.5 ± 0.8 faecal boli/15 min).

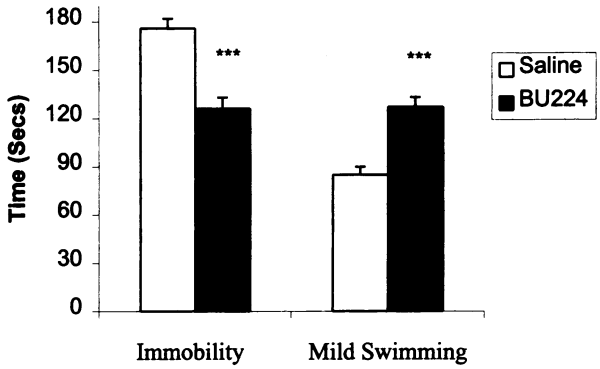

Figure 1. Effect of BU224 (10 mg kg^{-1}) on immobility and mild swimming behaviour of rats in the FST. Values as mean ± s.e.mean (n = 8). $^{***}P < 0.001$ *vs* Saline controls; Student's unpaired two-tailed t-test.

These data are further evidence of a role for I_2BS in modulating the behaviour of rats in the FST. The reduced immobility and increased time spent mild swimming following treatment of rats with BU224, indicates potential antidepressant activity for this compound.

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182P INDOMETHCIN ATTENUATES THE INHIBITORY EFFECTS OF CHOLECYSTOKININ ON FOOD INTAKE IN RATS

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We have previously demonstrated that i.p. administration of cholecystokinin (CCK) increases plasma levels of prostaglandin (PG) E2 in rats and that pretreatment with the COX inhibitor, ibuprofen, (which prevents the de novo synthesis of PGs) attenuates the hypophagia induced by CCK (Ebenezer & Romans, 2000). We have suggested that the suppressant effect of exogenous peripheral CCK on food intake in rats is, at least partially, mediated by a PG mechanism. This suggestion is further supported by the finding that the PG E2/E3 agonist, mistoprostal, decreases food intake in fasted rats in a dose-related manner (Romans & Ebenezer, 1999). The present study was undertaken to extend these observations.

Experiment 1. Male Wistar rats (n=8; b. wt. 300 - 380g) that were fasted for 22h a day, were injected i.p. with either vehicle (veh; 2%$^{w}/_{v}$ sodium carbonate) or indomethacin (indo; 2, 5 or 10 mg kg^{-1}) and presented with food, as described previously (Ebenezer, 1990). Food intake was measured at intervals over 120 min. A repeated measures design was used with each rat receiving all doses of veh and indo in a random fashion; 3 days separated successive trials.

Experiment 2. Male Wistar rats (n=38; b. wt. 300 - 380g) were divided into 4 equal groups. The animals were fasted for 22h a day. The rats received the following treatments: (a) Group 1 – veh followed by saline (sal), (b) Group 2 - veh followed by CCK (5 µg kg^{-1}), (c) Group 3 – indo (10 mg kg^{-1}) followed by sal, (d) Group 4 – indo (10 mg kg^{-1}) followed by CCK (5 µg kg^{-1}) Both injections were given i.p.; 15 min separated the injections. Immediately after the second injection, the rats were presented with food in experimental cages and intake measured.

The results from Experiment 1 show that indo (2 - 10 mg kg^{-1}) did not have any significant effects on food intake in fasted rats over the 120 min measurement period. For example, Mean ± s.e.mean cumulative food intake at 120 min: veh 8.9 ± 1.1 g; indo (2 mg kg^{-1}) 8.9 ± 1.3 g; indo (5 mg kg^{-1}) 6.7 ± 2.1 g; indo (10 mg kg^{-1}) 9.0 ± 1.4 g. The results of Experiment 2 is shown in Fig. 1. CCK (5 µg kg^{-1}) produced a significant reduction in food intake during the first 15 min after administration ($P<0.01$). Indo (10 mg kg^{-1}) significantly ($P<0.01$) attenuated the inhibitory effect of CCK on feeding.

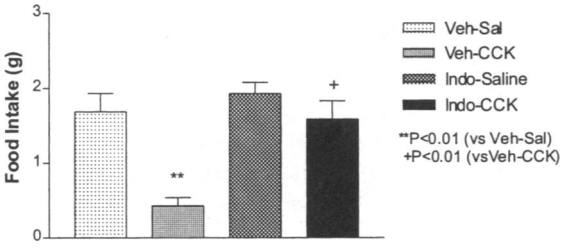

Fig.1. Effects of Indo on CCK-induced hypophagia.

The results of this study show that the COX inhibitor, indo, has no effects on food intake in fasted rats when administered on its own. However, it significantly attenuates the suppressant effect of exogenous peripheral CCK on food intake. These results extend previous observations with ibuprofen (Romans & Ebenezer, 2000) and add further support to the suggestion that the inhibitory actions of exogenous peripheral CCK on food intake in rats is, at least partially, mediated by a PG mechanism.

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183P EFFECTS OF MECAMYLAMINE AND RACLOPRIDE PRE-TREATMENT ON A RAT MODEL OF NICOTINE-SEEKING BEHAVIOUR

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Recently, pre-clinical studies have shown that re-exposure to nicotine is an important determinant of relapse. Aim of the present study is to investigate the role of treatment with the nicotinic receptor antagonist mecamylamine and with the dopamine D_2 subtype-selective receptor antagonist raclopride on a rat model of nicotine-seeking behaviour (Chiamulera C. *et al.*, 1996; Shaham, Y *et al.*, 1997).

Male Wistar rats (240-260g) were first trained to lever press for food as a reinforcer in operant chambers. Then, they underwent surgery for catheter implantation in the jugular vein under anaesthesia (mixture of chlordiazepoxide 9 mg kg^{-1} with ketamine 80 mg kg^{-1}, after pre-treatment with atropine 0.05 mg kg^{-1}). After a week of recovery, rats were trained to lever press for nicotine 0.03 mg kg^{-1} per infusion.

Rats that acquired intravenous (i.v.) nicotine self-administration (Fixed Ratio 2:nicotine 0.03 mg kg^{-1} per infusion, 1-h session per day) were able to maintain a stable responding for weeks (number of active lever presses did not vary more than 20% between three consecutive sessions). When exposed to saline infusions, rats decreased responding for nicotine-paired lever at low and stable values (4.07±1.52 means of active lever presses ± s.e.mean during last 1-h extinction session). Re-exposure to a single non-contingent priming dose of nicotine 0.015 mg kg^{-1} per infusion, delivered at the beginning of the relapse test session, induced a significant reinstatement of responding on nicotine-paired lever (16.10±3.70 means of active lever presses per 1-h relapse test session ± s.e.mean; $P<0.01$ vs last 1-h extinction session value, Student's t-test for paired data, $n=10$). In order to normalise responding to individual variability data were also expressed as Index of Relapse (I.o.R.) that is $\log_{10}$[active lever presses during the relapse test session/ active lever presses during the last extinction session]: 0.74±0.17 means of I.o.R ± s.e.mean.

In another experiment, mecamylamine 1, 2 mg kg^{-1} or saline (1 ml kg^{-1}) were given subcutaneously (s.c.) 30 min prior the relapse test session. Responding did not significantly differ among treatments (respectively 0.58±1.53, 0.52±1.37 and 0.35±0.07 means of I.o.R ± s.e.mean, ANOVA NS, n=4-5 per group of treatment). Dopamine D_2 antagonist raclopride, administered s.c. 30 min prior the test session, induced a significant reduction of reinstatement of responding at 0.1, 0.2, 0.4 mg kg^{-1} or saline (-0.34±0.09, -0.58±0.23 and -0.43±0.21 means of I.o.R ± s.e.mean respectively vs saline value of 0.50±0.07, Dunnett's test, $P<0.01$, n=4-6 per group of treatment).

These data show that antagonism of dopamine D_2 receptors, but not that of central nicotinic receptors, prevent nicotine-induced reinstatement of responding for nicotine-seeking behaviour in rats, suggesting a specific involvement of dopamine receptor modulation on relapse to smoking.

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184P A STUDY OF THE DIFFERENCES IN THE BEHAVIOUR OF DARK AGOUTI AND SPRAGUE-DAWLEY RATS IN THE ELEVATED PLUS-MAZE.

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The elevated plus-maze has been used extensively to examine putative anxiolytic agents (see Rodgers & Dalvi, 1997). In the course of studies using this behavioural paradigm we observed marked differences in the responses of untreated Dark Agouti (DA) and Sprague-Dawley (SD) rats. This communication reports on some of these observed differences and their interaction with the anxiolytic effect of diazepam.

Male DA (n = 8) and SD (n = 8) rats (220–360 g) were used. The plus-maze was constructed from black plastic, with 2 closed arms of height 10 cm, positioned 50 cm above the floor. The maze was illuminated by a single white 60 W light bulb and was cleaned with a weak cider vinegar solution (10 %) after each rat was tested. In randomised order, rats were placed in the central square facing an open arm, observed for 10 min and video-recorded for future analysis. Arm entries were counted as 4 paw entries only. Diazepam (1 mg kg^{-1} i.p.) or saline was administered to further groups of rats (n = 8, in each group) and behavioural observations were again made. Statistics were performed using GraphPad Prism (San Diego, USA). An unpaired t-test was used in the first study on open arm behaviour and 2-way analysis of variance used in the second study, where there were 2 variables. Ethological observations were examined by the Mann-Whitney U test and values are quoted as mean ± s.e. mean.

Overall activity levels, as indicated by the total number of arm entries, were lower but not statistically significantly different in DA compared to SD rats (SD: 18.8 ± 3.9; DA: 9.4 ± 1.4, p = 0.1). The % number of open compared to total arm entries did not differ significantly. However, the amount of time spent in the open arms as a % of total time spent in both open and closed arms was significantly lower in DA rats, compared to SD rats (SD: 17.7 ± 4.7; DA: 6.6 ± 1.8, p = 0.04). This suggested a higher 'anxiety' state in the DA rats, which was further exemplified by a number of ethological observations. The DA rats performed fewer head dips (SD: 17.1 ± 2.9; DA: 3.9 ± 0.8, p = 0.005) showed increased grooming behaviour (SD: 1.1 ± 0.5; DA: 3 ± 0.5, p = 0.03) and had an increased defecation bolus number (SD 0 ± 0; DA: 5.8 ± 1.1).

When we examined the effect of diazepam (1 mg kg^{-1}) on the behaviour of the 2 strains in the maze we found that handling plus injection altered the profile of plus-maze activity of the DA rats. As observed in the naïve animals, total numbers of arm entries were lower in the DA, compared to the SD, saline-treated groups. However, % time spent in the open arms did not differ between the two saline-treated groups. Diazepam administration had a similar anxiolytic effect in both strains, increasing the % time spent in the open arms by approximately 100 % in both groups, as compared to their respective control values (effect of treatment: F (1, 28) = 6.68, p < 0.05).

We conclude that, compared to SD rats, the DA strain shows a higher level of anxiety-like behaviour in the elevated plus-maze. However, acute handling appears to attenuate the anxiety, as has previously been shown in hooded rats following repeated handling (Brett & Pratt, 1990; File et al., 1992). Diazepam produced an anxiolytic response of similar magnitude in both strains, in contrast to the previous observations in chronically handled rats where the anxiolytic effect of benzodiazepines was abolished (Brett & Pratt, 1990; File et al., 1992).

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185P EFFECTS OF MILD UNCONDITIONED FOOTSHOCK ON *cfos* EXPRESSION IN THE PERIAQUEDUCTAL GREY OF RAT

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The midbrain periaqueductal grey (PAG) is involved in the integration of mammalian response to threatening stimuli. There are rostrocaudal and dorsoventral functional variations within the PAG (Carrive, 1993). Mild unconditioned footshock has been shown to increase *cfos* expression in the amygdala (Jeffrey *et al.*, 1998), whereas a higher intensity, repeated footshock is reported to enhance it in the amygdala (Pezzone *et al.*, 1992) as well as PAG (Pezzone *et al.*, 1993). The purpose of this experiment was to examine the effects of acute mild unconditioned electric footshock in the PAG using *cfos* expression as a marker of neuronal activation.

One group (No FS) of male Lister hooded rats (175-250g) was exposed to the footshock chamber for 10.5min but did not receive footshocks. The other group (V+FS) was administered (i.p.) vehicle (ethanol: Tween80: saline; 1:1:3), 20min prior to 10 electric footshocks (0.4mA for 1s, intershock interval = 1 min). Ninety minutes after exposure to the footshock chamber, all the rats were transcardially perfused with paraformalde-hyde (4%) under sodium pentobarbitone (60mg/kg, i.p.) anaesthesia. The cells expressing *fos* protein in 100µm coronal brain sections from the PAG were identified by immunoperoxidase staining using avidin-biotin, and diaminobenzidine as chromophore. The density of cells expressing fos like immunoreactivity (FLI) in the PAG was determined by manually counting brown-black stained nuclei using a micro-scope. The data were square root normalised and analysed using one-way ANOVA.

No significant change in FLI induced by footshock was observed when individual sections of the PAG were compared (Figure 1), although there was a trend towards an increased expression in the dorsal and lateral regions. However, when the data for each rostrocaudal region were pooled, there was a significant increase in the lateral PAG.

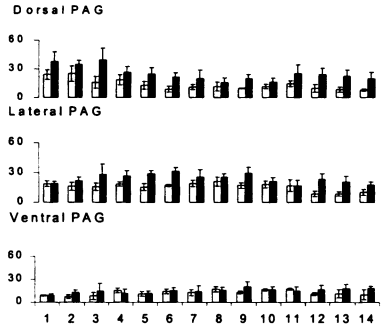

Figure 1

Y-axis represents FLI per 0.185mm^2. X-axis represents coronal sections from anterior to posterior PAG. Each bar represents mean ± SEM (n=4). Open bars = No FS, closed bars = V+FS. * $p<0.05$ one way ANOVA.

These data indicate that acute mild unconditioned footshock engages the PAG in the same manner as a more intense repetitive stimulus (Pezzone *et al.*, 1993).

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186P EFFECTS OF INTRACEREBROVENTRICULAR (icv) ADMINISTRATION OF WAY 100635 ON THE EFFECTS OF 8-OH-DPAT ON OPERANT FOOD INTAKE IN SATIATED AND FASTED PIGS

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It has previously been demonstrated that while the $5HT_{1A}$ agonist 8-hydroxy-2-(di-N-propylamino)-tetralin (8-OH-DPAT) increases food intake in non-deprived rats by an action at $5HT_{1A}$ autoreceptors (Fletcher et al., 1995), it decreases feeding in food-deprived rats (Ebenezer, 1992). However, it is not known whether 8-OH-DPAT suppresses food intake in fasted rats by an action at $5HT_{1A}$ receptors. Recently, and in general agreement with the results obtained in rats, we have shown that i.v. administration of 8-OH-DPAT, in the dose range 5 - 50 µg kg^{-1}, increases operant feeding in satiated pigs but decreases food intake in fasted animals (Ebenezer et al., 1999). The present study was undertaken to determine whether the hyperphagic and hypophagic effects of 8-OH-DPAT in satiated and fasted pigs, respectively, are mediated by central $5HT_{1A}$ receptors.

Prepubertal Large White pigs (male, b.wt. 35 - 40 kg) were housed in metabolism cages and trained to press operant panels for food and water reinforcements, as described previously (Ebenezer et al., 1999); a single food reinforcement weighed approximately 20g. The pigs were surgically prepared with a catheter in the jugular vein and a cannula in the lateral cerebral ventricle (Ebenezer et al., 1996). At 10.30h a buzzer sounded which signalled that the feeder was activated for 120 min (Expt. 1) or 90 min (Expt. 2). Expt. 1: Pigs (n=6) were injected icv at 11.45h with either saline (sal) or the selective $5HT_{1A}$ antagonist N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]-N-2-pyridinyl-cyclohexane carboxamide maleate (WAY 100635; 0.3 mg), and i.v. at 12 .00h with either sal or 8-OH-DPAT (25 µg kg^{-1}). Expt. 2: Pigs (n=7) were injected icv at 10.00h with either sal or WAY 100635 (0.3 mg) and i.v. at 10.15h with either sal or 8-OH-DPAT (25 µg kg^{-1}). In both experiments the pigs received all treatments in a repeated measures design and operant food intake was recorded on a data logger.

In Expt. 1, the pigs normally ate to satiety during the first 60 min of the feeding period. 8-OH-DPAT significantly increased operant food intake (mean±s.e.mean) from 40.0±22.0g (sal-sal) to 216.0±46.0g ($P<0.01$) during the 30 min period following the second injection. The hyperphagia produced by 8-OH-DPAT was completely abolished by icv pretreatment with WAY 100635 (Mean food intake±s.e.mean: 23.4±13.0g; $P<0.01$). ICV administration of WAY 100635 on its own had no effects on feeding (Mean food intake±s.e.mean: 13.4±10g). By contrast, in Expt. 2, 8-OH-DPAT significantly reduced food intake compared with controls during the first 30 min of the feeding period (Mean food intake ± s.e.mean: Sal-Sal. 1002.8±143.0g; Sal-8-OH-DPAT. 597.2±22.0g; $P<0.01$). The hypophagic effect of 8-OH-DPAT was completely abolished by icv pretreatment with WAY 100635 (Mean food intake±s.e. mean: 997.2±22.0g; $P<0.01$). WAY 100635 on its own did not have any effects on operant food intake in the hungry pigs (Mean food intake±s.e. mean: 980.0±100.3 g).

The results of the present study extend previous observations with 8-OH-DPAT in the pig (Ebenezer et al., 1999) and show that both the stimulant and depressant effects of the drug on feeding in satiated and hungry animals respectively, are mediated by central $5HT_{1A}$ receptors. While there is good evidence that 8-OH-DPAT increases food intake in satiated animals by an action at $5HT_{1A}$ autoreceptors (see Fletcher et al., 1995), it remains to be determined whether the hypophagic effect in hungry animals is mediated by $5HT_{1A}$ auto- or post-synaptic receptors.

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187P EFFECTS OF THYROTROPIN-RELEASING HORMONE-DEGRADING ECTOENZYME INHIBITORS ON THYROTROPIN-RELEASING HORMONE ACTIONS *IN VIVO*.

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Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) (EC 3.4.19.6) inhibitors may be valuable tools for enhancing central, therapeutic effects of TRH and for investigating the functions of TRH in the CNS (Kelly, 1995). To investigate this possibility, we conducted a preliminary study to assess the ability of two recently identified, TRH-DE inhibitors, pyroglutamyl-asparaginyl-proline-amide (Glp-Asn-ProNH_2, K_i = 17.5 μM) and Glp-Asn-Pro-7-amido-4-methyl coumarin (Glp-Asn-ProAMC, K_i = 0.97 μM) (Kelly *et al.*, 2000), to enhance TRH central actions *in vivo*.

Male Wistar rats (200-250 g) were administered vehicle (0.9% saline), TRH (5μg in 1μl) ± TRH-DE inhibitor (5μg in 1μl) by intracerebroventricular (i.c.v.) injection or 10 mg/kg of TRH ± 10 mg/kg inhibitor by intraperitoneal (i.p.) injection. Individual behaviours, including wet dog shakes (WDS) (Bennett *et al.*, 1989), were recorded by an observer blind to treatment at 5 min intervals for up to 45 min. Behaviours were summed to yield an overall activity count following i.p. injection. The affinities of peptides for TRH receptors in rat cortical membranes were determined in competition binding experiments using 6-8 nM [^{3}H]-3-MeHis-TRH. Non-specific binding was defined in the presence of 10 μM TRH.

Co-administration of Glp-Asn-ProAMC with TRH resulted in: (i) an 18-fold increase ($p<0.001$) in TRH-induced WDS following i.c.v. injection (Figure 1) and (ii) an enhancement of WDS responses to TRH 15-40 min after i.p. injection (2.4±1.1 vs 6.6±1.2, n=5, $p<0.05$ Student's t-test). Both results are consistent with protection of exogenously-administered TRH from degradation. Glp-Asn-ProNH_2 (i.p.) increased activity counts 3-fold 35-40 min post-treatment versus saline ($p<0.05$, n=5, repeated measures ANOVA and Dunnett test), suggesting a possible increase of central endogenous TRH.

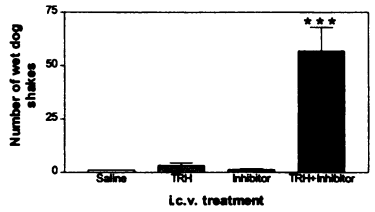

Figure 1. Effects of TRH±Glp-Asn-ProAMC on WDS. Data are expressed as mean ± S.E. of WDS/30 min (n=6). ***$p<0.001$ vs TRH, one-way ANOVA and Bonferroni test.

TRH competed for binding to [^{3}H]-3-MeHis-TRH-labelled TRH receptors in rat cortical membranes with a pK_i of 7.65 ± 0.17 (n=7). In comparison, the pK_i values for Glp-Asn-ProNH_2 and Glp-Asn-ProAMC were 5.15 ± 0.24 (n=6) and 4.32 ± 0.24 (n=3), respectively, supporting the interpretation that inhibition of TRH-DE underpins the observed behavioural effects. Data from this initial study provide the first demonstration that TRH-DE inhibitors can amplify central effects of TRH *in vivo* and thus may have investigative and therapeutic potential.

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188P CHARACTERISATION OF A 5-HT_{3B} RECEPTOR SUBUNIT ANTISERUM

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The 5-HT_3 receptor is a member of the cys-loop family of ligand-gated ion channels, and, in common with other members of this group, it consists of five subunits, each of which comprises of an N-terminal (extracellular) ligand-binding domain and a membrane-spanning domain. The 5-HT_3 receptor was for some years believed to form functional homopentamers *in vivo* as only one subunit was originally identified. Recently, however, a second subunit, the 5-HT_{3B} receptor subunit, has been cloned; unlike the A subunit this does not form functional receptors when expressed alone (Davies *et al.*, 1999). To examine the localization of this new subunit, we have developed a polyclonal antiserum and assessed its properties in recombinant homomeric (A or B only) and heteromeric (A+ B) receptors.

The antiserum was generated in rabbits as previously described (Spier *et al.*, 1999) against the peptide ^{27}PQDSALYHLSKQLLQKYH^{46}K which forms part of the N-terminal domain of the 5-HT_{3B} receptor subunit. To examine its specificity mouse 5-$HT_{3A(b)}$ and human 5-HT_{3B} receptor subunit DNAs were transfected into HEK293 cells using calcium phosphate precipitation. The cells were then cultured for 3 days and then either harvested for use in Western blots or fixed in 4% paraformaldehyde. The latter were used for labelling of extracellular protein moieties, where the antiserum was diluted in Tris buffered saline, whilst for labelling those that are intracellular 0.3% Triton-X-100 was added.

Expressed labelled 5-HT_3 receptors were visualized by indirect immunofluorescence using a biotinylated anti-rabbit secondary IgG and FITC fluorochrome as previously described (Spier *et al.* 1999).

Western blots revealed a single band at 53KDa for cells transfected with 5-HT_{3B} receptor subunit DNA, whereas there were no apparent bands for cells transfected with 5-HT_{3A} receptor subunit DNA or when using pre-immune serum (n=3). This is the expected molecular weight of the 5-HT_{3B} subunit. Labelling of HEK cells revealed a strong imunofluorescent signal in permeabilised cells transfected with 5-HT_{3B} receptor subunit DNA, but no signal in nonpermeabilised cells. Cells transfected with 5-HT_{3A} subunit DNA alone were not fluorescent, but in cells transfected with both 5-HT_{3A} and 5-HT_{3B} subunit DNA fluorescence was observed in both permeabilised and nonpermeabilised preparations. No fluorescence was observed when using pre-immune serum.

The results suggest that the new antiserum labels recombinant 5-HT_{3B} receptor subunits, and that the extracellular domain of these subunits can only reach the plasma membrane when in the presence of 5-HT_{3A} receptor subunits.

Supported by the Wellcome Trust.

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189P GENE GUN MEDIATED EXPRESSION OF YELLOW FLUORESCENT PROTEIN-LINKED 5-HT_{3A} RECEPTORS IN HEK293 CELLS AND BRAIN SLICES

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The 5-HT_3 receptor belongs to the family of ligand-gated channels that includes nicotinic acetylcholine, glycine and $GABA_A$ receptors; two 5-HT_3 receptor subunits, 5-HT_{3A} and 5-HT_{3B} have been cloned so far, the 5-HT_{3A} receptor subunit being unusual in that it can form functional homopentameric receptors. The properties of cloned neurotransmitter receptors such as these are usually examined in heterologous systems, such as *Xenopus* oocytes or non-neuronal cell lines, largely because of problems associated with transfecting DNA into neuronal tissue. In this study, however, we have used the Helios Gene Gun (Biorad) to insert DNA encoding yellow fluorescent protein (EYFP, a modified version of green fluorescent protein) linked to the 5-HT_{3A} receptor subunit DNA into HEK293 cells and organotypic slices of mammalian cerebellum in order to examine the efficacy of the Gene Gun as compared to a classical transfection technique, and to determine if it can be successfully used to transfect neurones.

Full length mouse 5-$HT_{3A(b)}$ receptor subunit DNA was inserted into the expression vector pEYFP-N1 (Clontech), and precipitated onto gold carriers (Ahn *et al.*,1999) The Gene Gun uses a low pressure helium pulse to sweep these microcarriers directly into HEK 293 cells grown on cover slips for 2-3 days or organotypic slices which had been grown on membrane supports for 1-2 weeks. Some HEK 293 cells were also transfected using calcium phosphate (Chen & Okayama,1988).

Examination of HEK293 cells after 3 days revealed successful transfection had been achieved both with calcium phosphate and the Gene Gun. Transfection efficiency for both procedures was variable, but examination of > 2000 cells in 3 different transfections suggested the transfection efficiency for both procedures was not significantly different (Student's t test): Gene Gun = 16.7 $\pm$ 3.6 %, calcium phosphate 13.3 $\pm$ 1.8% (mean $\pm$ SEM from 24 different images). The cells transfected with the Gene Gun in general had higher levels of fluorescence than those transfected with calcium phosphate, suggesting more rapid production of EYFP; in both groups of cells the receptors could be seen to cluster in the plasma membrane. Organotypic slices were observed after 24h, 3 days and 1 week and, although transfection efficiency was low (<0.05%), labelling of neuronal–like cells could be observed.

The results therefore suggest that the Helios Gene Gun provides a method of transfection that is similar in efficiency to calcium phosphate precipitation in HEK293 cells, and can be used to successfully transfect cells in brain slices; this method therefore provides a route to examine cloned ligand-gated ion channel receptor subunits in neuronal tissues.

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190P IMMUNOCYTOCHEMICAL LOCALIZATION OF GLUTAMATE RECEPTOR SUBTYPES IN THE LUMBAR REGION OF THE NEONATAL RAT SPINAL CORD

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Glutamate is the major excitatory neurotransmitter in the mammalian spinal cord. The actions of glutamate are mediated at different types of receptor termed ionotropic glutamate (iGlu) receptors and metabotropic glutamate (mGlu) receptor.
The neonatal rat spinal cord preparation has been used to characterise the actions of many novel compounds at mGlu receptors, as group I, II and III mGlu receptors are known to be present (for review of spinal cord pharmacology see Schoepp *et al*, 1999.) In order to elucidate the subtypes of mGlu and iGlu receptors present within this preparation, commercially available antibodies have been used for immunohisochemical localization.
Analysis was carried out on lumbar regions L3-L5 of spinal cords that were removed from male Wistar pups at postnatal day 1, 3 and 5 (n=8 per group). Spinal cords were then immersion fixed in 10% formalin and embedded into paraffin wax. Lumbar segments were then transversally sectioned (8 μm) using a sledge microtome and immunostaining protocol was carried out as described previously (Bond *et al.*, 2000). Briefly, sections were deparaffinised, rehydrated and endogenous peroxidase was quenched using H_2O_2. After incubation with pepsin and blocking with normal serum the primary antibody (obtained from Chemicon International, Inc.) was applied. Final antibody dilutions in antiserum were: mGlu5 (rabbit polyclonal; 1:500), mGlu1α (rabbit polyclonal 1:250), mGlu8 (guinea-pig polyclonal 1:1000), GluR2 (rabbit polyclonal 1 μg ml^{-1}), GluR2/3 (rabbit polyclonal 1 μg ml^{-1}), and GluR4 (rabbit polyclonal 2 μg ml^{-1}). All antibody incubations were carried out overnight. The primary antibody was detected using a biotinylated secondary antibody. Visualisation was carried out using a diaminobenzidine chromagen.

Light microscopic analysis showed immunoreactivity for mGluR1α in the ventral horn of the spinal cord on the cell bodies of laminae IX, and to a lesser extent with the mGlu8 antibody in the same region. There was strong immunoreactivity for mGlu5 (and to a lesser extent mGlu8 and mGlu1α) in the dorsal horn of the spinal cord. Immunocytochemical results with the iGlu receptor antibodies revealed the presence of GluR2/3 and GluR4 on the large motoneurones of laminae IX with little or no GluR2 staining in this region suggesting that both GluR3 and 4 subunits are expressed on motoneurones. GluR2 staining was primarily in the dorsal horn in contrast to results seen with GluR2/3 and GluR4. In all cases there was no staining in the absence of the primary antibody. There were no apparent differences in antibody staining with age.
The immunoreactivity observed with the mGlu5 antibody was similar to that observed by Vidnyanszky *et el*, (1994) in adult rat spinal cord. The staining of both mGlu1α and mGlu5 confirmed the presence of both group I mGlu receptor subtypes in the preparation and this is in agreement with previously reported pharmacological data using this preparation (Miller *et al*, 2000).
Immunoreactivity to mGlu8 in laminae I - II where afferent fibres enter the spinal cord via the dorsal root may be an indication that this receptor is expressed on primary afferent terminals. If so, one could conclude that at least part of the response observed with group III mGlu receptor agonists in neonatal rat spinal cord is mediated by the mGlu8 receptor in agreement with conclusions from previous pharmacological studies (Schoepp *et al.*, 1999).
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191P EFFECT OF BIA 3-202 ON CATECHOL-*O*-METHYLTRANSFERASE IN RAT LIVER AND ERYTHROCYTES

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The main action of catechol-*O*-methyltransferase (COMT) inhibitors in treating Parkinson's disease is to prevent *O*-methylation of L-DOPA peripherally, to improve its availability and brain penetration. The liver is endowed with one of the highest COMT activity and is believed to play an important role in the conversion of L-DOPA to 3-*O*-methyl-L-DOPA. Since erythrocytes are also endowed with considerable COMT activity and are more easily available than liver samples, it was hypothesised that they might be useful in the evaluation of new COMT inhibitors. Therefore, the aim of the present work was to evaluate the effect of BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone), a new reversible tight-binding COMT inhibitor with limited access to the brain (Vieira-Coelho et al., 2000), upon COMT activity in erythrocytes and liver. BIA 3-202 was given by gastric tube (0.3, 1,3,10 and 30 mg kg^{-1} in 0.5% carboxymethylcellulose) to overnight fasted male Wistar rats (230-250 g; Harlan, U.K.). Thereafter, 1 h after administration animals were anaesthetised with sodium pentobarbital (60 mg kg^{-1}) and 3 ml venous blood sample was collected and liver was removed. Soluble COMT (S-COMT) in erythrocytes was obtained by the method described by Schultz & Nissinen (1989). COMT activity in liver homogenates and samples of S-COMT from erythrocytes was evaluated by the ability to methylate adrenaline to metanephrine, as previously described (Vieira-Coelho & Soares-da-Silva, 1999). Results are arithmetic means with s.e.mean. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. A Michaelis-Menten behaviour was observed for S-COMT in erythrocytes from vehicle treated rats. Incubation of S-COMT assay mixture in the presence of increasing concentrations of adrenaline resulted in a concentration-dependent formation of metanephrine with a K_m and V_{max} values of 359±69 μM and 1.4±0.1 nmol mg $protein^{-1}$ h^{-1}, respectively. The kinetic parameters for liver COMT were as follows: K_m = 313±86 μM; V_{max} = 59±5 nmol mg $protein^{-1}$ h^{-1}. From these kinetics parameters, a saturating concentration of adrenaline (1000 μM) was chosen to use in inhibition studies. The basal S-COMT activity in erythrocytes (0.7±0.04 pmol mg $protein^{-1}$ h^{-1}) was 100 times less that observed in the liver (87.1±7.0 nmol mg $protein^{-1}$ h^{-1}). Despite differences in basal COMT activity in liver and erythrocytes, BIA 3-202 was endowed with a similar inhibitory profile upon COMT in liver and erythrocytes (fig 1.).

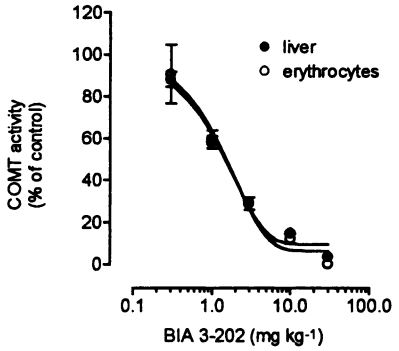

Figure 1. Effect of BIA 3-202 on liver and erythrocyte COMT activity.

In conclusion, COMT activity in erythrocytes can provide important information on the pharmacodynamic profile of COMT inhibitors.

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192P PHARMACOLOGICAL ANALYSIS OF CCR5 RECEPTORS USING [^{35}S]-GTPγS BINDING

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The chemokine receptors are members of the seven-transmembrane-spanning G-protein coupled receptor family and their activation results in T cell and macrophage migration and proliferation. One subtype (CCR5) serves as a co-receptor, in addition to CD4 receptors, for the human immunodeficiency virus (Alkhatib et al., 1996; 1997). The interaction of the receptor with a G-protein is a critical event in the mechanism of ligand action and subsequent signalling. The [^{35}S]GTPγS functional assay is a measure of this receptor/ G-protein interaction and subsequent GDP/GTP exchange. This provides a direct measure of ligand potency and maximal functional effect (efficacy) and is able to discriminate between full and partial agonists. The present study is the first to examine the potency and efficacy of MIP-1α and MIP-1β at CCR5 receptors using the [^{35}S]GTPγS binding assay.

Membranes were prepared from Chinese Hamster Ovary (CHO) cells stably expressing the human form of the CCR5 receptor (CHO/CCR5). Chemokine stimulated [^{35}S]GTPγS binding was carried out using a modification of the method described by Gardner et al., (1996)(1% BSA, 30μg membranes). In some experiments, cells were pretreated with pertussis toxin (100ng/ml) for 18 hours before preparation of membranes. Data were analysed using GraphPad Prism.

The chemokine MIP-1α dose dependently stimulated [^{35}S]GTPγS binding to CHO/CCR5 membranes with a maximal effect of 240±10 % over the basal level and an EC_{50} of 1.26 nM. Table 1 shows the effect of the chemokines MIP-1α, RANTES and MIP-1β on stimulating [^{35}S]GTPγS binding. There were no significant differences between the efficacies of the three ligands ($p>0.05$; Student's *t* test). RANTES was the most potent ligand and MIP-1β was the least potent; both were significantly different from MIP-1α ($p<0.05$; Student's *t* test). No response was seen with any of the chemokines when membranes were prepared from CHO/CCR5 cells pretreated with pertussis toxin.

Table 1. Chemokine potency and efficacy values at the CCR5 receptor defined using the [^{35}S]GTPγS binding assay.

Drug	pEC_{50} ±sem	EC_{50} (nM)	Efficacy (%MIP-1α)	n
RANTES	9.5 ± 0.1	0.32	94 ± 7	11
MIP-1α	8.9 ± 0.1	1.26	100	11
MIP-1β	8.4 ± 0.1	4.00	97± 8	11

These results show that all of the three chemokines tested, MIP-1α, RANTES and MIP-1β, act as full agonists at the human CCR5 receptor, with RANTES being the most potent. Treatment with pertussis toxin abolished the signalling of MIP-1α, RANTES and MIP-1β, indicating that CCR5 is coupling to $G_{i/o}$ G-protein subunits.

The financial support of the BBSRC is gratefully acknowledged.

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193P CHARACTERISATION OF THE HUMAN FcεRI α SUBUNIT PROMOTER REGION AND ITS MODULATION BY INTERLEUKIN-4 AND BY RECEPTOR ENGAGEMENT

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Immunoglobulin E (IgE) and its high affinity receptor (FcεRI) are at the centre of Type I (immediate) hypersensitivity reactions. In mouse, FcεRI is exclusively found as a $\alpha\beta\gamma_2$ tetramer, whereas the receptor is also expressed as a $\alpha\gamma_2$ trimer in human. FcεRIα contains two immunoglobulin-related domains, the second of which being required for IgE binding. In mouse, FcεRI is found only on mast cells and basophils, where triggering leads to the release of inflammatory mediators such as histamine, proteases and leukotrienes as well as of immunoregulatory and proinflammatory cytokines. In humans, FcεRI is additionally expressed on monocytes , epidermal Langerhans cells, eosinophils and platelets, where it is involved in antigen (allergen) presentation and in antibody-driven cellular cytotoxicity (ADCC) against helminthic parasites (Kinet, 1999).

To understand the differences between mouse and human regarding tissular distribution of FcεRI, we first reconstituted FcεRI-deficient mice by transgenesis with the human FcεRIα gene under the control of a 2.9 kb 5' region. In these animals, a functional "humanized" FcεRI was not only expressed on mast cells, as $\alpha\beta\gamma_2$ tetramer, but, like in humans, also on epidermal Langerhans cells, eosinophils (from *Schistosoma mansoni* infected mice) and on thioglycollate-elicited peritoneal macrophages, as $\alpha\gamma_2$ trimer (Dombrowicz, 1996, 1998). Taken together with these observations, the lack of expression in other cell types, such as neutrophils, splenocytes and thymocytes suggested that the transgene was able to direct expression of the hFcεRIα in a human tissue-specific manner. In this *in vitro* study, we have investigated the functional role of the 5' regulatory region of hFcεRIα spanning the sequence between –2901 bp and the start codon and its modulation by interleukin-4 and receptor aggregation. By transient transfections, we here show that the 2901 bp fragment is haematopoietic-specific (Cos-7 cells 1+/-0.4, J774 cells 8.7+/-2.8, RBL-2H3 cells 126.4+/- 44.5 n= 4) .

Using 10 additional deletion constructs, we identified positive regulatory regions localized between -117 and -205 bp, and between -502 and -864 bp. We also found positive activity between -1285 and -1582 bp, and a monocyte-specific region between -2455 and -2901 bp. Footprinting experiments showed that nuclear extracts from rat basophilic cells (RBL-2H3) were binding to the sequence 3' of -117 which contains two conserved 12 bp-motifs in the known FcRα genes. We confirmed this result by mobility shift assay with synthetic oligonucleotides corresponding to the two repeats and nuclear extracts from RBL-2H3 and murine monocytic cells (J774). HFcεRIα gene expression was more than doubled by 24 h IL-4 treatment (50 ng/ml), and we found that one of the two Stat6 sites present in the promoter was involved in this process. In addition, antigen stimulation (50 ng/ml DNP-HSA) increased luciferase activity by about 5 fold (n=9 unstimulated 50877+/- 21042, stimulated 235102+/- 40366 $p<0.01$), and no significant effect was obtained with monomeric IgE incubation (0.1μg/ml), confirming that upregulation of FcεRI by ligand-binding does not activate any pathways leading to FcεRIα subunit transcription.

We suggest that a direct mechanism is involved in receptor expression upregulation and since it is now clear that monomeric IgE, more than IL-4, is a major factor in regulating FcεRI expression this aspect will be further addressed.

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194P MONOCYTE CD163 EXPRESSION IS REGULATED BY GLUCOCORTICOIDS AND LIPOPOLYSACCHARIDE BUT NOT BY CHEMOKINES

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CD163 is a surface antigen expressed exclusively by cells of the monocyte/macrophage lineage. It has a Mr of 155kDa and is a member of the type B cysteine-rich scavenger receptor family. The protein is upregulated by glucocorticoids and IL-10 and downregulated by several pro-inflammatory mediators including γ-interferon (Sulahian *et al.*, 2000). The function of CD163 remains unclear, although it may have a role in monocyte adhesion events relating to late-stage inflammation.

The aim of this study was to study the regulation of blood monocyte CD163 expression by glucocorticoids *in vitro* in the context of bacterial lipopolysaccharide (LPS) or chemokines.

Blood mononuclear cells (MNC) were prepared from healthy volunteers by density centrifugation over histopaque. MNC were washed in phosphate-buffered saline (PBS) and $1x10^6$ cells suspended in RPMI 1640 ± 0.2% bovine serum albumin (RPMI+). Cells were then incubated at 37°C with either dexamethasone (DEX) or hydrocortisone (HC; 0-10μM) for 18h ± LPS (10nM), chemokines MCP-1, RANTES, IL-8 (10ng/ml) or γ-interferon (100IU/ml). Following incubation, MNC were washed twice with RPMI+ and reacted at $1x10^6$ cells/well in 96-well plates with anti-CD163 mAb Mac1.58 (1h 4°C). After washing, cells were stained with a FITC-goat anti-mouse IgG (30 min. 4°C). Finally, cells were fixed in 1% paraformaldehyde. In some experiments, steroids, chemokines, cytokines or LPS were incubated in citrated blood before incubation with antibodies as per the above procedure. Red cells were lysed and MNC fixed with lysing solution. (Becton Dickinson, Oxford). CD163 expression was analysed by flow cytometry (FACScan). Monocytes were identified by forward/side scatter profiles, and CD163 expression reported as FITC equivalent molecules per cell (FEMC), standardised to FITC-coated beads.

DEX treatment of MNC *in vitro* resulted in a dose-dependent increase in CD163 expression by monocytes, rising from a basal value of 5,000±150 to 81,200±4,500 FEMC at 10μM DEX ($p<0.001$, paired T-test, n=7) with a mean ED50 of $3x10^{-8}$M. HC-treated monocytes showed a lesser response; 3,700±180 to 53,300±3,500 FEMC at 10μM HC ($p<0.001$, paired T-test, n=7) with an ED50 of $5x10^{-7}$M. LPS alone caused a small reduction in expression from untreated levels (3,400±1,000 to 2,300±1,300 FEMC). In the presence of DEX (0.1μM) or HC (0.1μM) there was a significant elevation in CD163 expression over and above that with steroid alone. (DEX:43,800 ± 15,500;DEX + LPS:63,700 ± 21,400 FEMC n=3 HC : 9,100 ± 1,300; HC + LPS: 26,900 ± 2,800 FEMC n=6, $p<0.05$, paired T-test in both cases). MNC incubation with any chemokine in the presence or absence of DEX had no effect on monocyte CD163 expression. In conclusion, monocyte CD163 upregulation by glucocorticoids is amplified in the presence of LPS but not chemokines.

This induction could be physiologically important in late-stage inflammation where circulating HC levels are elevated and monocytes activated.

Sulahian T., Hogger P. et al Cytokine 2000,12; 1312-1321.

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195P THE PHARMACOLOGICAL EFFECTS OF THE CCR4 CHEMOKINE RECEPTOR LIGANDS MACROPHAGE DERIVED CHEMOKINE AND THYMUS AND ACTIVATION REGULATED CHEMOKINE ON HUMAN T CELLS

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T-helper type 2 (Th2) cells have been implicated in allergic diseases (Robinson *et al.*, 1992). The CCR4 receptor, which is differentially expressed on Th2 cells compared to Th1 (Bonecchi *et al.*, 1998), binds the chemokines TARC (Thymus and Activation Regulated Chemokine) and MDC (Macrophage Derived Chemokine), which have been shown to induce chemotaxis of Th2 cells (Imai *et al.*, 1999). Here we further examine the functional and pharmacological effects of these agonists on human T cells in terms of calcium fluxes and chemotaxis.

Naïve $CD45RO^-$ $CD8^-$ T cells were isolated from human peripheral blood. Cells were grown under activating conditions (RPMI plus 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine, 50μM β-mercaptoethanol, 1μg/ml PHA and 50ng/ml IL-2) or polarised to a Th1 (IL-12 (2ng/ml), anti-IL-4 (200ng/ml)) or Th2 (IL-4 (10ng/ml), anti-IL-12 (50ng/ml)) phenotype. Polarisation was characterised (by ELISA) by the relative production of IL-4, IL-5 and IFNγ (Romagnani, 1994). Calcium ($[Ca^{2+}]_i$) responses were determined by flow cytometry after loading with fluo-3AM (5μM). Chemotaxis was measured using a Boyden chamber with a collagen IV coated filter (5μm pore). Cells were resuspended in HEPES (20mM) buffered RPMI (pH 7.4) with BSA (1%) at 100,000 per well and incubated for 1h at 37°C, migrated cells were counted by microscopy. Data are expressed as the fold-increase over basal.

T cell polarisation was confirmed after day 14 of culture, following stimulation for 72h with concanavalin A (10μg/ml), as Th1 (IL-4: 0.7 ± 0.4pg/ml, IL-5: 0 ± 0pg/ml, IFNγ: 8900 ± 3560pg/ml) and Th2 (IL-4: 22.6 ± 13.9pg/ml, IL-5: 49 ± 39pg/ml, IFNγ: 434 ± 280pg/ml) (n = 5-7). The activated T cells produced a mixed cytokine profile of IL-4 (20.2 ± 6.8pg/ml), IL-5 (13.4 ± 11.2pg/ml) and IFNγ (5880 ± 2860pg/ml). CCR4 expression, determined by $[Ca^{2+}]_i$ response to MDC (10^{-7}M), peaked between 20 and 28 days of culture (maximum 42 ± 6%, day 28, n = 3). Subsequent calcium and chemotaxis measurements were made within these time-points. Concentration-effect (E/[A]) curves for $[Ca^{2+}]_i$ responses were constructed to MDC (Th2, $p[A]_{50}$ = 9.7 ± 0.1, n = 3; Th1, $p[A]_{50}$ = 9.8 ± 0.2, n = 3; activated, $p[A]_{50}$ = 9.9 ± 0.2, n = 3) and TARC (Th2, $p[A]_{50}$ = 9.2 ± 0.3, n = 3). Greater numbers of Th2 cells responded to MDC (55 ± 10%, n = 3) compared with either Th1 (13 ± 2%, n = 3) or activated T cells (21 ± 4%, n = 3). The magnitude of the $[Ca^{2+}]_i$ response to TARC was similar to that of MDC (50 ± 8% of Th2 cells responding, n = 3). Chemotaxis in response to MDC was also examined. The largest response was observed in Th2 (4.58 ± 0.22, at 10^{-8}M MDC, n = 4) compared to Th1 (1.65 ± 0.20, at 10^{-9}M MDC, n = 4) and activated T cells (1.43 ± 0.15, at 10^{-8}M MDC, n = 4). E/[A] curves constructed to TARC ($p[A]_{50}$ = 9.78 ± 0.14, n = 3) and MDC ($p[A]_{50}$ = 9.47 ± 0.13, n = 4) in Th2 cells were bell shaped.

These results summarise the pharmacological effects of MDC and TARC on human T cells in terms of chemotaxis and $[Ca^{2+}]_i$ responses. The greatest effects were seen in the Th2 cells, with both ligands demonstrating similar potencies and support previous evidence for the presence of the CCR4 receptor on Th2 cells.

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196P COX-1-DERIVED PGE_2: A COMPARISON BETWEEN HUMAN AND RAT BLOOD

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Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the production of prostaglandins (PGs) by inhibiting cyclooxygenase (COX) enzymes. As PGE_2 is a major product of COX-2 at inflammatory sites it is often used as a marker of the activities of NSAIDs against COX-2 *in vivo*. Here, we have studied the production of PGE_2 in human and rat blood. Our results cast doubts on the validity of PGE_2 as a marker in the profiling of NSAIDs' activity and selectivity in the rat.

Male Wistar rats (250-280 g) were anaesthetised with pentobarbitone sodium and blood (RB) collected via the abdominal aorta. Blood was also collected from healthy human volunteers (HB) via the antecubital vein. In one set of experiments (**exp1**), heparinised RB and HB was aliquoted into 96-well plates (100 ul/well) and treated with vehicle (VHC, 0.1 % DMSO in DMEM), aspirin (ASA, 0.1 mM) or the selective COX-2 inhibitor DFP (0.01 mM; Leblanc *et al.*, 1999). Following incubation for 30 min (37 °C, 95% O_2-5% CO_2), Ca^{2+} ionophore A23187 (50 μM) was added and the blood incubated for further 15 min. The plates were then spun and the plasma obtained was stored (–40 °C) until measurement of PGE_2 by radioimmunoassay. In a second set of experiments (**exp2**), the protocol above was repeated on RB, rat platelet rich plasma (RPRP) and rat washed leukocytes (RWL, 8 x 10^6 ml^{-1} in DMEM+10% FCS). RPRP was obtained by centrifuging heparinised RB at 200 g for 7 min. Leukocytes were isolated by dextran sedimentation of erythrocytes. Briefly, 0.8 parts of 3.5% dextran (M.W. 250000) in saline were added to 1 part of RB and incubated (45 min, 37 °C). Following sedimentation of the red cells, the upper leucocyte-containing layer was washed in PBS to remove platelets. Contaminating erythrocytes were eliminated by hypotonic lysis.

In exp1 RB produced PGE_2 in response to A23187, and this was inhibited by aspirin but not by the COX-2 selective inhibitor DFP. HB produced only negligible amounts of PGE_2. Exp2 demonstrated that the production of PGE_2 in RB in response to A23187 was found in platelets but not in leukocytes.

Table 1. Effect of vehicle (VHC), aspirin (ASA) or DFP on PGE_2 production in exp1 and exp2. Data represent ng ml^{-1} and are expressed as mean ± s.e.m. of 4 separate experiments.

	Treatment		
Exp1	VHC	ASA	DFP
RB	24±0.9	2.3±0.2	20.5±0.6
HB	1.6±0.5	<0.07	1.3±0.4
Exp2			
RB	25.5±5	6.8±2.2	24.8±4.1
RPRP	24.2±3.9	0.6±0.5	21.9±2.6
RWL	<0.07	<0.07	<0.07

In conclusion, our results show that RB has the capacity to produce copious amounts of COX-1-derived PGE_2 from platelets. As platelets have a prominent role in inflammation (see Klinger, 1997), platelet-derived PGE_2 may contribute to the inflammatory process in rats. This observation may explain why some authors (e.g. Wallace *et al.*, 1998 and 1999) have found that COX-2 selective inhibitors need to be given at non-selective, COX-1 inhibiting, doses to be anti-inflammatory in the rat. Most importantly, the differences found between human and rat blood suggest that extrapolations to humans of results from NSAIDs testing in the rat should only be made very cautiously.

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197P COMPARISON OF THE EFFECTS OF THE SELECTIVE COX-2 INHIBITOR, DFP, AND INDOMETHACIN ON LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS IN THE RAT MESENTERY

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In acute animal studies COX-2 selective inhibitors cause markedly less gastrointestinal injury than standard NSAIDs (Masferrer *et al.*, 1994). Similar experiments have suggested that increased adherence of leukocytes to the vascular endothelium and the consequent accumulation of neutrophils in the mucosa are critical events underlying the gastric toxicity of NSAIDs (Wallace *et al.*, 1993). Thus, the aim of this study was to compare the effects of a traditional, non-selective NSAID, indomethacin, and a highly selective COX-2 inhibitor, DFP (Leblanc et al., 1999), on leukocyte-endothelial cell interactions.

Male Sprague-Dawley rats (250g) were anaesthetized (sodium thiopentobarbital, 120 mg kg^{-1}, i.p.) and the abdomen opened. Intravital video microscopy was used to monitor leukocyte rolling, leukocyte-endothelial cell adhesion and leukocyte emigration in postcapillary venules of the externalised mesentery (Tailor *et al.*, 1997) before and 15, 30 and 60 min after administration of indomethacin or DFP (both 20 mg kg^{-1}, i.v.).

Indomethacin significantly increased the number of rolling leukocytes (Figure 1A), reduced leukocyte rolling velocity (Figure 1B) and increased leukocyte adhesion to the endothelium (Figure 1C). The COX-2 selective inhibitor DFP did not induce any of those effects. Neither indomethacin nor DFP induced leukocyte emigration into the interstitial tissue over the time analysed.

Our results suggest that in the normal gut selective inhibition of COX-2, unlike non-selective COX inhibition, does not induce inflammatory events. This lack of pro-inflammatory activity may partly underlie the gastric sparing action of COX-2-selective drugs and substantiates the notion that COX-1 and not COX-2 is active in 'non-inflamed' conditions.

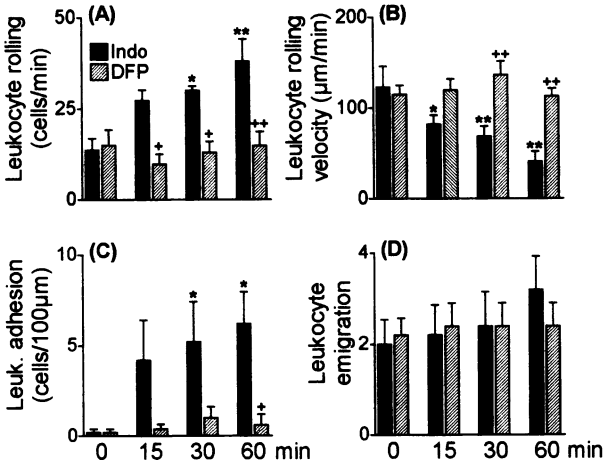

Fig 1. Effects of indomethacin or DFP on leukocyte-endothelial cell interactions. Results expressed as mean ± s.e.m. of 5 experiments (* $p<0.05$ and ** $p<0.01$ vs respective basal value, repeated measures-ANOVA + Dunnet's test; + $p<0.05$ and ++ $p<0.01$ vs respective value in indomethacin treated rats, ANOVA + Tukey test).

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198P ML3000, A BALANCED INHIBITOR OF CYCLOOXYGENASE AND LIPOXYGENASE, INHIBITS PLATELET/POLYMORPHONUCLEAR LEUKOCYTE TRANSCELLULAR METABOLISM AND ADHESION

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Functional and metabolic interactions between platelets and polymorphonuclear leukocytes (PMN) are relevant to several pathological conditions such as inflammation and thrombosis. These cells cooperate in processing arachidonic acid (AA) and AA-derived metabolites. *In vitro*, activated platelets increase the production of 5-LO metabolites by providing free AA to PMN. Moreover, they synthesise LTC4 and 5-12diHETE by metabolising PMN-derived LTA4 and 5-HETE, respectively. Adhesion through the specific adhesive molecules P-selectin and Mac-1 regulates PMN recruitment on activated platelets at site of vascular damage or inflammation (Evangelista *et al.*, 1996). Production of LO transcellular metabolites requires direct cell-cell contact (Maugeri *et al.*, 1994); on the other hand, the activity of Mac-1 may be regulated by these metabolites (Capodici C *et al*, 1998).

We investigated the effect of the polysubstituted pyrrolizine ML3000, a balanced inhibitor of cyclooxygenase (CO) and 5-LO (Laufer *et al.*, 1994), on the production of LO metabolites and cell-cell adhesion in mixed human PMN/platelet population *in vitro*. The production of LO metabolites was evaluated in mixed cell suspensions (platelets/PMN, $2x10^8/1x10^7$/ml) stimulated with 10 µM A23187, under stirring at 1,000 rpm, for 5 min at 37°C. LTB4, LTC4, and 5-12diHETE were determined by HPLC. PMN-platelet adhesion was evaluated by double colour cytofluorimetry using hydroethydin-labelled PMN (10^7/ml) and fluorescein-labelled, thrombin-stimulated, PFA-fixed platelets ($2x10^8$/ml; Evangelista *et al.*, 1996). The formation of platelet-PMN conjugates in mixed cell suspensions, incubated for 2 min at 37°C under stirring at 1,000 rpm, was quantified as percentage of PMN displaying the platelet fluorescence marker (%(+)PMN), and as number of platelets bound to PMN (N°PLT/100PMN). Cell suspensions were pre-incubated for 15 min at RT with ML3000 or its solvent DMSO (control).

Control mixed cell suspensions produced 125±8, 320±40, and 248±34 ng/ml of LTB4, LTC4 and 5-12diHETE, respectively, (mean±sem n=4). ML3000 concentration-dependently reduced the synthesis of all AA metabolites. At 10 µM, the production of LTB4 and 5-12diHETE was reduced to 6.4±2.2 and 1.4±0.8 ng/ml, while LTC4 was undetectable. Interestingly, in the same range of concentrations, ML3000 inhibited PMN adhesion to activated platelets. At 10 µM, the %(+)PMN was reduced from 55±8 to 32±4, and the N°PLT/100PMN from 157±52 to 50±11 (n=3). The inhibitory effect of ML3000 on platelet-PMN adhesion was reproduced by the 5-LO specific inhibitor BAY-X1005, but not by the CO inhibitor diclofenac, suggesting the anti-adhesive activity of ML3000 be mainly mediated by its anti-5-LO activity.

Inhibition of metabolic and adhesive interactions between PMN and platelets may contribute to the anti-inflammatory activity of ML3000 and extend its pharmacological potential to pathological settings, such as thrombosis and vascular injury, for which the relevance of platelet-PMN interaction is recognised.

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199P ANTI-INFLAMMATORY ACTIVITY ON ADJUVANT-INDUCED ARTHRITIS IN RATS OF OCTANORDAMMARANE TRITERPENES FROM RESIN EXTRACTS OF *COMMIPHORA KUA*

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We previously reported purification of six myeloperoxidase (MPO) inhibitory molecules (four triterpenes and two lignans) from *C. kua* (family Burseraceae) resin, a traditional Kenyan anti-inflammatory remedy (Battu *et al.*, 1999) and recently showed their anti-inflammatory actions on carrageenan-induced rat paw acute inflammation (Battu *et al.*, 2000). We now report the effects of the four purified triterpenes, mansumbinone (M1) mansumbinoic acid (M2), 16(S),20(R)-Dihydroxydammar-24-en-3-one (M3) and 3β,16(S),20(R)-Trihydroxydammar-24-ene (M4) (Provan & Waterman, 1986) on adjuvant-induced rat arthritis and on cyclooxygenase (COX) activity.

Drugs suspended in acacia mucilage were administered daily (*p.o.*) to male Wistar rats (200–250g) during adjuvant arthritis induced by injecting heat killed *M. tuberculosis* in liquid paraffin (100µl, 5mg.ml^{-1}) into a hindpaw (Duwiejua *et al*, 1993). Inflammatory oedema was monitored by measuring hindpaw thickness in both injected and non-injected paws. Neutrophil infiltration was monitored using neutrophil elastase (NE) as a marker (Tanaka *et al*, 1990). COX inhibition was measured spectrofluorimetrically using malondialdehyde formation (Dannhardt *et al.*, 1998). Data was analysed statistically using the paired *t*-test (Two-tailed). All of the purified molecules were anti-inflammatory and M4 was the most active in reducing oedema and neutrophil infiltration in the non-injected paws (Fig.1). The equipotent molar ratios as COX inhibitors of the molecules Indo : M2 : M4 : M3 : M1 were 1.0 : 2.3 : 13.0 : 290.3 : 1078.7 respectively. Our previous (Battu *et al.*, 1999, 2000) and current studies thus show that M2 and M4 are potent inhibitors of both MPO and COX, and suppress both acute and chronic inflammation.

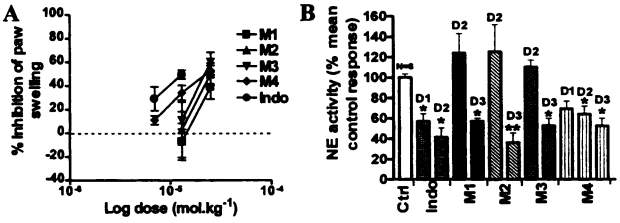

Figure 1: Dose-effect curves of test drugs (Mol.kg^{-1} × 10^{-5} : D1=0.7; D2=1.3; D3=2.5) on non-injected inflamed paws in adjuvant arthritic rats: (**A**) Swelling; (**B**) Neutrophil infiltration (mean ± s.e.m, n=3; *p<0.05, **p<0.01; Ctrl=vehicle; Indo=Indomethacin).

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200P REGULATION OF EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE BY THE UREMIC CATABOLITE METHYLGUANIDINE (MG)

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Monocyte function changes have been recognised as a key factor responsible for immunological disorders associated with uremia (Deenitchina *et al.*, 1995). We have previously demonstrated that the uraemic toxin MG is able to inhibit plasma TNFα levels in LPS-treated rats (Autore *et al.*, 1999), to reduce significantly TNFα release by LPS-stimulated J774.A1 macrophages (Autore *et al.* 1999) and to inhibit NO production via constitutive and inducible isoforms of NO synthase (Sorrentino *et al.*, 1997).

In this study we have simulated a condition similar to that expected in uraemia by pre-exposing J774.A1 cells to MG (0.1-10 mM), prior to activation with LPS (6x10^3 u ml^{-1} 24^{-1} h), and examined the effect of MG treatment on the expression of the inducible L-arginine-NO pathway. MG was added to the cells 18 h or 30 min before or 24 h after LPS challenge. Western blot analysis, performed using a monoclonal antibody for murine iNOS on whole cell lysates, revealed a marked and significant reduction in iNOS protein expression in cells pre-incubated with MG (0.1-10 mM). This effect was both concentration- and time-related. In contrast, when MG was added after LPS challenge (24 h) iNOS expression remained virtually unaltered (Table 1). This is the first demonstration that MG inhibits iNOS expression and this finding could have significant implications in unravelling the complex mechanism(s) involved in uraemic patient immunodeficiency where an increase in endogenous guanidino compounds like MG is observed (Yokozawa *et al.*, 1990; Mendes-Ribeiro *et al.*, 1996).

Table 1. Time- and concentration-dependent effect of methylguanidine (MG) on LPS-induced iNOS expression in J774.A1 macrophages. Data represents means±s.e.m and is expressed as % of inhibition (n=5)

MG mM	Before LPS		After LPS
	18 h	30 min	24 h
0.1	33.75±4.9	34.28±7.0	0
1.0	27.74±2.7	48.00±11.1	0
10	60.80±3.9	88.72±5.5	0

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201P NERVE GROWTH FACTOR (NGF) INDUCES EXTRAVASCULAR ACCUMULATION OF MACROPHAGES AND LYMPHOCYTES, IN ADDITION TO NEUTROPHILS, IN RAT DORSAL SKIN

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NGF is known as an important growth factor, involved in the development of nerves in the neonatal animal. However, there is increasing interest in the role of NGF in pain and inflammation. Notably, NGF is a mediator of thermal hyperalgesia (Lewin and Mendell *et al.* 1993), which is now known to have a neutrophil- dependent component (Bennett *et al.* 1998). The aim of this study was to investigate the histopathological changes after of an intradermal (i.d.) injection of NGF into rat dorsal skin.

Male Wistar rats (230-250g) were anaesthetised with sodium pentobarbitone (60mg/kg) and their dorsal skin shaved. Injection of NGF (40pmol/100µl, i.d.) and Tyrode (vehicle control 100µl, i.d.) was performed. Inflammatory cell infiltrates were measured 5 hours after NGF administration. After this time point, animals were killed. Skin samples were removed and stored in 10% neutral buffer formalin for fixing. Once fixed, samples were embedded in paraffin wax, sectioned at 3µm and haematoxylin and eosin (H & E) stained. Inflammatory cell accumulation (neutrophils, macrophages and lymphocytes) was quantified by taking cell counts. Pathological changes of the skin were also noted. These included oedema, fibrin deposition, smooth muscle proliferation and muscle ischemia.

A significant increase of neutrophils and macrophages is seen in the skin at 3 and 5 hours after NGF administration compared to uninjected and Tyrode control sites. Lymphocyte accumulation into the skin significantly increases 5 hours after NGF administration.

Cell counts /0.025mm^2	Uninjected	Tyrodes	NGF
Neutrophils	0.00±0.00 (12)	0.00±0.00 (12)	12.27±1.17 ***(12)
Macrophages	9.89±0.67 (12)	8.11±0.5 (12)	30.69±2.52 ***(12)
Lymphocytes	1.25±0.45 (12)	1.20±0.60 (12)	4.88±0.96 ***(12)

Table 1: Cellular effect of NGF administration into rat dorsal skin after 5 hours. Results (mean ± s.e.mean of (n)) are expressed as inflammatory cell counts/0.025mm^2 of skin tissue. *P<0.05, ***P<0.001 compared to Tyrode injected control skin sites.

Pathological observations showed an increase in oedema and fibrin deposition after NGF administration compared to Tyrode control. The smooth muscle of the skin demonstrated a significant increase in accretion formation and basophilic inclusion formation after NGF administration (P<0.05, n=12).

These findings demonstrate that NGF administration causes a significant accumulation of inflammatory cells, that include macrophages and lymphocytes, in addition to neutrophils. Other consequences of NGF administration (i.e. increased fibrin deposition) suggest NGF capable of enhancing wound healing. We have also shown here that NGF has a profound effect on skin smooth muscle by causing possible regeneration and muscle ischemia.

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202P CAPSAICIN-MEDIATED NEUROGENIC VASODILATATION IN NEUROKININ-1, NK_1, RECEPTOR KNOCKOUT MICE

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Capsaicin induces tachykinin NK_1 receptor-dependent neurogenic oedema formation in the mouse ear and this model has been used to determine the absence of NK_1 receptors in NK_1 knockout mice (Bozic *et al.*, 1996). We have now simultaneously measured neurogenic vasodilatation and oedema formation in the ear and investigated for the identity of the vasodilator mediator.

Male CD1 mice (30g) were used in capsaicin dose-response studies. Sv129+C57BL/6 mice (both sexes, 25-40g), wild-type and NK_1 receptor knockout, were used in other experiments. Anaesthesia was induced by urethane (100µl/10g; 25% w/v). Oedema formation was measured by the accumulation of ^{125}I-albumin, injected i.v., and blood flow by laser Doppler flowmetry. Responses were measured for 1h after topical application of capsaicin to one ear and ethanol (vehicle control) to the contralateral ear. Results are shown as mean ± s.e.mean and statistical analysis by ANOVA plus Bonferroni's multiple comparisons test.

Capsaicin at doses of 0.2 - 200µg/ear produced significant vasodilatation in CD1 mice, whereas a dose of 2µg/ear was required to induce oedema formation, see Table 1. In a separate series of experiments, capsaicin induced significant oedema in wild-type Sv129+C57BL/6 mice, but no oedema was observed in NK_1 receptor knockout mice, as expected (Cao *et al.*, 1999).

However, significant neurogenic vasodilatation (p<0.05) was observed in both wild-type and NK_1 knockout mice, and remained unaltered in wild-type mice in the presence of the NK_1 antagonist SR140333 (480nmol/kg), but not observed in NK_1 knockout mice pretreated with the CGRP antagonist $CGRP_{8-37}$ (400nmol/kg).

Table 1. Neurogenic vasodilatation and oedema induced by capsaicin (0.02-200µg/ear) in CD1 mice; *=p<0.05, **=p<0.01, ***=p<0.001 compared to control values

Capsaicin (µg/ear)	Area under blood flow curve (mm^2)	Plasma extravasation (µl/mg tissue)
Control (n=27)	164.31 + 31.85	0.031 + 0.001
0.02 (n=6)	131.68 + 23.62	0.025 + 0.001
0.2 (n=10)	811.24+243.74**	0.041 + 0.008
2 (n=9)	798.96+206.27*	0.095 + 0.005**
20 (n=7)	701.79+127.48**	0.115 + 0.011***
200 (n=10)	793.61+182.51**	0.095 + 0.008**

The results show that the dose of capsaicin necessary to induce oedema is greater than that which produces vasodilatation. They also suggest that CGRP, but not substance P, is the mediator of the increased blood flow.

A.D. Grant is supported by a BHF studentship

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203P INVOLVEMENT OF FPR IN THE ANTI-INFLAMMATORY ACTION OF ANNEXIN-1

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A recent *in vitro* study with human neutrophils has suggested that annexin 1 (ANX-A1)-derived peptides interact with the receptor for formyl-Met-Leu-Phe (fMLP), termed FPR, to down-regulate cell activation (Walther *et al.*, 2000). We have assessed here the *in vivo* relevance of this hypothesis.

Human polymorphonuclear cells (PMN) were prepared by centrifugation on a gradient of histopaque (1.077 and 1.119 g/l; Sigma Chem. Co., Poole, UK). Cells were incubated with peptide Ac2-26 (Getting *et al.*, 1997) or with fMLP (Sigma) for 15 min at 37°C. In some cases, the FPR antagonists Boc1 (N-t-butoxycarbonyl-fMLP; Sigma) or Boc2 (N-t-butoxycarbonyl-fPLPLP; Sigma) were added to cells for 5 min prior to the agonists. L-selectin (CD62L) expression on the cell surface was measured by flow cytometry, using a selective monoclonal antibody (clone FMC46 Serotec Ltd., Oxford, UK). Cell chemotaxis was determined with 96 well plates equipped with a 3 μm-pore filter (Receptor Technology Ltd., Adderbury, UK), using 10^5 PMN and a 90 min incubation period. *In vivo*, peptide Ac2-26 (200 μg) was injected i.v. to mice alone or together with 50 μg Boc1 or Boc2, 15 min prior to zymosan (1 mg i.p.; Getting *et al.*, 1997). FPR gene knocked-out (KO; Gao *et al.*, 1999) and C57Bl/6 mice (body weight ~25-28 g) received peptide Ac2-26 (100 or 200 μg i.v.) prior to zymosan (1 mg i.p.). In all cases, PMN influx into the peritoneal cavity was measured 4 h later. Statistical differences were analysed by analysis of variance followed by Bonferroni test.

Peptide Ac2-26 reduced L-selectin expression on human PMN in a concentration-dependent manner, with a significant effect at 20 μgml^{-1} (6.6 μM), and a maximal reduction of 55±5% at 200 μgml^{-1} (66 μM) (n=6 experiments, $P<0.05$ as determined on original values). FMLP (0.1 μM) produced a marked reduction in L-selectin expression (from 50 to 90% depending on the donor), reverted by addition of 50 μgml^{-1} Boc1 or Boc2 (n=3, $P<0.05$). The FPR antagonists also inhibited the effect of peptide Ac2-26 or ANX-A1. In the chemotaxis assay, peptide Ac2-26 produced maximal PMN migration at 100 μgml^{-1} (33 μM): $25\pm5x10^3$ cells, vs. a basal outflow of $3\pm1x10^3$ PMN (n=4; $P<0.05$). PMN pre-incubation with 0.1 μM fMLP, but not with 20-100 μgml^{-1} peptide Ac2-26, almost abolished the chemotactic response to the peptide (4 ± 0.5 $x10^3$ PMN, n=4). *In vivo*, zymosan (1 mg) produced a PMN migration of $12.9\pm2.5x10^6$ PMN per mouse (n=10), and this was attenuated by ~30% by i.v. injection with Ac2-26 (100 μg), and this was prevented by co-injection of 50 μg Boc1 or Boc2 (n=6-12, $P<0.05$ in both cases). In the FPR KO mice, treatment with peptide Ac2-26 (100 or 200 μg i.v.) was not able to modulate PMN migration into the peritoneal cavity, whereas a 15±5% and 40±6% inhibition were calculated in wild type mice (n=8).

In conclusion, these data indicate an involvement for FPR in the effects of ANX-A1-derived peptides. The nature of the FPR–ANX-A1 interaction remains elusive. Comparison of peptide Ac2-26 and fMLP effects on PMN functions as well as on intracellular signalling is required to clarify this point.

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204P EFFECT OF THE HMG-CoA REDUCTASE INHIBITOR, PRAVASTATIN, ON SEPHADEX-INDUCED AIRWAY INFLAMMATION IN THE RAT.

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It has been shown previously that inhibitors of HMG-CoA reductase, such as pravastatin, not only block cholesterol synthesis but also suppress lymphocyte function (Tatsuno *et al* 1997). Furthermore pravastatin has been reported to significantly decrease incidence of clinical severe acute rejection episodes in transplants, an effect that is attributed to immuno-suppression (Katznelson *et al* 1995). We have presented data showing that T cells play a role in orchestrating Sephadex induced pulmonary eosinophilia in the rat (Underwood *et al* 2000). The aim of this experiment was to determine the effect of pravastatin on Sephadex induced airway inflammation.

Male Sprague Dawley rats (380-400 g, n=8) were orally dosed with vehicle (Veh) (0.5% methyl cellulose (w/v) and 0.2% tween80 (v/v), 2 ml kg^{-1}) or compound (3, 10 and 30 mg kg^{-1}) 24 and 1 hour prior to and 6 hours after saline (Sal) (1 ml kg^{-1}, i.t.) or Sephadex (Seph) (5 mg kg^{-1}). Pravastatin (Pra) was used at a dose previously shown to inhibit HMG-CoA reductase (Koga *et al* 1992). Budesonide (3 mg kg^{-1}) was included as a positive control using the same dosing regime. Saline or Sephadex instillation was performed while the animals were anaesthetised with halothane (4% in oxygen for 4 minutes). Twenty-four hours after challenge the animals were culled with an overdose of pentobarbitone (200 mg kg^{-1}, i.p.) and the lungs were, weighed, perfused, chopped and digested. Total white cell counts and differential counts were performed on the lung tissue digest samples, T-cells, CD^{4+} and CD^{8+}, were counted by flow cytometry as previously described (Underwood *et al* 1997)
Statistical analysis was by Students t test or one-way analysis of variance with correction for multiple comparisons. Values are expressed as mean ± s. e. mean.

Table 1 Effect of Pravastatin on Sephadex induced airway inflammation.

Treatment	Eosinophils cells 10^3 mg^{-1}	$CD4^+$ cells 10^3 mg^{-1}	$CD8^+$ cells 10^3 mg^{-1}	Weight mg
Veh / Sal	0.63±0.09	0.67±0.12	1.05±0.07	1.07±0.04
Pra 30 / Sal	0.48±0.09	0.60±0.18	0.92±0.08	1.11±0.02
Veh / Seph	1.26±0.28*	0.63±0.14	0.68±0.08	1.19±0.06
Pra 3 / Seph	1.45±0.41	0.61±0.17	0.69±0.07	1.15±0.06
Pra 10 / Seph	1.44±0.21	0.54±0.05	0.53±0.05	1.28±0.05
Pra 30 / Seph	1.30±0.24	0.40±0.07	0.44±0.03*	1.39±0.06*
Bud 3 / Seph	0.46±0.15*	0.10±0.20*	0.17±0.03**	1.02±0.04

** $p < 0.05$, ** $p < 0.01$ compared to relevant control group.*

Sephadex administration caused significant eosinophilia, a increase in lung weight but did not alter lymphocyte number (Table 1). Pravastatin caused a dose dependant decrease in T cell counts but did not alter eosinophil number and significantl increased lung weight. Budesonide significantly reduce eosinophil and lymphocyte numbers and reduced the lun weight. In this model of airway inflammation pravastatin di reduce the number of T cells in the lung tissue, but did not alte eosinophil number. However, an effect of pravastatin on tissu eosinophilia at a dose that has a greater impact on T cells canno be ruled out. In conclusion the lack of effect of pravastatin o Sephadex induced eosinophilia and the adverse effect on lun tissue weight suggests that pravastatin would not be an effectiv anti-asthma therapy.

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205P EFFECTS OF A RANGE OF PRO- AND ANTI-INFLAMMATORY AGENTS UPON ADENYLATE CYCLASE SENSITISATION AND MUSCARINIC M2 REGULATION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Muscarinic M2 receptors are constitutively expressed in human cultured airway smooth muscle (ASM) cells (Widdop *et al.*, 1993) where they inhibit β_2-adrenoceptor-mediated cyclic AMP (cAMP) formation via negative regulation of adenylate cyclase (AC). During airway inflammation, a range of pro- and anti-inflammatory agents have been reported to be elevated in the airways including Interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), RANTES, eotaxin and tumour growth factor-beta (TGF-β). The aim of this work was to study the effects of chronic exposure to these agents upon M2 function and AC activity in human cultured ASM cells.

Culture of human ASM cells and determination of [^{3}H]-cAMP formation was performed as previously described (Billington *et al.*, 1999). T-tests / ANOVA plus Bonferroni's multiple comparison test were used as appropriate.

As widely reported, the direct activator of AC, forskolin (FSK)(10min, 10μM), significantly increased cAMP formation (6.2 ± 0.8 fold cf basal, n=20, p<0.001). This response was inhibited following 10min pretreatment with the muscarinic agonist, carbachol (CCh)(1μM) (38.9 ± 3.7% inhibition cf FSK alone, n=20, p<0.01). Interestingly, 18h pre-exposure to the pro-inflammatory cytokine IL-1β (400pg/ml) caused a large augmentation of FSK (10min, 10μM)-induced cAMP formation (3.4 ± 0.4 fold cf FSK, n=7, p<0.001). However, despite 18h exposure to IL-1β, acute addition of CCh (10min, 1μM) still produced an inhibition of FSK–induced cAMP formation (44.2± 3.6 % inhibition cf IL-1β+FSK, n=4, p<0.005). The magnitude of this effect in % terms was similar to that observed in the absence of IL-1β, suggesting that the M2 receptor is still functional under these conditions.

TNF-α (18h, 10ng/ml) was also observed to augment FSK-induced cAMP formation (2.1 ± 0.4 fold cf FSK, n=7, p<0.05) and, as with IL-1β, functionality of the M2 receptor appeared to be maintained: % reduction with CCh (10min, 1μM) was 16.9 ± 3.5 cf TNF-α+FSK, n=7, p<0.05. Chronic exposure to the pro-inflammatory chemokines RANTES or eotaxin or to the anti-inflammatory growth factor TGF-β (all 18h, 20ng/ml) produced no significant effect on FSK-induced cAMP formation (89.4 ± 6.1; 85.3 ± 8.1 and 130 ± 35.8% of response to FSK alone respectively; all n=4, p=ns). The M2-mediated inhibition of cAMP formation in human ASM cells was maintained following chronic exposure to these agents (41.3 ± 3.4; 39.5 ± 2.5 and 31.75 ± 6.6 % inhibition cf FSK + agent respectively; all n=4, p<0.05).

In summary, M2 receptors are constitutively expressed in human ASM cells. Heterologous desensitisation of receptor-mediated effects on cAMP function was not observed despite chronic exposure to a range of pro- and anti-inflammatory mediators. However, the data presented do demonstrate the ability of IL-1β and TNF-α to sensitise AC. This could represent a homeostatic mechanism to promote ASM relaxation during inflammation.

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206P BRADYKININ B_2 RECEPTOR-INDUCED CONTRACTION OF HUMAN ISOLATED GALLBLADDER IS INCREASED BY INFLAMMATION

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It has previously been reported that stimulation of bradykinin (BK) B_2 receptors evokes inflammatory events in a number of experimental models (Geppetti, 1993). Furthermore, it has been suggested that expression of B_2 is increased under certain inflammatory conditions (Schmidlin et al., 1998). In this study we investigated the ability of BK to contract human isolated gallbladder (GB) and the role of inflammation on this response. The study was approved by the regional ethical committee. Sections (approx. 100 x 20 mm) of human isolated GB were obtained from patients (45–74 yrs) undergoing routine surgical procedure, and placed in 5 ml organ baths containing an oxygenated and gassed Krebs buffer solution, maintained at 37°C and at a tension of 1.5 g. Samples were taken from gallbladders with (INF, n = 9) or without (NINF, n = 7) morphological signs of inflammation. After an initial stabilisation period (90 min), three reproducible cumulative concentration-response curves were performed with BK (0.1 nM – 3 μM) or carbachol (10 nM-30 μM). Results are expressed as % KCl 120 mM. Statistical analysis was performed by means of Student's *t*-test. BK was more potent and efficacious than carbachol either in INF or in NINF tissues (Fig. 1). Response to KCl (120 mM) was not different in NINF (235 ± 47 mg) and INF (216 ± 50 mg). Maximum response to BK (3 μM) was higher in INF than in NINF tissues (Fig. 1). In contrast, the response to carbachol was not different in INF and NINF conditions (Fig. 1). The contraction produced by BK (1 μM) in INF tissue (166 ± 19%) was reduced by the BK B_2 receptor antagonist HOE140 (47 ± 5%, P<0.05, n = 5), but not by indomethacin (130 ± 17, P>0.05, n = 5). Similar findings were observed in NINF tissue (data not shown, n = 5-7).

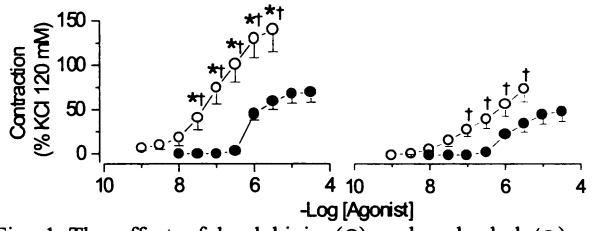

Fig. 1 The effect of bradykinin (○) and carbachol (●) on human isolated gallbladder in inflamed (INF, left panel) and non-inflamed (NINF, right panel) tissue. P < 0.05 * vs. non-inflamed tissue, † bradykinin vs. carbachol (n = 5 – 9).

We showed for the first time that BK contracts the isolated human gallbladder *via* activation of B_2 receptors and without the involvement of prostanoid generation. We showed also that BK is more potent and efficacious than the muscarinic agent, carbachol. Finally, we demonstrated that the contractile response to BK is greater in tissues showing morphological signs of inflammation. It remains to be determined whether or not this increased response is due to greater BK B_2 receptor expression. BK B_2 receptor antagonists may be beneficial in the treatment of biliary colic.

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207P SIGNAL TRANSDUCTION OF HUMAN Y_5 NEUROPEPTIDE Y RECEPTORS

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Y_5 neuropeptide Y (NPY) receptors mediate a number of physiologically important functions, but little is known about their signal transduction. Therefore, we have studied for a number of signalling pathways, which have been implicated for other NPY receptor subtypes, whether they are activated by Y_5 receptor stimulation.

Our studies are based on HEC-1B cells which had been stably transfected with cloned human Y_5 receptors (Moser et al. 2000); in some cases non-transfected parental HEC-1B cells were studied for comparison. Cyclic AMP accumulation, elevations of intracellular Ca^{2+} concentrations, activation of phospholipase C and of isoforms of protein kinase C (PKC) and of ERK, JNK and p38 forms of mitogen-activated protein kinases were determined as previously described (Kribben et al. 1997; Michel et al. 1992; Taguchi et al. 1998; Keffel et al. 2000). In the PKC and MAPK experiments, effects of 100 nM of the phorbol ester phorbol-12-myristate-13-acetate (PMA) were studied for comparison. Data are means ± s.e.means.

NPY concentration-dependently inhibited 1 μM forskolin-stimulated cyclic AMP accumulation (-log EC_{50} 8.88 ± 0.25; n = 4-5) in transfected but not in non-transfected HEC-1B cells with almost complete inhibition at 100 nM NPY, and this was blocked by overnight pre-treatment of the cells with 100 ng ml^{-1} pertussis toxin. In concentrations up to 100 nM NPY caused only small elevations of intracellular Ca^{2+}, which were similar in transfected and non-transfected cells (n = 5-6). NPY (100 nM) did not increase inositol phosphate accumulation (14 ± 8% over basal, n = 5). Upon stimulation with 100 nM NPY for 1, 5 and 10 min we did not detect consistent activation of the classical PKC isoforms PKCα, $PKC\beta_I$ and $PKC\beta_{II}$ and PKCγ, the new PKC isoforms PKCδ, PKCε, PKCη and PKCθ and the atypical PKC isoforms PKCι and PKCξ whereas most of them were activated by PMA at the 10 min time point. While treatment with 100 nM PMA for 10 min significantly ($p < 0.05$ in a two-tailed, one-sample t-test) activated ERK, 46 kDa JNK, 54 kDa JNK and p38 forms of mitogen-activated protein kinases to 166 ± 19% (n = 15), 232 ± 36 (n = 18), 127 ± 13 (n = 20) and 193 ± 32% (n = 20) of time-matched control values, neither kinase family was consistently activated by 100 nM NPY over a time course from 1 to 60 min (n = 8-13).

We conclude that, at least upon expression in HEC-1B cells, the signal transduction of Y_5 NPY receptors is limited to inhibition of cyclic AMP accumulation.

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208P INVOLVEMENT OF MULTIPLE SIGNALLING PATHWAYS DURING STIMULATION OF RENAL DOPAMINE D_1-LIKE RECEPTORS

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The activation of D_1-like receptors located on the proximal tubules causes inhibition of tubular sodium reabsorption by inhibiting Na/H exchange and Na^+-K^+ ATPase activities. The D_1-like receptors are linked to multiple cellular signalling pathways: the adenylyl cyclase-protein kinase A system, the phospholipase C-protein kinase C system, and phospholipase A_2 (Jose et al., 1996; Lokhandwala et al., 1998). The present study examined the effect of D_1–like dopamine agonists on Na^+-K^+ ATPase activity in OK cells and the transduction pathways involved. OK cells (ATCC 1840-CRL) grown on polycarbonate filters were mounted in Ussing chambers and connected to an automatic voltage current clamp that allowed continuous monitoring of short circuit current (I_{sc}, μA/cm^2). Amphotericin B was added to the apical bath to increase the sodium delivered to Na^+-K^+ ATPase to the saturating level. Under these conditions, a decrease in I_{sc} is assumed to represent the sodium transport mediated by Na^+-K^+ ATPase (Gomes et al., 2000). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. Amphotericin B (0.1 - 1.0 μg ml^{-1}) added from the apical cell border of the monolayer induced a concentration dependent decrease in I_{sc}, the maximum effect being attained at 1.0 μg ml^{-1}. This effect was prevented by ouabain (100 μM; 80.6±2.4 % reduction) and substitution of sodium (92.9±0.6 % reduction) or potassium (87.6±2.4 % reduction) by choline chloride and caesium chloride, respectively. The D_1-like receptor agonist SKF 38393 produced a concentration dependent inhibition of the amphotericin B-induced I_{sc} decrease with an IC_{50} value of 130 (91, 186) nM. The effect of 1 μM dopamine was completely abolished by pre-treatment of cells with SKF 83566 (1 μM), a D_1-like receptor antagonist. Activation of PKA with cAMP (0.1-0.5 mM) reduced the response to amphotericin B (1.0 μg ml^{-1}), in a concentration-dependent manner. The specific PKA antagonist, H-89 (10 μM), abolished the effect of both cAMP and SKF 38393. Pre-treatment of OK cells with cholera toxin (500 ng ml^{-1}, overnight) markedly attenuated the amphotericin B-induced decrease in I_{sc} (42.0±3.1 % reduction) and enhanced the forskolin stimulated accumulation of cyclic AMP (95±18 vs. 168±14 % increase over basal values). Altogether, these results suggest the involvement of a G_s class of proteins. Activation of PKC with PDBu (10-1000 nM) reduced the response to amphotericin B (1.0 μg ml^{-1}), in a concentration-dependent manner, with an IC_{50} value of 197 (24, 1630) nM. The inactive analogue PDDC (1000 nM) was devoid of effect. Down regulation of PKA and PKC with overnight exposure of cyclic AMP (100 μM) and PDBu (100 nM), respectively, abolished the inhibitory effects of SKF 38393 (300 nM) and that of their corresponding controls (cyclic AMP 200 μM and PDBu 100 nM). The phospholipase C inhibitor, U-73,122 (3 μM), markedly attenuated the inhibitory effect of 300 nM SKF 38393 (28.8±5.2 % vs. 8.4±11.8 % decrease). The cytochrome P450 inhibitor, ethoxyresorufin (100 nM), failed to alter the inhibitory effect of SKF 38393 (300 nM). In conclusion, it is suggested that the D_1-mediated inhibition of Na^+-K^+ ATPase activity in OK cells involves both the adenylyl cyclase-PKA system and the phospholipase C-PKC system.

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209P STAUROSPORINE-INDUCED CELL DEATH IN MADIN-DARBY CANINE KIDNEY CELLS

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We have recently reported that several protein kinase C inhibitors including staurosporine (STAU) cause cell death in Madin-Darby canine kidney (MDCK) cells (Yang et al. 1998). The present study was designed to investigate the underlying mechanisms.

MDCK cells (cultured in the presence of 0.5% fetal bovine serum) were treated with STAU or vehicle for 20-24 h. Cell numbers in the dish (and sometimes also those floating in the culture medium) were counted. The percentage of viable dead cells was assessed by trypan blue exclusion. The percentage of apoptotic cells was assessed primarily by staining with Hoe 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole; 5 $\mu g\ ml^{-1}$; Alexandrov et al. 1999). Activation of the JNK and p38 forms of mitogen-activated protein kinases was measured by immunoblotting of their tyrosine-phosphorylated forms (Alexandrov et al. 1998). Data are means ± s.e. mean. Statistical significance of differences was assessed by paired t-tests or repeated measures ANOVA followed by Dunnett's multiple comparison test with $p<0.05$ considered significant.

STAU (10-1000 nM) treatment concentration-dependently reduced the number of MDCK cells/dish (control $53.3\pm2.8 \times 10^4$, 1000 nM STAU $25.3\pm1.2 \times 10^4$; n=8; $p<0.01$) and concomitantly increased the percentage of apoptotic cells (from 6.0±0.5% to 89.0±0.9%; $p<0.01$). A separate series of experiments confirmed the induction of apoptosis by 1000 nM staurosporine using DNA-laddering and the TUNEL technique. Additional experiments using 100 nM STAU demonstrated that the loss of MDCK cells in the dish was at least partly due to cell detachment, since the number of MDCK cells floating in the medium was increased from 2.7±0.5 to $11.4\pm1.8 \times 10^4$ cells/dish (n=10, $p<0.001$). Almost all detached cells stained positively with Hoe 33342, i.e. were apoptotic, and approximately 30% of them were unable to exclude trypan blue.

Treatment with STAU (1000 nM) activated the 46 and 54 kDa forms of JNK and p38 to 1915±727%, 3960±2167% and 893±361% of control, respectively (n=16, $p<0.001$ each).

Based on the proposed roles for JNK and p38 in the induction of apoptosis, we used the p38 inhibitor SB 203,580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) to determine whether p38 might mediate STAU effects on MDCK cells. In the presence of 1000 nM STAU, SB 203,580 (1-10 µM) concentration-dependently reduced the percentage of Hoe 33342-positive MDCK cells from 71.7±2.2% to 46.8±1.7% (n=6; $p<0.01$). However, the STAU-induced reduction of MDCK cell number (from 35.1±1.3 to 25.6±0.8, n=12; $p<0.001$) was not affected by up to 10 µM SB 203,580.

We conclude that STAU-induced reduction of MDCK cell number involves apoptosis. This is accompanied by activation of the JNK and p38 forms of mitogen-activated protein kinases and by cell detachment. A p38 inhibitor reduces STAU-induced apoptosis but does not affect reductions of cell number.

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210P DUALISTIC ATP-DEPENDENT ACTION OF NEW SYNTHESIZED 3-4-DIHYDRO-2H-1,4-BENZOXAZINE DERIVATIVES AND CROMAKALIM ON MUSCLE KATP CHANNEL

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Potassium channel openers (KCO) comprise a structurally diverse molecules showing a broad spectrum of applications (Logman and Hamilton, 1992). These molecules exerts their action by opening the ATP-sensitive K^+ channels (KATP), which is widely distributed in the tissues. However, to date molecules selective for skeletal muscle KATP channels were not developed. Preceding experiments have shown that cromakalim (crom.), a benzopyran derivative, restores the resting potential of depolarised muscle fibres of humans and animals affected by periodic paralyses by opening the muscular KATP channel (Tricarico *et al.*, 1999). However, the use of crom. in neuromuscular disorders is limited by the side effects. Searching for molecules more potent than crom. in activating the muscular KATP channel, new 3-4-dihydro-2H-1,4-benzoxazine derivatives were synthesised. All the resulting molecules had an electron-withdrawing group at position C6 of the 3-4-dihydro-2H-1,4-benzoxazine ring, which is essential for conferring KATP channel agonist properties. Two novel modifications were performed; first, an aminopiridine substituent was introduced at position C3 that increases the steric hindrance and hydrogen bond capability of the molecules. Second, the replacement of one hydrogen atom with short methyl and long chain ethyl and propil alkyl groups, at position C2 of 3-4-dihydro-2H- 1,4- benzoxazine ring was explored. This would increase the lipophilic characteristic as well as the steric hindrance of the molecules

Experiments were performed "in vitro" by patch-clamp technique to evaluate the effects of different concentrations of the compounds on macropatch KATP currents from rat skeletal muscle fibres enzimatically isolated, in the presence or absence of internal ATP (100 M). In the presence of ATP, the benzoxazine derivatives ($1\cdot10^{-10}$M-$1\cdot10^{-5}$M) and crom. ($1\cdot10^{-10}$M-$2\cdot10^{-4}$M) enhanced the KATP currents. The order of potency of the molecules as agonists was the propil ($DE_{50}=1.6\cdot10^{-8}$M)>ethyl($DE_{50}=1.1\cdot10^{-7}$M)>crom.($DE_{50}=2.6\cdot10^{-5}$M) > methyl ($DE_{50}=5.8\cdot10^{-5}$M). In the absence of internal ATP, the benzoxazine derivatives ($1\cdot10^{-11}$M-$1\cdot10^{-4}$M) and crom. ($1\cdot10^{-11}$M-$1\cdot10^{-4}$M) inhibited the KATP currents with a biphasic dose-response curves. The order of potency as antagonists was crom.($IC_{50}=1.1\cdot10^{-10}$M)≥methyl ($IC_{50}=3.6\cdot10^{-10}$M)>ethyl ($IC_{50}=7.1\cdot10^{-10}$M) > propil($IC_{50}=1.7\cdot10^{-9}$M). These results suggest that the new benzoxazine derivatives and crom. exert their action on muscle KATP channel by binding on two sites, one stimulatory ATP-dependent site, and one inhibitory ATP-independent site. This phenomenon may explains some controversial effects of KCO such as the antiarrhythmic/proarrhythmic activity (Tosaki *et al.*, 1993) supported by cofin M.U.R.S.T. 98-2000.

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211P Gi PROTEIN COUPLED D_2-LIKE RECEPTORS STIMULATE ATP-SENSITIVE K^+ CHANNELS THROUGH A CYCLIC AMP-INDEPENDENT PATHWAY IN OK CELLS

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We have recently shown that independent activation of D_1–like and D_2–like dopamine receptors in OK cells markedly attenuated decreases in short circuit current (I_{sc}, $\mu A\ cm^2$) induced by amphotericin B (Gomes & Soares-da-Silva, 1999). Classically, a decrease in I_{sc} induced by amphotericin B represents the sodium transport mediated by Na^+-K^+ ATPase. The effects resulting from D_1–like, but not D_2–like, receptor stimulation were accompanied by increases in cAMP, suggesting the involvement of cAMP-dependent and cAMP-independent pathways, respectively. The present study was aimed to evaluate in more detail the effect of D_2–like dopamine agonists on amphotericin B-induced I_{sc} changes in OK cells and the transduction pathways involved. OK cells (ATCC 1840-CRL) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Minimum Essential Medium supplemented with 10% foetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 $\mu g\ ml^{-1}$ amphotericin B and 100 $\mu g\ ml^{-1}$ streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 μg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. Amphotericin B (0.1 - 3.0 $\mu g\ ml^{-1}$) added from the apical cell border of the monolayer induced a concentration dependent decrease in I_{sc}. The maximum effect of amphotericin B was attained at 1.0 $\mu g\ ml^{-1}$, this being prevented by ouabain and substitution of sodium or potassium by choline chloride and caesium chloride, respectively (Gomes & Soares-da-Silva, this meeting). The D_2-like receptor agonist quinerolane (Jose et al., 1992) produced a concentration dependent inhibition of the amphotericin B-induced I_{sc} decrease with an IC_{50} of 252 nM. The effect of 1 μM quinerolane was completely abolished by pretreatment of cells with 1 μM S-sulpiride, a D_2-like receptor antagonist. Blockade of K^+ channels with tetraethylammonium (1 mM, applied from the basal side) or glibenclamide (10 μM) had no significant effect the amphotericin B-induced I_{sc} decrease, but prevented the effects of quinerolane. Pre-treatment of OK cells with pertussis toxin (100 ng ml^{-1}, overnight) also abolished the inhibitory effect of quinerolane (1 μM) on amphotericin B-induced I_{sc} decrease, which suggested the involvement of Gi proteins. Quinerolane (1 μM) affected neither the basal levels of cyclic AMP nor the forskolin (3 μM)-induced increase in cyclic AMP levels (251±29 vs 205±21 % increase). Similar results were obtained in cells pretreated with pertussis toxin. It is concluded that stimulation of D_2-like receptors coupled to Gi proteins leads to stimulation of ATP-sensitive K^+ channels in OK cells, by a cyclic AMP-independent pathway.

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212P HIV-1 Gp120 PROTEIN MODULATES CRF SYNTHESIS AND RELEASE VIA THE STIMULATION OF ITS mRNA FROM THE RAT HYPOTHALAMUS *IN VITRO*: INVOLVEMENT OF *i*NOS.

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Hypothalamo-pituitary-adrenal (HPA) axis alterations are frequently observed during human immunodeficiency virus type I (HIV-1) infection. It is suggested that these alterations are due to the central actions of the virus envelope protein Gp120 (Lortholary *et al*, 1996).

In this study, we examined whether the HIV-1 Gp120 protein can modulate corticotropin releasing factor (CRF) secretion by using the incubation of rat hypothalamic explants as an *in vitro* model. Released and intra-hypothalamic CRF were measured by RIA.

Treatment of the hypothalamic fragments with recombinant Gp120 resulted in a time and concentration dependent increase in CRF release. The maximal dose of 10 nM Gp120 increased CRF release by 56.4% after 1h (control 61.2 ± 5 pg/ml, treatment 96.6 ± 6 pg/ml, $p < 0.01$), and 78.4% after 3h (control 100.6 ± 9 pg/ml, treatment 179.5 ± 6 pg/ml, $p < 0.01$), as compared with their respective controls. The intra-hypothalamic amount of CRF was also increased by 54.7% and 77.3% *vs.* controls after 1 (control 1527 ± 92 pg/hypothalamus, treatment 2362 ± 295, $p < 0.01$) and 3h (controls 1598 ± 118, treatment 2834 ± 332, $p < 0.05$) respectively. This effect is not a consequence of Gp120 toxicity on the hypothalamic neurons, since the citotoxicity index, expressed as released *vs.* intra-hypothalamic LDH activity ratio, was below 5% both in controls and treatments, with no statistical difference between the two groups. Moreover, the action of Gp120 was blocked by pretreatment with cycloheximide, suggesting that the viral protein modulates CRF secretion *via* an increase in its synthesis.

We also investigated the effects of Gp120 on CRF gene expression. RNase protection analysis of total RNA isolated from the explants indicated that 10 nM Gp120 significantly increases CRF mRNA in a time-dependent manner. Furthermore, Gp120 did not modify CRF mRNA stability, suggesting that the viral protein modulates CRF gene expression at the transcriptional level. Analysis of the mechanisms that mediate Gp120-induced CRF synthesis was conducted. The incubation of the explants with recombinant interleukin-1 (IL-1) type I receptor antagonist did not antagonize the actions of Gp120, at 1 and 3h, indicating that the effect of the latter is independent of IL-1-mediated mechanisms. The involvement of some second messenger pathways was also investigated. Specific inhibitors of cAMP-PKA, cyclooxygenase or heme-oxygenase pathways failed to antagonize the Gp120-induced increase in CRF production. By contrast, incubation with aminoguanidine, a selective inhibitor of inducible nitric oxide synthase (iNOS), blocked CRF release and its mRNA accumulation, stimulated by Gp120, whereas selective inhibitors of endothelial and neuronal NOS had no effect.

These results indicate that Gp120 directly stimulates CRF gene expression and peptide synthesis from the rat hypothalamus *in vitro*, *via* the activation of iNOS. Therefore, the actions of this viral protein on the HPA axis may, in part, reflect its ability to modulate CRF synthesis.

Lortholary O, *et al* (1996) J. Clin. Endocrin. Metab. 2, 791-796

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213P IDENTIFICATION OF KEY RESIDUES INVOLVED IN BINDING OF [^{125}I]-PROLACTIN-RELEASING PEPTIDE (PrRP) TO GPR10

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GPR10 is a novel G-protein coupled receptor that is a human counterpart of rat UHR-1 (Marchese et al., 1995). Human prolactin-releasing peptide (PrRP) has been identified as an endogenous ligand for GPR10 and exists in two forms of 20 and 31 amino acids: PrRP-31 and PrRP-20 (Hinuma et al., 1998). In this study we have used site directed mutagenesis to identify key amino acid residues in the receptor which we suggest play important roles in binding of PrRP.

Point mutations to alanine were introduced by the QuickChange PCR-based mutagenesis kit (Stratagene) as follows: Q141A, E212A, E213A, N298A, D302A, and Q317A using the wild type expression vector, pCDN-GPR10 as the template for mutagenesis. The accuracy of all PCR-derived sequences was confirmed by dideoxy sequencing of the mutant plasmids. Wild-type and mutant GPR10 receptors were then transiently transfected into HEK293 cells and cell surface expression was confirmed by His tagging. Radioligand binding assays by saturation analysis using [^{125}I]-PrRP-20 on membranes from these cells were performed as previously described (Langmead et al., 2000). All results are the mean ± s.e.m. of at least 3 independent experiments. In membranes from cells transiently expressing wild type GPR10, specific binding of [^{125}I]-PrRP-20 represented ~90% of total binding and both high and low affinity binding sites could be detected. The K_D and B_{max} values were very similar to values for [^{125}I]-PrRP-20 binding to the stable GPR10 cell line (Langmead *et al.*, 2000). Individual K_D and B_{max} values for each cell line are shown in Table 1. GPR10-E212A exhibits 2 site binding with ~80% specific binding. GPR10-Q141A and GPR10-N298A show ~75% and ~60% specific binding respectively, but binding to the lower affinity site is indistinct and effectively abolished. GPR10-E213A, GPR10-D302A and GPR10-Q317A show no specific binding.

GPR10	K_{D1} (nM)	B_{max1} (fmol mg^{-1})	K_{D2} (nM)	B_{max2} (fmol mg^{-1})
wild-type	0.050 ± 0.012	2605 ± 1072	1.55 ± 0.52	6275 ± 800
Q141A	0.050 ± 0.004	519 ± 43	-	-
E212A	0.0036 ± 0.0007	421 ± 90	0.75 ± 0.14	6196 ± 1602
E213A	-	-	-	-
N298A	0.085 ± 0.018	251 ± 25	-	-
D302A	-	-	-	-
Q317A	-	-	-	-

Table 1. K_D & B_{max} values for [^{125}I]-PrRP-20 binding to GPR10 mutants.

These results suggest that the amino acid residues identified play crucial roles in binding of PrRP to GPR10. Mutations of E213, D302 or Q317 completely abolish specific binding, whereas mutations at Q141, E212 or N298 greatly reduce B_{max} values of the higher affinity site and, for the latter two, abolish binding to the lower affinity site.

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214P C-TERMINUS TRUNCATION OF THE RAT A_{2B} ADENOSINE RECEPTOR PROMOTES ARRESTIN-INDEPENDENT SURFACE RECEPTOR LOSS FOLLOWING PROLONGED AGONIST EXPOSURE

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The distal C-terminus tail of the rat A_{2B} adenosine receptor is required for rapid agonist-induced desensitization and internalization (Matharu *et al*; this meeting). Here we investigated the role of this part of the receptor in mediating long term agonist-dependent desensitization and internalization responses. Chinese Hamster Ovary cells were stably transfected with Wild Type (WT) or Q325stop receptor (last 7 amino acids deleted), as described elsewhere (Matharu *et al.*, this meeting).

Desensitization was measured in intact cells cultured in 24-well dishes. Cells were pretreated with 10μM NECA, washed with PBS to remove the agonist and rechallenged with 10μM NECA for 20 minutes, in the presence of 100 μM Ro201724 as phosphodiesterase inhibitor. The amount of cAMP accumulated during the agonist challenge was measured (pmoles cAMP/mg total protein), and the difference of cAMP produced with and without agonist pretreatment was used as a measure of functional desensitization. Values quoted are means ± s.e.mean. In contrast to shorter periods of agonist exposure (Matharu *et al.*, this meeting), NECA pretreatment (10 μM; 24hrs) led to a similar reduction in WT and Q325stop A_{2B} adenosine receptor responsiveness as compared with cells not pretreated with NECA (27.2 ± 13.6 % and 32.7 ± 8.6 % of control response remaining in WT and Q325stop cells respectively; n=4).

Internalization of surface receptor was quantified by an immunosorbent assay (ELISA; Daunt *et al.* 1997), making use of the HA-epitope tag. After 24 h exposure to 10 μM NECA, similar levels of receptor internalization were observed in WT (69.4 ± 6.7 %) and Q325 stop (56.8 ± 12.7 %) cells (n=5). Imaging of WT and Q325stop cells by confocal microscopy fully supported this finding. To further investigate the mechanism of receptor internalization cells were transiently transfected with dominant negative mutant forms of arrestin-2 (319-410; arr-DNM) and dynamin (K44A; dyn-DNM). Overexpression of these DNM proteins abolished agonist-(NECA; 10 μM; 24 hr) induced WT receptor internalization (53.1 ± 5.8 %, 11.3 ± 2.8 % and 8.3 ± 4.5 % loss of surface receptor in control, arr-DNM and dyn-DNM expressing cells respectively; n=4). Interestingly in Q325 stop cells arr-DNM expression failed to prevent loss of surface receptor whilst the dyn-DNM did abolish receptor sequestration (40.9 ± 3.4 %, 33.5 ± 4.2 % and 8.8 ± 3.7 % loss of surface receptor in control, arr-DNM and dyn-DNM expressing cells respectively; n=4).

These experiments demonstrate that the distal C-terminus tail of the A_{2B} adenosine receptor is not required for long term agonist-induced desensitization and internalization. Removal of the distal C-terminus tail of the receptor abolishes arrestin-dependent agonist-induced loss of surface receptor, suggesting that the mutant receptor internalizes via an arrestin-independent mechanism.

Daunt *et al.* (1997) Mol. Pharmacol., 51, 711-720.

215P ON THE CHRONIC REGULATION OF GAP JUNCTION PROTEIN CONNEXIN 43 (Cx43) EXPRESSION

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Gap junction channels providing intercellular electrical and chemical communication are dodecameric proteins consisting of certain connexin isoforms. Cx43 is one of the most abundant connexins, typically expressed in the heart. These proteins are synthetized in the rough endoplasmic reticulum, transferred to the Golgi apparatus, oligomerized to hexameric connexons and then incorporated into the membrane, where they dock to a connexon of the adjacent cell (Dhein, 1998). Half-life time of Cx43 is ca. 1.5 hours (Darrow et al., 1995, 1996). At present, there is only little known on the chronic regulation of the expression of these connexins. Thus, we wanted to investigate, whether stimulation of protein kinase C (PKC) with 0.1 µM phorbol-12,13-didecanoate (PDD), of protein kinase A (PKA) with 1 mM dibutyryl-cAMP (dBcAMP), of adenylylcyclase (AC) with 10 µM forskolin, or of TNFα-receptor with 10 U/ml TNFα, leads to alteration of Cx43 expression. Cultures of HeLa cells stably transfected with rat Cx43 (HeLa-rCx43, kindly provided by Prof. Willecke, Dept. of Genetics, Univ. Bonn, Germany) were exposed for 24 hours to vehicle or to one of these stimuli in absence or presence of 10 µM of the p38 MAP kinase inhibitor SB203580 or 5 µg/ml brefeldin A (BFA, blockade of protein transfer from endoplasmic reticulum) or 2 µM monensin (blockade of transfer of connexins from Golgi). Each experiment was repeated three times. After exposure, cells were either submitted to Cx43 immunohistology or after lysis to SDS-PAGE and Western blot. We found that Cx43 specific immunofluorescence was significantly enhanced by PDD (17±2), dBcAMP (15,6±3,6), forskolin (15,6±2,1) and TNFα (11,2±1,6) as compared to vehicle control (9.1±1,9 densoitometric units in image analysis). These immunohistological results were in parallel to the Western blots. The increase in Cx43 expression could be completely suppressed by SB203580. BFA and monensin had no clear effect on Cx43 in Western blot, but immunohistology revealed that the increase in Cx43 expression by PDD, forskolin, dBcAMP and TNFα was restricted to the perinuclear area in cells treated with BFA or monensin. We conclude that chronic stimulation of PKC, PKA, or of TNFα receptors can lead to enhanced expression of Cx43 via a p38 MAP kinase dependent pathway. Experiments using BFA indicate, that there may be enhanced synthesis or storage in the endoplasmic reticulum and the sensitivity to monensin shows that the newly synthetized connexins may be transferred to the Golgi apparatus. Thus, gap junction channel expression is not static but can be dynamically regulated in HeLa-rCx43 cells.

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216P CHARACTERISATION OF SUBSTANCE P INDUCED CALCIUM RELEASE BY USING THE NK_1 ANTAGONISTS L-733,060, GR205171 AND MK-0869 IN HUMAN NK_1-CHO CELLS.

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The aim of this study was to characterise substance P-induced intracellular calcium increase in CHO cells expressing the human NK_1 receptor (hNK_1-CHO cells) by using the potent and selective NK_1 receptor antagonists L-733,060 (Seabrook *et al.*, 1996), GR205171 (Gardner *et al.*, 1996) and MK-0869 (Tattersall *et al.*, 2000). After 30min of labelling with the cytoplasmic calcium indicator Fluo-4 AM (2µM), hNK_1-CHO cells (Lundstrom *et al.*, 1998) were washed and incubated in the absence or presence of the antagonists for 60min, at 37°C in Hank's balanced salts with 20mM Hepes, and then non-cumulative concentration-response curves of substance P (SP) (2pM-300nM) were performed. Fluorescence was monitored using a FLIPR (λ_{EX}=488nm, λ_{EM}=510-570nm). Data are expressed as mean±s.e.mean.

SP produced a concentration-dependent increase in cytosolic calcium concentration. The SP effect was biphasic in nature with a fast response (peak) followed by a slow response (plateau). The SP pEC_{50} for the fast response was 10.32±0.07 (n=9), whereas the pEC_{50} of the slow response (measured after 210s from the agonist addition) was 8.88±0.07. The latter had a maximal response of 67.4±3.4% in respect to the response of the fast component. The slow component of NK_1 receptor stimulation by SP was completely abolished by EGTA (5mM) suggesting that extracellular calcium plays a role in the slow response to SP stimulation. L-733,060 (L, 10-100nM; n=3) right shifted in parallel the agonist curves both for the fast and slow response, with no depression of agonist maximal effect. The antagonism was competitive as measured by Schild analysis, since slope factors were not significantly different from one (slope=1.12±0.08, slope=0.93±0.09 for the antagonism of the fast and the slow response, respectively). Therefore, constraining the slope factors to unity, the antagonist pK_B values were 8.70±0.05 and 8.09±0.08 for the fast and slow response to SP, respectively. These values were significantly different (P<0.01). Both GR205171 (GR, 0.3-3nM; n=3) and MK-0869 (MK, 1-10nM; n=3) produced a non-surmountable antagonism of SP-induced fast response that was characterised by a right shift and a concentration-dependent reduction of the maximal response to the agonist. However, the effect of the two antagonists was more pronounced on the slow response of SP in respect to the fast one. 1nM GR and 3nM MK completely abolished the slow response to SP, whereas induced only 24.9±0.9% and 10.2±6.0% of reduction of the maximal effect of the agonist fast component, respectively. Combined dose-ratio analysis (Shankley *et al.*, 1998) between L (50nM) and GR (1nM) or L (50nM) and MK (5nM) revealed an additive effect (syntopic) of the antagonists in the fast response. Statistical analysis for the additive model gave t-test values of 0.13±0.08 (d.f.=11) for GR and 0.14±0.26 (d.f.=11) for MK, which were not significantly different from zero, suggesting that GR and MK are competitive in respect to L. Hence, the unsurmountable effect of GR and MK on SP receptor stimulation is probably due to a pseudo-irreversible phenomenon rather than a non-competitive action (Hale *et al.*, 1998). L also protected the slow phase of SP response from the suppression induced by GR and MK (65.8±2.0% and 61.1±2.5% slow response for L+GR and L+M respectively versus L alone). Therefore, it is suggested that also the effect of GR and MK on the slow component is receptor-mediated.

The observation that L is slightly more active on the fast component, whereas GR and MK were markedly more active on the slow phase of SP response in hNK_1-CHO cells indicates that these two phases, whilst both being activated by the same agonist, can be selectively differentiated by antagonists.

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217P CHARACTERIZATION OF σ_1 RECOGNITION SITES IN NG 108-15 CELLS BY RECEPTOR BINDING AND Ca^{2+} MOBILIZATION

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The term "sigma" (σ) is used to refer to a unique class of binding sites heterogeneously distributed in the nervous sys-tem and in peripheral organs that may serve as receptors for an as-yet-unidentified endogenous ligand. σ recognition sites have been classified into at least two types, termed σ_1 and σ_2; σ_1 recognition sites display preferential affinity for (+) benzo-morphans. Recently, sigma 1 receptor has been cloned (Han-ner et al., 1996). The amino acid sequence does not resemble that of any mammalian protein, contains one putative trans-membrane domain and has 60% homology to a yeast C7-C8 sterol isomerase. The functional role of σ recognition sites and the cellular mechanisms responsible for the effects produced by σ-site ligands have not been clearly determined, although these compounds may act as neuromodulators.

In this study σ recognition sites expressed in the mouse neuroblastoma x rat glioma hybrid cell line NG 108-15 were characterized by receptor binding and ability to modulate intra-cellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) or Ca^{2+} influx in viable cells. NG 108-15 cell membranes possess high affinity binding sites for the σ_1 receptor ligands [^{3}H](+)-pentazocine (K_d= 6.0±0.7nM; values are expressed as menan ± S.E.) and [^{3}H](+)-N-allylnormetazocine (NANM ; K_d=33.3±4.2nM). Saturation analysis of [^{3}H]1,3-di-O-tolylguanidine (DTG) binding, in the presence of 200 nM (+)-NANM to mask the σ_1 sites, confirmed the occurrence of σ_2 recognition sites in this cell line (K_d=16.7±2.1nM). Moreover, NG 108-15 viable cells bind the σ_1 receptor ligand [^{3}H](+)-pentazocine (K_d=32 ± 3.5nM). The affinity toward sigma 1 receptor of several typical sigma ligands was assessed in homogenates of NG 108-15 cells giving the following order of potency: haloperidol > (+) pentazocine >(-) pentazocine > DTG > (+) -3 -(3-hydroxy-phenyl)-*N*-(1-propyl)piperidine (PPP). The affinity of the sigma 1 antagonist NE 100 (Okuyama S. et. al., 1993) was evaluated in homogenates as well as in viable cells. The affinity of this ligand was significantly higher in homogenates (K_i=0.015±0.003nM) than in viable cells (K_i=782±86nM), suggesting a possible lower affinity for σ sites expressed in the plasma membrane. [γ^{35}S]GTP binding (100 pM) to Gα subunit of G proteins was not altered in presence of (+)pentazocine 10^{-5}M compared to controls and at different concentrations of GDP (10^{-7}-10^{-4}M). Therefore, these data exclude the involvement of G proteins in (+) pentazocine interaction with sigma binding sites in NG 108-15 cells. The Ca^{2+} sensitive dye, fluo 3, was used to measure intracellular calcium ($[Ca^{2+}]_i$) in this cell line. (+)-pentazocine (10^{-9}-10^{-6}M) did not modify $[Ca^{2+}]_i$ when applied alone. However, this compound inhibited the increase in $[Ca^{2+}]_i$ provoked by depolarization with KCl (75 mM) in a concentration-dependent manner (EC_{50}=26±2.9nM). The sigma 1 antagonist Ne 100 (10^{-6}M) was unable to abolish this effect. (+)Pentazocine, added after bradikinin (100 nM), increased $[Ca^{2+}]_i$ either in presence or in absence of extracellular Ca^{2+} (EC_{50}=129±25nM and EC_{50}=0.30±0.07nM, respectively). This effect was antagonized by NE 100 (10^{-6} M).

These results suggest that, in NG 108-15 cells, (+)pentazocine may modulate the Ca^{2+} influx during depolarization of viable cells and Ca^{2+} release from internal storage interacting with two different receptor populations, the first insensitive and the second sensitive to the sigma antagonist NE 100.

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218P THE p38 MITOGEN-ACTIVATED PROTEIN KINASE INHIBITOR SB203580 CAN ANTAGONISE $P2X_7$ RECEPTOR-MEDIATED RESPONSES.

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SB203580 is reported as a selective inhibitor of the p38 mitogen-activated protein kinase (p38 MAPK) and has been used to implicate this kinase in inflammatory disease (Young et al.,1997). Since it interacts with the ATP binding site of p38 MAPK (Young et al., 1997) we have investigated its actions at another ATP-binding protein, the $P2X_7$ receptor, and report that SB203580 can antagonise certain $P2X_7$ receptor mediated effects.

Studies were performed using HEK293 cells stably transfected with rat, human or mouse $P2X_7$ receptors (Chessell *et al.*, 1998). Receptor function was determined at 22°C in electrophysiological studies (Chessel et al., 1998) or by measuring 2'-and 3'-O-(benzoylbenzoyl)-ATP (BzATP) stimulated ethidium (Michel *et al.*, 2000) or ^{45}Ca accumulation (Michel *et al.*, 1998). The accumulation studies were performed on cells grown in 96 well plates using buffer (pH 7.4) containing, (in mM), 10 Hepes, 10 glucose, 5 KCl, 0.5 $CaCl_2$ and either 280mM sucrose (sucrose buffer) or 140mM NaCl (NaCl buffer). Data are mean±s.e.mean of 4-8 experiments. pIC_{50} values were estimated against an EC_{50} concentration of BzATP.

Following a 20min preincubation, SB203580 blocked BzATP-stimulated ethidium accumulation in sucrose (pIC_{50} 5.7±0.1) or NaCl buffer (pIC_{50} 6.0±0.1). In contrast, SB203580 (30μM) had no effect at rat $P2X_7$ receptors and only reduced responses at mouse receptors by 32±8%. At human $P2X_7$ receptors, SB203580 was effective without pre-incubation (pIC_{50}=6.1±0.1; NaCl buffer) suggesting a rapid onset of action. The effects of 10μM SB203580 reversed within 2mins of washout. SB203580 (10μM) also blocked BzATP-stimulated ^{45}Ca accumulation at human $P2X_7$ receptors but was less effective in electrophysiology studies.

Thus, inward currents activated by 30μM BzATP were not affected by 10μM SB203580 (78±7% of control P>0.05 Student's T-test) and those to 100μM BzATP were even increased (162±12% of control; P<0.05). The rise times of inward currents to BzATP (30 and 100μM) were increased approximately 2 fold by 10μM SB203580 (i.e. responses exhibited a slower onset).

SB203580 was also examined at chimeric $P2X_7$ receptors in which amino acids (a.a) 1-255 of one species orthologue were ligated to a.a 256-595 of a second species orthologue (Thompson *et al.*, 2001). SB203580 blocked BzATP-stimulated ethidium accumulation at the human-rat chimera (pIC_{50}=5.6±0.1 in sucrose buffer) but even 30μM SB203580 had no effect at the rat-human chimera.

In conclusion, SB203580 can block BzATP-stimulated ethidium and ^{45}Ca flux through the human $P2X_7$ receptor but has no effect at rat or mouse orthologues. The effects at the human $P2X_7$ receptor are rapid and reversible suggesting a direct effect on the receptor rather than an effect mediated through p38 MAPK. Interestingly, SB203580 was less effective at blocking inward currents suggesting that, like calmidazolium (Virginio *et al.*, 1997), it may discriminate between different forms of the $P2X_7$ receptor. The ability of SB203580 to selectively block human-rat chimeric receptors suggests that it interacts with the N-terminal 255 amino acids of the human $P2X_7$ receptor. Finally, these, and other results (Birkenkamp *et al.*, 2000), suggest caution in the use of SB203580 as a specific p38 MAPK inhibitor.

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219P STIMULATION OF MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS VIA $P2Y_1$ AND $P2Y_T$ RECEPTORS IN BRAIN CAPILLARY ENDOTHELIAL CELLS

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The $P2Y_1$ receptor is activated by adenosine tri- and diphosphates and couples to activation of phospholipase C_β (PLC_β) leading to mobilisation of Ca^{2+} from intracellular stores (Barnard *et al.*, 1997). This receptor is present on a clonal line (B10) of rat brain capillary endothelial cells (Foelde *et al.*, 1995). However, this cell line also expresses an additional, yet uncloned P2Y receptor subtype, which is coupled to inhibition of adenylyl cylase upon activation by the same agonists (Webb *et al.*, 1996). This receptor in many ways resembles in its properties, the platelet $P2Y_T$ receptor (Simon *et al.*, 2001). The aim of this study was to investigate the role of the $P2Y_1$ and $P2Y_T$ receptors, endogeneously expressed in B10 cells, in the activation of downstream events such as mitogen-activated protein (MAP) kinase cascades. These pathways have important implications in both regulation of transcription factor activation as well as in functions such as cell differentiation and apoptosis.

B10 cells, in which the only P2Y subtypes detectable are the $P2Y_1$ and $P2Y_T$ receptors, were cultured to confluence, serum starved for 4 h prior to incubation with agonists. Stimulation of particular MAP kinases from whole cell extracts was detected by Western blotting using antibodies specific for the phosphorylated and hence active forms of extracellular signal-regulated kinases (ERK1 and ERK2), stress-activated protein (SAP) kinases and p38 (n=3).

The potent agonist 2-methylthioADP (2-MeSADP, 300 nM) at both $P2Y_1$ and $P2Y_T$ receptors, induced a strong but only transient activation of ERK1 and ERK2 (maximal response at 10 min). A third, yet uncharacterised ERK isoform was also transiently activated by 2-MeSADP. Phosphorylation of the ERKs induced by 2-MeSADP (0.1-100 nM) was concentration-dependent. ADP, ATP (0.1- 10 μM) and 2-MeSATP (10-1000 nM) were also agonists and induced transient phoshorylation of all ERKs detected, although with much lower potency than 2-MeSADP.

2-MeSADP (300 nM) induced a transient increase in the phosphorylation of the p45 and p46 SAPK isoforms, reaching a peak by 10 min and returning to basal levels by 30 min. A strong phosphorylation with a similar kinetic profile of a third SAPK isoform (p43) was also observed. However, this SAPK isoform was not recognised by the phospho-independent antibodies specific to ERK1 and ERK2, p38 or indeed for the SAP kinases, suggesting B10 cells may express an uncharacterised member of the MAP kinase family. The phosphorylation level of the p54 SAPK isoform and all p38 kinases was unaffected by treatment with 2-MeSADP.

The 2-MeSADP-induced ERK activities were only partially blocked by either the $P2Y_1$ receptor selective antagonist, adenosine-2'-phosphate-5'-phosphate (A2P5P, 10 μM) or the $P2Y_T$ receptor selective antagonist 2-propylthioadenosine-5'-(β,γ-difluoro methylene) triphosphonate (AR-C 66096, 1 μM). However, complete blockade of the 2-MeSADP-induced phosphorylation of ERKs was achieved by co-addition of both antagonists.

Activation of ERKs by 2-MeSADP (5 nM) was unaffected by either of the PKC inhibitors, Gö 6976 (10 nM) or Ro31-8220 (50 nM). However, the agonist-induced phosphorylation of the ERK isoforms in the presence of 1 μM AR-C 66096, was abolished by the PI-3 kinase inhibitor, LY 294002 (100 μM), and partially inhibited by PP1 (200 nM), a selective Src inhibitor. In contrast, in the presence of the $P2Y_1$ receptor antagonist, A2P5P (10 μM), the 2MeSADP-induced ERK activity was unaffected by PP1 and partially inhibited by the PI-3 kinase inhibitor.

This suggests that the P2Y receptor subtypes in B10 cells utilise distinct transduction mechanisms to stimulate the ERK pathway.

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220P CORRELATION BETWEEN EFFECTS ON [^{35}S]GTPγS BINDING AND LOW/HIGH AFFINITY RATIOS FOR THE HUMAN 5-HT_{1A} RECEPTOR TRANSFECTED IN HeLa CELLS

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In CHO cells expressing the human 5–HT_{1A}-serotonergic receptor, the selective agonist [^{3}H]8-OH-DPAT labels the receptor coupled to a G protein, whereas [^{3}H]spiperone labels the free receptor (Sundaram et al., 1993). Furthermore, affinities of a range of ligands for [^{3}H]8-OH-DPAT and [^{3}H]spiperone indicated that agonists preferentially label the coupled state whereas antagonists label both states with equal affinity (Sundaram et al., 1995). Spiperone binds to the free receptor with a higher affinity suggesting that this ligand is an inverse agonist (McLoughlin and Strange, 2000). To verify this hypothesis, we have evaluated the affinity for [^{3}H]8-OH-DPAT and [^{3}H]spiperone of WAY 100635 and a number of newly synthesized 5–HT_{1A} ligands. The effects of these compounds on [^{35}S]GTPγS binding were also evaluated.

Affinity of tested compounds for [^{3}H]8-OH-DPAT and their effects on [^{35}S]GTPγS binding in HeLa cell membranes stably expressing the cloned human 5-HT_{1A} receptor were evaluated according to the method previously reported (Testa et al., 1999). Affinity for [^{3}H]spiperone binding in HeLa cell membranes was evaluated with the same procedure utilised for [^{3}H]8-OH-DPAT. Ratio of affinities were evaluated as R = Ki [^{3}H]spiperone / Ki [^{3}H]8-OH-DPAT.

The tested compounds were classified as agonists, partial agonists, neutral antagonists and inverse agonists on the basis of the results obtained in the [^{35}S]GTPγS binding. As summarized by some examples shown in the table, agonists and partial agonists showed R>3, whereas no marked differences were observed between R values of neutral antagonists and inverse agonists. It is concluded that [^{35}S]GTPγS binding describes more accurately the nature of the interaction of ligands with the 5–HT_{1A} receptor.

Compound	[^{35}S]GTPγS	[^{3}H]spiperone	[^{3}H]8-OH-DPAT	R
5-HT	100	22.04	3.00	7.3
8-OH-DPAT	100	32.35	3.44	9.4
Buspirone	77	59.43	19.56	3.0
NAN 190	21	4.41	0.98	4.5
WAY 100635	0	0.11	0.39	0.3
Rec 27/0061*	-29	0.48	0.60	0.8
Rec 27/0224**	-33	0.27	0.19	1.4
Spiperone	-25	7.87	38.44	0.2

The data were calculated from 2 to 3 exp. which agree within ≤ 15%.

Table 1: Data on [^{35}S]GTPγS binding are expressed as % of max 5-HT stimulation. Affinities for [^{3}H]ligands are Ki (nM).

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221P PHARMACOLOGY OF A NOVEL α4β3δ $GABA_A$ RECEPTOR CELL LINE

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The novel $GABA_A$ receptor δ subunit has been found to co-assemble with the α4 subunit with a restricted distribution in the thalamus and hippocampus, δ being primarily extra-synaptic. The α4 subunit is most homologous to the α6 subunit, both produce receptors which are insensitive to diazepam when expressed with β3γ2, but have high affinity for the benzodiazepines Ro15-4513 and bretazenil (Wisden *et al.*, 1991). The role of the δ subunit is currently unclear, as expression in recombinant systems has proved problematic. Previous reports have been contradictory regarding δ sensitivity to neurosteroids (Vicini *et al.*, 1996, Mihalek *et al.*, 1999).

We investigated the pharmacology of the human α4β3δ receptor using a stable cell line generated in mouse Ltk^- cells and the whole-cell patch-clamp technique (for all experiments $n \geq 4$). The α4β3δ receptor exhibited high affinity for GABA 0.5 ± 0.03μM compared to 2.6 ± 0.05μM at α4β3γ2. Efficacy of the partial agonist piperidine-4-sulphonate was higher on α4β3δ than α4β3γ2 (73.2 ± 3.5% of a maximum GABA response, compared to 39.5 ± 9.7%). The agonist THIP was observed to behave as a 'super-agonist' relative to GABA (α4β3δ 162.5 ± 7.4% compared to 66.8 ± 3.0% at α4β3γ2). The δ subunit additionally conferred much slower desensitization to the GABA response with desensitization rate constants of 4.8 ± 0.5 s for α4β3δ and 2.5 ± 0.2 s for α4β3γ2.

The receptor showed low sensitivity for the cation zinc (3.1 ± 0.5μM at α4β3δ and 2.0 ± 0.2μM at α4β3γ2) confirming the inclusion of the δ subunit. α4β3δ receptors were more sensitive to inhibition by the cation lanthanum, with 300μM resulting in 67.0 ± 3.5% inhibition of a GABA EC_{50} at α4β3δ receptors compared to 40.5 ± 0.9% at α4β3γ2. A large degree of potentiation of the GABA EC_{20} was observed by the neurosteroids 5α-pregnan-3α-ol-20-one (312.9 ± 37.3% at α4β3δ compared to 61.2 ± 3.7% at α4β3γ2) and alphaxalone (371.8 ± 42.8% at α4β3δ and 128.1 ± 22.7% at α4β3γ2). The inhibitory steroid pregnenalone sulphate inhibited the GABA EC_{50} with similar IC_{50}'s of 1.2μM and 508nM at α4β3δ and α4β3γ2 respectively. Sensitivity of the GABA EC_{20} to potentiation by a barbiturate was noted, with pentobarbital eliciting 390.0 ± 41.1% at α4β3δ and 260.0 ± 62.7% at α4β3γ2 receptors. As previously observed, the benzodiazepine Ro15-4513 potentiated α4β3γ2 receptors, with a maximum potentiation of 39.5 ± 3.1%, but did not effect α4β3δ (-3.3 ± 1.9%).

The α4β3δ $GABA_A$ receptor not only has a unique distribution within the brain, but also a distinct pharmacology and receptor kinetic profile.

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222P REDUCTION BY GABAPENTIN OF K^+-EVOKED RELEASE OF [^{3}H]-GLUTAMATE FROM THE TRIGEMINAL NUCLEUS OF THE STREPTOZOTOCIN-TREATED RAT

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Gabapentin reduces the symptoms of neuropathic pain in streptozotocin-treated diabetic rats (Field *et al.*, 1999). Recently we showed that gabapentin can inhibit a facilitatory effect of substance P on glutamate release in rat trigeminal slices (Maneuf *et al.*, 2000), and we have now examined the effect of gabapentin on glutamate release in the trigeminal slice from the streptozotocin-treated rat.

Slices of the caudal trigeminal nucleus were prepared from male Hooded Lister rats (300g initially) 4 weeks after treatment with streptozotocin (50mg kg^{-1} i.p., n=48). Prior to sacrifice the presence of static allodynia was confirmed using the von Frey filament method and blood glucose was measured. Slices were incubated in artificial cerebrospinal fluid (aCSF) containing [^{3}H]-glutamate for 30min before transfer to the perfusion apparatus. After a 40-min wash aliquots were collected every 5min for 60min. A 5-min pulse of aCSF containing 30mM K^+ was applied to evoke the release of [^{3}H]-glutamate, with gabapentin, substance P or vehicle present 5 min before and during the high K^+ pulse. Results are expressed as fractional rate of release, with the amplitude of the peak of glutamate release in the presence of gabapentin expressed as a percentage of that in the presence of vehicle. The experiments were conducted in normal (11mM) and in high (30mM) glucose concentrations.
Compared to the normal level of around 8mM, blood glucose was increased in 38 of the streptozotocin-treated rats (28% >33mM, 44% 20-33mM, 10% 10-20mM). Interestingly, all the animals showed a significant degree of allodynia, that did not appear to correlate with blood glucose (52% of rats responded to 3.63gf, 40% to the 2gf hair, 8% to 1.5gf; median response level in controls is 11.75gf).

In the normal rat up to 100μM gabapentin is ordinarily unable to affect release of glutamate from the trigeminal slice, but after streptozotocin treatment, 30μM gabapentin reduced K^+-evoked release of [^{3}H]-glutamate either in normal (3.46±0.42% to 2.8±0.22%, Tukey's $p<0.05$) or high-glucose aCSF (3.31±0.076% to 2.51±0.14, Tukey's $p<0.05$). The uptake of glutamate in slices from streptozotocin-treated rats was reduced in normal glucose (41.7% of control), whereas high glucose restored uptake to normal (84.7% of control). The addition of 1μM substance P caused an increase in evoked release of glutamate in both normal (3.46±0.42% to 4.44±0.48%, Tukey's $p<0.05$) and high glucose (3.31±0.076% to 4.64±0.29%, Tukey's $p<0.05$) that was blocked by gabapentin (30μM) in both normal and high glucose conditions.

In conclusion, after streptozotocin gabapentin is able to reduce the release of glutamate in the trigeminal. It is interesting to speculate that this effect may be of relevance to the anti-hyperalgesic/allodynic actions of gabapentin.

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223P INVESTIGATION OF $GABA_A$ RECEPTORS IN CEREBELLAR PURKINJE NEURONS USING MICE LACKING THE α1 OR β2 SUBUNITS

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Cerebellar Purkinje neurons have been shown to express a limited repertoire of GABA-A subunits, predominantly consisting of α1β2γ2 receptors (Laurie et al., 1992). These cells then provide an ideal candidate for study of the effects of transgenic elimination of the α1 or the β2 subunits.

Whole-cell patch-clamp recordings were made from dissociated cerebellar Purkinje neurons isolated from both the α1-/- and β2-/- mice, at 14-17 days of age, and compared to those of wild type litter mates. Recordings from wild type neurons revealed the presence of robust GABA-mediated currents in all cells tested with a mean amplitude of 2982 ± 271 pA (n=30) in response to 1mM GABA application. GABA EC_{20} currents were enhanced 148 ± 8% by the benzodiazepine modulator chlordiazepoxide at 3μM (n=20), and 182 ± 13% by the α1 selective compound, zolpidem at an α1-selective concentration of 100nM (n=17). In contrast 33/52 of the cells from the α1-/- mice did not produce a response to 1mM GABA. In those cells that did respond to GABA, the currents were significantly reduced in amplitude, (mean of 52 cells, 257 ± 54 pA). Of those cells with GABA currents large enough to measure an EC_{20} response, 5 were studied using the benzodiazepine modulators. The potentiation by chlordiazepoxide was identical to that in wild type animals (138 ± 35%), however the effect of 100nM zolpidem was markedly reduced (34 ± 17%) suggesting that these receptors contained either α2 or α3 subunits.

Similar experiments using the β2 -/- animals revealed a different profile. In contrast to the α1 -/- mice, every cell responded to GABA; however, the mean current amplitude was reduced compared to wild type (1234 ± 144 pA (n=13)). Like the α1 -/- mice, the potentiation by chlordiazepoxide was similar to wild type (104 ± 17% (n=6)); however there was marked potentiation produced by 100nM zolpidem (108 ± 15% (n=5). The effect of zolpidem was significantly reduced compared to wild type mice ($p<0.01$) suggesting that the receptors present contain a mixed population of α1βxγ2 and α2βxγ2. To determine the β-isoform present in the receptors expressed in the β2 -/- mice, the effects of the β-subunit selective agents loreclezole and etomidate were investigated. These compounds selectively potentiate GABA responses mediated by receptors containing a β2 or β3 subunit, but not β1 containing receptors (Wafford *et al.*, 1994, Belleli *et al.*, 1997). Robust potentiation of the GABA EC_{20} by both modulators was observed in both the wild type and β2 -/- mice suggesting that the remaining receptors expressed in Purkinje contained predominantly β3 subunits.

The data provides evidence that the major GABA subtype in Purkinje neurons is α1β2γ2, which is not compensated for in the α1-/- mice. In the β2-/- mice, a proportion of α1β3γ2 is present, suggesting β3 can partially substitute for β2.

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224P MUTANT-SPECIFIC MEXILETINE BLOCK OF MYOPATHIC SODIUM CHANNELS EXPLAINED BY DIFFERENCES IN GATING.

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Hyperkalemic periodic paralysis (hyperPP), paramyotonia congenita (PC), and potassium-aggravated myotonias (PAM) are allelic muscle disorders due to mutations in the α-subunit of the skeletal muscle Na^+ channel (SkM1), which result in an increased Na^+ current (I_{Na}) inducing myotonia or paralysis. Mexiletine (Mex) is a Na^+ channel blocker useful to counteract myotonia. A few studies have shown that myopathic Na^+ channel mutants present different sensitivities to Mex. Here, we ask whether such differences are due to mutation-induced differences in drug binding affinities or in channel gating. Wild-type (WT) and mutant (A1156T for hyperPP, R1448C for PC, and G1306E for PAM) human SkM1 channel α-subunits were transiently expressed in tsA201 cells and I_{Na} was recorded using the whole-cell patch-clamp method. The R(-) enantiomers of Mex and a new derivative (Me7, α-[(2-methylphenoxy)methyl]-benzene methanamine) were applied at final concentration nearby the patched cell. Tonic block (TB) of I_{Na} was measured 3 minutes after drug exposure by applying a single test-pulse from -120 to -20 mV, while use-dependent block (UDB) was evaluated by applying a train of test-pulses at 10 Hz (Desaphy *et al.*, 1999). The IC_{50}s were calculated by fitting the concentration-response relationships with a first-order binding function (Table 1). For both compounds and for all channel types, I_{Na} recorded before and after drug application inactivate with the same time constants, suggesting the lack of open channel block by the drugs. TB and UDB were also determined at a holding potential (h.p.) of -180 mV. In this condition, the IC_{50}s were similar for the 4 alleles ($p<0.05$), being about 800 and 250 μM for TB and UDB by Mex, and 300 and 47 μM for TB and UDB by Me7, respectively. This indicates that resting and fast-inactivated channel binding affinities do not differ between WT and mutant channels. Thus, the different IC_{50}s measured at -120 mV may be due to differences in channel gating. We evaluated the voltage-dependence of channel fast inactivation using a standard double-pulse protocol. The half-maximum inactivation potentials [in mV: -79.6 ± 1.4 (n=38) for WT, -81.4 ± 2.0 (n=21) for A1156T, -66.8 ± 2.0 (n=31) for G1306E, and -90.9 ± 1.7 (n=44) for R1148C] were linearly correlated ($p<0.05$) with the IC_{50}s calculated at -120 mV. This indicates that apparent differences in channel sensitivity to drugs reflect differences in the proportion of resting and fast-inactivated channels at the h.p. of -120 mV. In conclusion, gating differences between Na^+ channel mutants underlie variations in sensitivity to Mex and Me7. (supported by Italian Telethon project #1208)

Table 1. IC_{50}s are given in μM

	Mex TB	Mex UDB	Me7 TB	Me7 UDB
WT	236	37	47	8
A1156T	269	41	47	8
R1148C	48	7	7	1
G1306E	642	130	130	27

Desaphy *et al.*, *Br. J. Pharmacol.* **128:**1165-1174, 1999.

225P ISOSTERIC DERIVATIVES OF MEXILETINE: NEW POTENT USE-DEPENDENT BLOCKERS OF SKELETAL MUSCLE SODIUM CHANNELS WITH POTENTIAL ANTIMYOTONIC ACTIVITY

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Mexiletine is among the few drugs clinically effective in the symptomatic treatment of myotonic syndromes for its ability to reduce the high frequency discharges of action potentials typical of the disease, by blocking voltage-gated sodium channels in an use-dependent manner. However, its use is restricted by various side effects (Rüdel *et al.*, 1994). New analogs of mexiletine were synthesised in order to design more potent and/or tissue-selective use-dependent sodium channel blockers. Previous studies have shown that an increased distance between the chiral carbon atom and the terminal amino group as in 3-(2,6-dimethylphenoxy)-2-methylpropanamine (Me2) and a higher lipophilicity due to the presence of a phenyl group on the asymmetric centre as in α-[(2,6-dimethylphenoxy)methyl]benzenemethanamine (Me4) enhanced the potency and/or the use-dependent behaviour of drug (De Luca *et al.*, 1997, 2000).

The present study was aimed at evaluating the effect of the isosteric substitution of oxygen atom with a sulphur one on Me2 and Me4 to see whether a further increase in lipophilicity could improve the drug activity. The two tio-derivatives obtained, TioMe2 and TioMe4, were tested *in vitro* as enantiomers on sodium current of single fibres of frog semitendinosus muscle by vaseline-gap voltage clamp method (De Luca *et al.*, 2000). The block of peak sodium currents (I_{Namax}) produced by each drug was evaluated by using depolarising steps from -100 to -20 mV at 0.3 (tonic block) and 10 Hz (use-dependent block) frequencies, as previously described (De Luca *et al.*, 2000). Statistical significance of difference between mean values was estimated by unpaired Student's t-test.

Compared to Me2 (De Luca *et al.*, 1997), the maximal half-concentrations (IC_{50}) for tonic block of S(+)-TioMe2 (mean ± s.e.m.: 5.6 ± 0.7 μM, n= 6 frogs/14 fibres) is 20fold lower while the tonic block produced by S(+)-TioMe4 (IC_{50}=11.7 ± 2.6 μM, n= 4/10) is similar to that observed with Me4 (DeLuca *et al.*, 2000). The isosteric substitution maintains the use-dependent behaviour of drug since, at the end of 10Hz frequency stimulation, the IC_{50} values for use-dependent block of S(+)-TioMe2 and of S(+)-TioMe4 are 1.9 ± 0.2 μM (n= 6/14) and 4.7 ± 1.0 μM (n= 4/10) respectively. As Me2 but not Me4 (De Luca *et al.*, 1997, 2000), the tio-derivatives of mexiletine are stereoselective, the S(+)-enantiomer being 2fold more potent than the R(-)-one.

The present data corroborate the role of the lipophilicity for the potency of use-dependent sodium channel blockers and suggest that the tested tio-derivatives of mexiletine might be therapeutically interesting potent drugs to solve the hyperexcitability of myotonic syndromes. (Supported by Italian Telethon project #1208).

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226P MUTATIONAL ANALYSIS OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST BINDING SITES

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Our current model of nicotinic acetylcholine receptors (AChRs) holds that the two agonist binding sites are formed at subunit interfaces. In the foetal muscle receptor (subunit composition $2\alpha_1\beta_1\gamma\delta$), the ACh binding sites are at the α/γ and α/δ interfaces. Similarly, in the main subtypes of heteromeric neuronal AChRs (compositions $2\alpha_3\beta_4$ and $2\alpha_4\beta_2$) agonist binding sites are formed at α/β interfaces. Results from affinity labelling and mutagenesis studies combined with sequence comparisons suggest that each subunit folds according to the same basic pattern and that the binding sites receive contributions from residues in the clockwise face of the α subunits (assuming the orientation set out in figure 1) and from the anticlockwise face of γ, δ, β_2 or β_4 (Prince and Sine, 1998). (Figure 1).

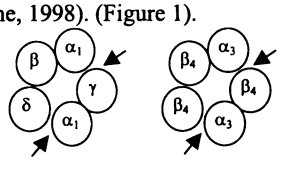

Figure 1. Subunit compositions of the foetal muscle (left) and $\alpha_3\beta_4$ (right) AChRs. The arrows indicate binding sites for ACh.

In this study we aimed to test the model set out above by making a Tyr->Phe mutation at position 93 of the α_3 subunit (Y93F) and the linearly equivalent position in β_4 (Y71F). In other α subunits Y93F causes a dramatic reduction in agonist affinity, suggesting that this residue is located in the agonist binding site and is thus on the clockwise face of the subunit (reviewed in Prince and Sine, 1998). According to the model of the AChR binding sites set out above, β_4Y71 should be in the clockwise face of β_4 and thus should not contribute to an agonist binding site. To our knowledge, the effects of mutating residues equivalent to Y93 in non-α subunits have not been previously examined.

Nicotinic acetylcholine receptors were expressed in stage V/VI *Xenopus* oocytes and responses to nicotine and DMPP (dimethylphenylpiperazinium) measured under two electrode voltage clamp as described by Gerzanich *et al* (1998). Three different combinations of subunits were expressed: $\alpha_3\beta_4$ (wild-type); α_3Y93Fβ_4; $\alpha_3\beta_4$Y71F. Concentration response curves were fitted to the Hill equation using Prism 3.0 (GraphPad Software). The fitted EC_{50} values for each combination are summarised in table 1 and are expressed as mean ± SEM of 3 determinations. Statistical comparisons were made using Students unpaired t-test.

Table 1. Parameters for the activation of wild-type and mutant $\alpha_3\beta_4$ ACh receptors.

	EC_{50} (μM)	
	Nicotine	DMPP
$\alpha_3\beta_4$	97 ± 23	11 ± 0.6
α_3Y93Fβ_4	512 ± 42*	192 ± 44*
$\alpha_3\beta_4$Y71F	57 ± 8	12 ± 0.8

*Log EC_{50} significantly different from wild-type ($P<0.05$)

Our results demonstrate that as observed previously in the α_1 and α_7 subunits (reviewed in Prince and Sine, 1998) α_3Y93F causes a significant reduction in apparent agonist affinity. In contrast, the equivalent mutation in β_4, Y71F, was without effect. These data support the hypothesis that in the α subunits, Y93 contributes to the agonist binding site but that the equivalent residue in β_4 is not in the binding domain.

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227P COMPARISON OF THE BINDING OF [^{3}H]NC(1-13)NH_2, [^{3}H]NCOH AND [^{125}I]Y^{14}NCOH TO RECOMBINANT HUMAN NOCICEPTIN RECEPTORS EXPRESSED IN CHINESE HAMSTER OVARY CELLS.

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Nociceptin (NC) is the endogenous ligand for the nociceptin receptor (NCR) (Calo et al., 2000). Characterisation of the actions of this receptor have been hampered by the lack of selective ligands leading to a paucity of radioligands for characteriation of the receptor. Classic binding studies of this receptor utilise [^{3}H]NC(1-17)OH or [^{125}I]Y^{14}NC(1-17)OH (Calo et al., 2000) or very recently [^{3}H]Ac-RYYRWK-NH_2 (Thomsen et al., 2000). NC(1-13)NH_2 is the minimal fragment that retains full biological activity and in this study we have characterised [^{3}H]NC(1-13)NH_2 in a series of binding studies and compared the data with [^{3}H]NC(1-17)OH and [^{125}I]Y^{14}NC(1-17)OH.

Binding studies were performed using membranes (~2-15μg) prepared from CHO cells expressing the human NCR essentially as described (Okawa et al., 1999) for 30mins at room temperature with ~0.2nM [^{3}H]NC(1-17)OH, 3-4pM [^{125}I]Y^{14}NC(1-17)OH, ~0.5nM [^{3}H]NC(1-13)NH_2 and displacing ligands in various combinations. In displacement studies the concentration of displacer producing 50% displacement was corrected for the competing mass of radioligand to yield K_i. Data are mean±s.e.mean (n≥3)

The binding of [^{3}H]NC(1-17)OH, [^{125}I]Y^{14}NC(1-17)OH and [^{3}H]NC(1-13)NH_2 was concentration dependent and saturable with B_{max} and pK_D values of 1348±44fmol. mg protein^{-1} and 10.06±0.04 (n=6), 1169±76fmol. mg protein^{-1} and 10.45±0.06 (n=3) and 1043±58fmol. mg protein^{-1} and 10.35±0.03 (n=7) respectively. pK_i values of a range of ligands active at NCR and opioid receptors (Calo et al., 2000) are shown in table 1.

Table 1. Comparison of [^{3}H]NC(1-17)OH, [^{3}H]NC(1-13)NH_2 and [^{125}I]Y^{14}NC(1-17)OH in displacement studies. Values are pK_i. NalBzOH=Naloxone benzoylhydrazone.

Displacer	[^{3}H]NC	[^{3}H](1-13)	[^{125}I]NC
NC	Not done	9.91±0.03	Not done
NC(1-13)NH_2	10.28±0.02	Not done	10.47±0.07
NC(1-9)NH_2	7.91±0.09	7.59±0.04	8.33±0.02
[Nphe^1]NC(1-13)NH_2	8.48±0.03	8.52±0.14	8.72±0.01
Ro65-6570	8.27±0.05	8.12±0.02	8.22±0.02
J-113397	8.64±0.02	8.57±0.06	8.61±0.03
Dynorphin A	8.15±0.02	7.71±0.07	8.90±0.09
NalBzOH	8.18±0.06	7.83±0.08	8.68±0.07

There is agreement between values obtained using [^{3}H]NC and the novel [^{3}H]NC(1-13)NH_2. In general, displacement studies with [^{125}I]Y^{14}NC yields pK_i values with slightly higher affinity and this difference is more pronounced for NC(1-9)NH_2, Dynorphin A and NalBzOH. We cannot explain this discrepancy. [^{3}H]NC(1-13)NH_2 may provide a useful alternative to full sequence [^{3}H]/[^{125}I] forms of NC.

Peptides were kindly synthesised by Dr R Guerrini, Univeristy of Ferrara, Italy.

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228P EFFECTS OF Ro 64-6198 IN NOCICEPTIN/ORPHANIN FQ-SENSITIVE ISOLATED TISSUES

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Nociceptin/orphanin FQ (NC) modulates various biological functions by selectively activating a G protein coupled receptor named OP_4. Recently a compound of non peptide nature, Ro 64-6198, has been identified as a potent and selective agonist of human recombinant OP_4 receptors [Jenck et al., 2000]. In this study we investigated the in vitro pharmacological profile of Ro 64-6198 on three NC sensitive preparations, namely the mouse (mVD) and rat (rVD) vas deferens, and the guinea pig ileum (gpI).

Tissues were taken from male Swiss mice (25-30 g), albino guinea pigs (300-350 g), and Sprague Dawley rats (250-300 g). The tissues were prepared according to Bigoni et al. (1999); they were suspended in 5 ml organ baths containing oxygenated Krebs solution and continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 msec duration and 0.05 Hz frequency. The electrically evoked contractions were measured isotonically with strain gauge transducers. Cumulative concentration-response curves (crc) to NC or Ro 64-6198 were performed (0.5 log unit steps). When required, receptor antagonists were added to the medium 15 min before performing agonist crc. Ro 64-6198 was kindly supplied by F. Hoffmann-LaRoche AG, Basel (Dr. F. Jenck), all other drugs were prepared and purified in house. Data are expressed as means ± s.e.m. of n experiments.

In the three isolated tissues, Ro 64-6198 inhibited in a concentration-dependent manner the electrically evoked twitches. In the rVD and mVD the maximal effect of Ro 64-6198 was similar to that induced by NC (about 80% inhibition), however the non peptide agonist was 2 fold less potent than the natural peptide. In the gpI, Ro 64-6198 (pEC_{50} 6.21) showed about 100 fold lower potency than NC (pEC_{50} 8.10) but significantly higher maximal effects (88 ± 1% and 63 ± 3%, respectively, $n \geq 5$, $p < 0.05$ Student t test). In the three tissues the inhibitory effect of Ro 64-6198 has been pharmacologically characterised using the selective OP_4 antagonists [Nphe^1]NC(1-13)NH_2 (30 μM) and J-113397 (0.3 μM). In the rVD, [Nphe^1]NC(1-13)NH_2 and J-113397 displace to the right the crc to Ro 64-6198 without modifying the maximal effects and showing pK_B values (6.30 and 8.05, respectively, $n \geq 5$) similar to those obtained against NC (6.16 and 7.77). In the other preparations neither [Nphe^1]NC(1-13)NH_2 or J-113397 modified the crc to Ro 64-6198.

These results demonstrated that the pharmacological profile of Ro 64-6198 is tissue and/or species dependent. Ro 64-6198 behaves as a potent and selective OP_4 agonist in the rVD but not in the other preparations. These findings may possibly explain why when tested in vivo Ro 64-6198 mimics in the rat but not in the mouse the clear anxiolytic effect elicited by NC [Jenck et al., 1997; Jenck et al., 2000].

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229P ELECTROPHYSIOLOGICAL STUDIES AT CHIMERIC HUMAN AND RAT $P2X_7$ RECEPTORS.

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Recent studies on P2X receptors have identified conserved regions important for ATP binding and gating (Ennion *et al.*, 2000). These regions are conserved between subtypes, yet the potency of ATP varies across P2X receptors, and across species orthologues (Collo et al., 1996;Chessell *et al.*, 1998). This suggests additional residues may contribute to agonist potency. In this study we have constructed chimeric $P2X_7$ receptors of human and rat orthologues to gain further insight into regions that contribute to the species differences in agonist potency between $P2X_7$ receptors.

Rat (R) and human (H) $P2X_7$ receptor cDNAs were bisected at a unique Bgl II restriction site corresponding to amino acid (a.a.) 255 within the putative extracellular domain. Corresponding N- and C-termini were exchanged, ligated and subcloned directionally into pcDNA3.1 to produce cDNAs encoding HR $P2X_7$ and RH $P2X_7$ chimeric receptors. These were stably transfected into HEK293 cells and whole cell recordings (Chessell et al., 1998) made in extracellular buffer containing either NaCl or Na glutamate (both with 0.5mM Ca^{2+} and no added Mg^{2+}). Inward currents were measured at a holding potential of –90mV. Concentration-effect curves (CEC) to ATP or BzATP (2'-&3'-O-(benzoylbenzoyl)-ATP) were determined using a 2s application of agonist every 30s. Data are mean±s.e.mean of 4-14 experiments.

In NaCl buffer, BzATP pEC_{50} values were 5.1±0.1 (R), 5.3±0.1 (HR), 4.2±0.1 (H) and 4.4±0.1 (RH). At HR receptors the Hill slope of the CEC (0.8±0.1) was less than at H (1.8±0.2), R (2.4±0.3) or RH (1.9±0.1) receptors and the CEC appeared biphasic (see below). CEC were also determined using Na glutamate external solution which, as demonstrated previously, increases agonist potency enabling determination of CEC maxima. pEC_{50} values for BzATP in Na glutamate were 6.5±0.1 (R), 6.5±0.1 (HR), 5.2±0.1 (H) and 5.1±0.1 (RH). ATP possessed low potency at R, H and RH receptors in both NaCl and Na glutamate buffer. EC_{50} values were approximately 1mM although CEC did not reach a clear maximum. ATP was a more potent agonist at HR receptors. However, as with BzATP, the CEC was biphasic. In 24/26 cells (NaCl buffer), a plateau in the CEC was obtained with either 30μM ATP or 10μM BzATP (current=1882±219 pA; pEC_{50} for "high-potency" component: ATP 5.4±0.1, BzATP 6.0±0.1). At higher agonist concentrations, a second component of the CEC was observed which did not reach a clearly defined plateau (maximal current 3960±398pA at 3mM ATP or 100μM BzATP).

In a previous study using chimeric receptors (Thompson *et al.*, 2000) we found that regions of the receptor preceding a.a. 255 were major determinants of agonist potency at human and mouse $P2X_7$ receptors and contributed to the lower potency of BzATP for mouse than for human $P2X_7$ receptors. In contrast, in this study, residues after a.a. 255 seem to determine the high potency of agonists at rat $P2X_7$ receptors. It is not known whether the differences in potency reflect differences in agonist affinity or the gating properties of the receptor. With the HR chimera, agonist potency for the "high-potency" component of the CEC was higher than at H or R receptors suggesting that separate regions in the H and R receptors may interact in an additive/synergistic manner to increase agonist potency.

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230P THE PROTECTIVE EFFECT OF ETHANOL AGAINST gp120-INDUCED CYTOTOXICITY INVOLVES THE INHIBITION OF A Ca^{2+}-ACTIVATED NO/cGMP PATHWAY

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HIV-1 coat glycoprotein gp120 induces death of human CHP100 neuroblastoma cells via a mechanism which involves abnormal activation of N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Corasaniti et al., 1995). It is well established that acute exposure to ethanol reduces the excytotoxic effects elicited by excessive stimulation of NMDA receptors in primary neuronal cell cultures (see Wirkner et al., 1999). Here we report the original observation that acute exposure to ethanol reduces intracellular calcium concentration ($[Ca^{2+}]_i$), attenuates gp120-dependent increase in nitric oxide (NO) levels, and inhibits the cytotoxic effects induced by gp120 in CHP100 neuroblastoma cell cultures (Corasaniti et al., 1995). Cell viability was estimated by trypan blue exclusion. Intracellular free calcium concentration ($[Ca^{2+}]_i$) was detected fluorimetrically, by measuring changes in intracellular Fura2/AM fluorescence intensity. NO levels were determined by Griess reaction and cGMP production was assessed by enzyme immune assay (EIA). Statistical analysis was performed by using ANOVA test.

In agreement with our previously published data (Maccarrone et al., 1998), a brief exposure (20 min) of CHP100 cells to 10 pM gp120 induced a significant ($P<0.001$ *vs* control; n=6) cytotoxic effect that was reduced by a pre-incubation (10 min beforehand) with 0.1% (17 mM) ethanol (16.7 ± 0.66% cell death; $P<0.01$ *vs* gp120; n=6). Higher ethanol concentrations, up to 0.3% (51 mM) and 0.5% (86 mM), conferred greater protection (9.6 ± 0.4% and 8.7 ± 0.33%, respectively; n=6 experiments per group) against gp120-elicited cell death ($P<0.01$ *vs* gp120). Moreover, addition of gp120 (10 pM) to CHP100 cells resulted in a 135% enhancement in $[Ca^{2+}]_i$ ($P<0.001$ *vs* control; n=6). In the presence of 0.1%, 0.3% and 0.5% ethanol, $[Ca^{2+}]_i$ was significantly lower than that detected in cell suspensions treated with the viral protein alone, with a decrease of 13% ($P<0.05$ *vs* gp120; n=6), 18% ($P<0.01$ *vs* gp120; n=6) and 24% ($P<0.001$ *vs* gp120; n=6), respectively. Similarly, addition of gp120 (10 pM) led to a significant enhancement of NO_2^- production with a 195% increase ($P<0.01$ *vs* control), which was significantly attenuated ($P<0.05$ *vs* gp120; n=6 sets of experiments) by pretreatment with 17 mM (-40%), 51 mM (-43%) and 86 mM (-45%) ethanol. The increase in NO_2^- induced by gp120 (10 pM; n=6) was paralleled by a significant ($P<0.001$ *vs* control) rise of cGMP levels (424%). When 0.1% ethanol was added 10 min before incubation with the viral protein, we observed a 41.5% ($P<0.001$ *vs* gp120) decrease in cGMP production. Pretreatment with 0.3% and 0.5% ethanol produced a further reduction of gp120-induced cGMP accumulation, calculated as 69.8 and 72.7%, respectively ($P<0.001$ *vs* gp120).

These findings demonstrate that an acute exposure to ethanol, within a range of clinically relevant concentrations (20-100 mM), prevents gp120-induced CHP100 cell death by inhibiting a Ca^{2+}-activated NO/cGMP pathway.

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231P CHARACTERISATION OF THE β-ADRENOCEPTOR SUBTYPES IN URINARY BLADDER OF THE PIG

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β-adrenoceptors mediate relaxation of bladder detrusor muscle in several species. This relaxation may be mediated via β_1, β_2, β_3-receptors or a mixture of these subtypes (Oshita et al., 1997). This study investigates the characterisation of β-adrenoceptor subtypes in pig urinary bladder.

Cell membranes were obtained from bladder dome of female pig, with the mucosal and serosal layers removed. Tissues were pulverized and homogenized. Homogenates were centrifuged twice at 45,000 ×g for 10 minutes at 4°C. Saturation experiments were conducted with 7 different concentrations (0.25 to 16 nM) of [^{3}H]DHA. Competition experiments with [^{3}H]DHA used different concentrations of unlabelled antagonists (ICI 118551 0.1nM-1μM and CGP 20712A 0.1nM-2mM) to determine the drug affinity. Tissue homogenates were incubated (37°C) with 1.3nM [^{3}H]DHA. Nonspecific binding represented [^{3}H]DHA bound in the presence of 1μM of unlabelled propranolol. In functional experiments, pig detrusor strips (dome region) were set up in gassed Krebs solution at 37°C and concentration-relaxation curves to β-agonists (isoprenaline, salbutamol, CGP12177 and BRL37344) were obtained in the absence and presence of antagonists. Antagonist affinity (pK_B values) and Schild plots were constructed from shifts of concentration-relaxation curves to β-agonists.

In competition binding, displacement of [^{3}H]DHA by the β_1-selective antagonist CGP20712A best fitted binding to a single receptor with a low affinity (pKi=4.84), suggesting that β_1-receptors were not present. Displacement binding with ICI 118551 (β_2-selective antagonist) best fitted a two site model with only 26% of binding sites having a high affinity (pKi=8.77) (β_2-receptor s).

In functional studies *in vitro*, isoprenaline (non-selective β-agonist) and salbutamol (β_2 -selective agonist) demonstrated relaxation with high potency. However CGP12177 and BRL37344 (β_3 -selective agonists) had a low potency on the bladder and the concentration-response curves were shallow with Hill slopes of 0.47 and 0.04, respectively.

Propranolol antagonised concentration-relaxation curves to isoprenaline with high affinity (pK_B=9.31±0.12). CGP20712A(β_1-selective antagonist) did not antagonise these responses below 30μM, and atenolol (β_1-selective antagonist) antagonised responses with a low affinity (pK_B=6.13±0.08). ICI118551 (β_2-selective antagonist) antagonised responses to isoprenaline with a high affinity (pK_B=7.9±0.07), but the Schild slope was low (0.62±0.03) suggesting that responses were mediated by more than one β-receptor. ICI118551 had the same effects on concentration-relaxation curves to isoprenaline after β_1-receptors had been blocked by 1μM CGP20712A. ICI118551 antagonised responses to salbutamol with high affinity (pK_B=8.7±0.08), but analysis of the Schild slope was low (0.77±0.20). The Schild plot for SR59230A (β_3-selective antagonist) was biphasic, apparent pK_B values for 3-10nM SR59230A being 8.6± 0.08 and those for 30nM-1μM being 7.7±0.1.

These *in vitro* data suggest that β-mediated responses of the pig bladder are predominantly mediated via the β_2-subtype but the data also suggests a contribution from β_3-adrenoceptors.

Oshita et al.,(1997) *Br J Pharmacol.*,;122:1720-4

232P ET-1 SENSITIZES ACTIONS OF VASOCONSTRICTOR AGENTS IN RAT ISOLATED PERFUSED LUNGS

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Previous studies have shown that endothelin-1 (ET-1) can increase the sensitivity of the contractile apparatus to Ca^{2+} in permeabilized rings of rat pulmonary artery (Evans *et al.*, 1999). In the present study we have investigated whether ET-1 can similarly sensitise the pulmonary vascular bed in perfused rat lungs, which contains fine resistance vessels.

Male Wistar rats (280-340g) were anaesthetised with pentobarbital sodium (100 mg/kg, I.P.) and heparinized (500 IU, I.V.). The pulmonary artery was cannulated, perfused with Krebs solution at 37°C, aerated by 20% O_2, 75% N_2 and 5% CO_2 and pulmonary perfusion pressure (PPP) recorded via a pressure transducer (Lal *et al.*, 1994). Lungs were artificially ventilated with room air at 28 strokes/min and equilibrated for 15 min before adding drugs. Preliminary experiments studied the optimal ET-1 concentration (0.3–3 nM) and infusion time (15-60 min) for sensitization. These were found to be ET-1 1nM, the sub-threshold concentration, for 30 min (n=7). Dose-responses to phenylephrine (PHE) and angiotensin II (ANG II) were recorded before and after infusing ET-1 (1nM) for 30 min. Antagonists, which did not affect basal PPP, were infused for 15 minutes before injection of PHE and ANG II. All data shown are mean ± s.e.mean. Data were analysed by unpaired Student's t-test or paired t-test and significance was accepted if $P < 0.05$.

ET-1 shifted PHE and ANG II response-curves to the left (figure 1). Responses to 10 nmoles PHE were increased from 2.96 ± 0.25 mmHg to 3.86 ± 0.45 mmHg ($p<0.05$, n=12). Responses to 10 pmoles ANG II were increased from 0.99 ± 0.17 mmHg to 2.57 ± 0.42 mmHg (n=8, $p<0.05$). Time-matched studies showed that PHE responses

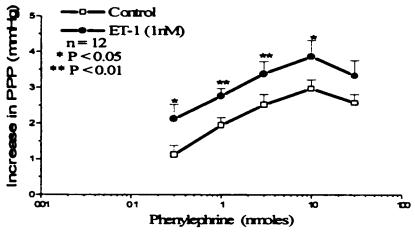

Figure 1 Effect of ET-1 (1nM) on PHE responses in rat isolated perfused lungs.

repeated after 30 min were not significantly different to controls. The selective ET_A receptor antagonist, PD156707 (5μM) and the selective ET_B receptor antagonist, BQ788 (5μM) reversed the potentiated responses to PHE and ANG II by ET-1. PPP responses to 10 nmoles PHE and 10 pmoles ANG II with ET-1 were reduced by PD156707 (5μM) from 3.86 ± 0.45 mmHg to 1.91 ± 0.26mmHg (n=4-12, $P<0.01$), and 2.57 ± 0.42 mmHg to 0.95 ± 0.24mmHg (n=4-8, $P<0.01$), respectively. BQ788 (5μM) shifted PPP responses to 10 nmoles PHE and 10 pmoles ANG II with ET-1 from 3.86 ± 0.45 mmHg to 2.01 ± 0.57mmHg (n=5-12, $P<0.05$) and 2.57 ± 0.42 mmHg to 0.93 ± 0.33mmHg (n=5-8, $P<0.05$), respectively. These results suggest that ET-1 can sensitize the pulmonary resistance vessels to vasoconstrictors and this action involves both ET_A and ET_B receptors.

Q. Yu holds a University studentship and ORS award.

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233P RELAXATION TO BRADYKININ IN BOVINE PULMONARY SUPERNUMERARY ARTERIES CAN BE MEDIATED BY BOTH A NITRIC OXIDE -DEPENDENT AND -INDEPENDENT MECHANISM

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The aim of the present study was to determine the relative contribution of nitric oxide, PGI2 and potassium channels in bradykinin-induced relaxation of bovine supernumerary arteries.

Bovine lungs were obtained fresh from the local abattoir. Segments of supernumerary arteries (diameter 0.5 - 1 mm) were dissected from the lung and freed of surrounding connective tissue. The vessels were then weighed and suspended between stainless steel hooks in Krebs-Henseleit buffer (37°C) under a tension of 1 g and gassed with a mixture of O2:CO2 95%/5% v/v). In some rings the endothelium was removed by abrading the luminal surface with forceps. The tissues were allowed to equilibrate for 1 hour then contracted with U46619 (0.3 μM). Concentration response curves for bradykinin-induced relaxation were constructed. Paired tissues acted as time controls. Relaxations are expressed as % decrease of the U46619-induced tone. Results are means ± s.e. mean. The significance of differences was determined using Student's t-test.

In endothelium-intact, but not denuded rings, bradykinin (100 pM-100 nM) produced a concentration-dependent relaxation (pEC50, 9.6±0.1; maximum relaxation (Rmax.), 100 %, n=25), which was unaffected by preincubation (40 min) with the cyclooxygenase inhibitor indomethacin (10 μM), the guanylyl cyclase inhibitor ODQ (10 μM, Brunner *et al.*, 1996). Preincubation with the nitric oxide synthase inhibitor inhibitor L-NAME (100 μM, 40 min) reduced the tissue sensitivity to bradykinin without inhibiting the maximum relaxation (pEC50, 9.0±0.3, $p<0.05$; Rmax., 87±9 %, n=13). The response in the presence of indomethacin and L-NAME was similar to that of L-NAME alone.

In rings preincubated with indomethacin and L-NAME the bradykinin-induced relaxation was abolished by increasing the extracellular potassium concentration to 30 mM. Blockade of large-conductance calcium-sensitive (BKCa) and voltage-dependent (Kv) potassium channels with charybdotoxin (ChTX, 100 nM) inhibited the maximum relaxation (Rmax., 48±12 %, n=4). Blockade of BKCa alone with iberiotoxin (IbTX, 100 nM) reduced the tissue sensitivity (pEC50, 7.9±0.5; $P< 0.05$, n=4) but did not impair the maximum relaxation. Blockade of the small-conductance calcium-sensitive potassium channels (SKCa) with apamin (100 nM) did not alter the response, but the combination of apamin and ChTX abolished the relaxation. The combination of apamin and IbTX inhibited the maximum relaxation (Rmax., 33±12 %, n=4).

Although preincubated with L-NAME/ODQ did not prevent bradykinin-induced relaxation, the addition of ODQ (10 μM)/L-NAME (100 μM) to preconstricted rings relaxed with bradykinin (100 μM) reversed the bradykinin-induced relaxations (% reversal of relaxation, ODQ, 129±7; L-NAME, 124±6, n=5)

These results suggest that the nitric oxide/guanylyl cyclase pathway can mediate relaxation. However, when this pathway is blocked, by preincubation with L-NAME/ODQ, a compensatory mechanism involving an EDHF-activation of BKCa, SKCa and Kv mediates relaxation to bradykinin.

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234P THE ACUTE EFFECTS OF *d*-FENFLURAMINE ON PULMONARY PRESSOR RESPONSES TO 5HT AND HYPOXIA IN THE BLOOD-PERFUSED, ISOLATED RAT LUNG PREPARATION

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Chronic treatment with the 5-hydroxytryptamine (5HT)-releasing agent, *d*-fenfluramine (*d*F), is associated with the development of pulmonary hypertension (PH) in man (Abenhaim *et al.* 1996). In the present study, we have investigated the acute effects of *d*F on the changes in pulmonary arterial pressure (ΔPpa) evoked both by 5HT, a monoamine implicated in PH, and by hypoxic pulmonary vasoconstriction (HPV) in the isolated lung preparation of Wistar rats. For comparison, these studies were also performed using the fawn hooded (FH) rat, which has a 5HT storage defect and an increased susceptibility to spontaneous PH (Sato *et al.*, 1992).

Male Wistar and FH rats (8-12 weeks, n=6), were anaesthetised (sodium pentobarbitone, 60 $mg.kg^{-1}$, i.p.), the lungs were isolated *in situ*, ventilated (air + 5% CO_2) and perfused with autologous blood at constant flow (20 $mL.min^{-1}$, 37°C). The effect of cumulative doses of *d*F (4-100 μM perfusate concentration) on basal Ppa and ΔPpa to 5HT (10 μM) and HPV (hypoxic ventilation 2% O_2 +5% CO_2) were determined. Results are presented as mean ΔPpa ± s.e.mean mm Hg) from the pre-dose baseline. Statistical analysis employed a mixed linear model with autoregressive correlation structure followed by Dunnett's test. Table 1 shows the dose-related and sustained increases to *d*F alone (4-100 μM), upon which the responses to 5HT and hypoxia were superimposed. In the presence of increasing doses of *d*F up to 40 μM, responses to 5HT and hypoxia were significantly increased in both strains. After the highest dose of *d*F (100 μM), responses to 5HT were reduced to levels similar to those obtained prior to *d*F dosing while those to hypoxia remained elevated.

The finding that vasoconstrictor responses to 5HT but not to hypoxia were selectively attenuated by acute *d*F endorses similar sensitivities of the 5HT-evoked responses seen with chronic *d*F dosing (Marriott *et al.*, 1998). This selective action of dF could reflect its postulated ability to enter cells via the 5HT transporter (Rothman *et al*, 1999), subsequently evoke the release of 5HT and cause a desensitisation of the 5HT receptor(s) responsible for pulmonary vasoconstriction.

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Marriott, H.M., Emery, C.J., Poole, R. *et al.* (1998) *Eur. Resp. J.* **23**, 141S.
Rothman, R.B., Ayestas, M.A., Dersch, C.M *et al.* (1999) *Circulation* **100**, 869-875.

Table 1. Effect of *d*F on Ppa and 5HT- and hypoxia-induced pressure changes in vessels of isolated lungs from Wistar and fawn hooded rats.

Cumulative	Ppa responses to *d*F		ΔPpa responses to 5HT (10 μM)		ΔPpa responses to hypoxia (2% O_2 + 5% CO_2)	
*d*F (μM)	Fawn hooded	Wistar	Fawn hooded	Wistar	Fawn hooded	Wistar
0	-	-	7.1 ± 0.6	5.6 ± 0.3	14.4 ± 1.3	8.7 ± 1.8
4	4.0 ± 0.3	4.2 ± 0.2	8.9 ± 0.9*	7.8 ± 0.7**	18.0 ± 1.3*	13.9 ± 1.5**
40	7.8 ± 0.4	8.1 ± 0.2	11.9 ± 1.6***	9.0 ± 1.1***	21.9 ± 1.4***	18.4 ± 1.9***
100	12.7 ± 1.1	13.4 ± 0.6	9.1 ± 1.7	5.4 ± 0.9	19.7 ± 1.8*	17.0 ± 1.5***

Values are mean ± s.e.mean Ppa or ΔPpa in mm Hg (n=6). ***$p<0.001$, **$p<0.01$, *$p<0.05$ compared to values obtained prior to *d*F treatment.

235P 5-HT MODULINE IS AN ENDOGENOUS MODULATOR OF 5-HT_{1B}-MEDIATED CONTRACTION IN RABBIT PULMONARY RESISTANCE ARTERIES

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5-HT-moduline (5-HTm) is a tetrapeptide (Leu-Ser-Ala-Leu) recently identified in cerebral tissue which acts as a specific endogenous antagonist to 5-$HT_{1B/1D}$ receptors with a high affinity (Massot et al., 1996). We have previously shown that the 5-HT_{1B} receptor plays a critical role in the control of human pulmonary vascular tone (Morecroft et al., 1999). Here we have investigated the influence of 5-HT-moduline on responses to the 5-HT_1 receptor agonist, 5-carboxamidotryptamine maleate (5-CT) in isolated rabbit pulmonary resistance arteries (PRAs), taken from male white New Zealand rabbits (3.5kg). We also examined the effect of bestatin, which inhibits the aminopeptidase believed to cleave the peptide into two dipeptides in vivo.

PRAs (~200-300μ i.d) mounted in wire myographs were bathed in Krebs solution and bubbled with 16% O_2 / 5% CO_2 balance N_2. A transmural pressure equivalent to 12-16mmHg was applied to the tissue to give values similar to that in vivo. Cumulative concentration response curves to 5-CT (10^{-8}-10^{-4}M), 5-CT plus 5-HTm (10^{-5}M) and 5-CT plus 5-HTm plus bestatin (10^{-6}M) were constructed.

pEC_{50} values and maximum responses (Emax, % response to 50mM KCl) to 5-CT are summarised in table 1. We observed a significant reduction to Emax in the presence of 5-HTm. Athough 5-HTm greatly reduced the maximum response there was no significant effect on the pEC_{50} values.

Preliminary experiments indicate that bestatin further potentiates the effect observed with 5-HTm and completely abolished the contraction in one experiment. These findings also show that bestatin has no inhibitory effects on the 5-CT responses on its own.

This study demonstrates the ability of 5-HTm to modulate 5-HT_1 receptor responses in rabbit PRAs and provides the first evidence of a role for 5-HTm in the peripheral vasculature.

Table 1. Maximum responses (Emax) & pEC_{50} values (±SEM) obtained with 5-CT in rabbit PRAs

	Emax	pEC_{50}	n
5-CT	88.76±5.98*	5.56±0.20	10
5-CT+5-HTm	51.11±14.74*	4.28±0.79	10
5-CT+5-HTm + bestatin	15.17±13.49	4.55±1.85	4

Statistical comparisons were made using a Students unpaired t-test (*P<0.05) compared to control. n= number of animals

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236P ROLE OF SENSORY NEUROPEPTIDES IN *PARIETARIA JUDAICA*-INDUCED AIRWAY HYPERRESPONSIVENESS IN SENSITIZED RABBIT

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Airway hyperresponsiveness (AHR) in response to spasmogens is a hallmark of asthma (O'Byrne PM and Inman MD., 2000). C-fibres have obtained considerable attention in the context of airway hyperresponsiveness. Among the different inflammatory mediators involved in asthma, tachykinins, such as substance P and neurokinin A, localized in capsaicin-sensitive sensory C-fibres, might be involved in the pathogenesis of this disease (Barnes PJ, *et al.*, 1991). Direct evidence for the involvement of endogenous tachykinins in human allergic airway disease, however, has thus far not been obtained. The purpose of this study is to investigate the role of capsaicin-sensitive sensory C-fibres and tachykinins in an allergic rabbit model to Par j1, a major allergen of *parietaria judaica* pollen, the most active among pollens distributed around the Mediterranean area (D'Amato G, *et al.*, 1996).

In spontaneously ventilated and anaesthetized rabbits [diazepam (25 mg/kg i.p.) and subsequently administered Hypnorm ® (0.4 ml/kg; a mixture of fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg/ml, i.m.). airway responsiveness to histamine was determined by exposing *parietaria judaica* allergic rabbits to cumulative concentrations of aero-solised histamine (1.25 - 80 mg ml^{-1}; 2 min per concentration), dis-solved in saline, and administered directly to the lungs *via* an endo-tracheal tube, after pre-treatment with either capsaicin or tachykinin antagonists. The provocation concentrations (PC) of histamine which produced a 50% increase in lung resistance (R_L) (PC_{50}) or a 35% fall in dynamic compliance (C_{dyn}) (PC_{35}) were determined for each animal by linear interpolation and used as indices of airway responsiveness.Bronchoalveolar lavage was performed immediately following histamine challenge and total and differential cells counts were performed.

Bartlett's test for homogeneity of variances was used on all data to determine whether parametric or non-parametric statistics were to be applied. For the lung function studies, statistical analysis was performed on log_{10} transformed data (PC_{50} and PC_{35}) in order to normalize the distribution of the data and to allow the application of parametric statistics. The paired t-test was used for the histamine lung function data before and after allergen challenge. Bronchoalveolar lavage cell data were analysed using the Kruskall-Wallis one-way analysis of variance. Results were considered significant if P <0.05.

Par j1, major allergen of *parietaria judaica*, induced AHR to inhaled histamine, in Par j1 immunised rabbits, [PC_{50} (mg/ml): 39.39 ± 2.06 ; 10.29 ± 1.12 (n=8) and PC_{35} (mg/ml): 36.84 ± 1.7 ; 9.44 ± 0.86 (n=8), prior to and 24 h after Par j1 challenge respectively]. Capsaicin pre-treatment (total dose of 80 mg/kg s.c. administered over a 3-day period), completely inhibited the AHR achieved 24 h following antigen exposure (P< 0.01). Pre-treatment with SR48968, an NK_2 receptor antagonist, significantly reduced the antigen induced AHR (P< 0.05), while pre-treatment with SR140333 and SR142801, NK_1 and NK_3 receptor antagonists respectively, did not significantly modify it. Bronchoalveolar lavage fluid obtained from vehicle and capsaicin-treated rabbits challenged with Par j1 showed no significantly differences either in total and differential cell counts between these two groups.

Parietaria judaica-induced AHR in immunised rabbits was shown to be inhibited by pre-treatment with capsaicin, an effect that is not related to an action on the associated pulmonary infiltration of eosinophils. The involvement of NK_2 receptor stimulation in this phenomenon, suggests that NK_2 receptor antagonists may be useful for investigating mechanisms of bronchopulmonary alterations in asthmatic patients.

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237P DIFFERENTIAL EXPRESSION OF mRNA FOR ION CHANNELS AND TRANSPORTERS IN CYSTIC FIBROSIS (CF) AND NON-CF HUMAN LUNG SAMPLES.

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Cystic fibrosis (CF) is an inherited disease, and arises as a result of genetic mutation in the cystic fibrosis transmembrane regulator (CFTR) chloride channel in all areas of the body. In the lung, this causes increased sodium absorption and decreased chloride and bicarbonate secretion, leading to insufficient fluid transport into the airways. In this study, we have measured the levels of mRNA for 2 ion channels and a transporter, which may play a pivotal role in fluid transport, in order to investigate their relative expression in CF lung.

Sections of human lung (tertiary/quaternary bronchus and parenchyma) were obtained from patients with CF (n=25) or non-smoking, non-CF (normal) patients (n=17). All tissue samples were obtained through medically qualified intermediaries with the informed consent of the donor, and with approval of the local ethics committee. Tissues were snap frozen in liquid nitrogen and stored at -80°C until use. After extraction of total RNA, the levels of expression of mRNA for CFTR, AE2 (anion exchanger type 2) and ENACα (amiloride-sensitive epithelial sodium channel, alpha sub-unit, which is the most predominant ENAC sub-unit in human lung) were determined by quantitative reverse transcription-polymerase chain reaction using a sequence detection system (ABI PRISM™ 7700). The level of a ubiquitously expressed housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) was determined in the same reaction and was used to provide an internal marker of mRNA integrity within the experiment (see Bowen *et al.* (2000) for methods). Statistical analyses used an unpaired t-test with Bonferroni correction, with $p<0.05$ taken to indicate significance.

When normalised to the levels of mRNA for GAPDH, the levels of both CFTR and AE2 were significantly increased in CF bronchus, but there was no significant difference in the levels of ENACα (Table 1). There were no significant differences in the levels of mRNA for these genes between CF and normal lung parenchyma.

	Bronchus		Parenchyma	
Target	Log Copy No.	Fold Increase	Log Copy No.	Fold Increase
AE2	2.1±0.1	3.4**	1.8±0.1	NSD
CFTR	4.0±0.1	2.4*	4.0±0.1	NSD
ENAC	4.6±0.1	NSD	4.5±0.1	NSD

Table 1. Expression of mRNA in normal and CF airways. Data are expressed as log copy number (mean ± s.e.mean) in CF lung, and fold increase in mRNA copy number compared to normal lung (normalised to GAPDH). * indicates $p<0.05$, ** indicates $p<0.01$, NSD indicates no significant difference. Log copy numbers are per 100ng of total RNA.

In summary, we have shown that mRNA for CFTR and AE2 are significantly increased in CF bronchus. This may represent an attempt to correct the deficient ion and fluid transport seen in CF airways, and is consistent with Lee *et al.* (1999) who reported CFTR-mediated upregulation of AE2 function. The lack of differential regulation of ENACα in CF lung may support the theory that loss of CFTR regulates ENACα at the level of channel gating, by increasing open channel probability, rather than increases in channel number (Stutts *et al.*, 1997).

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238P S-HEXYL-GLUTATHIONE CHANGES RECEPTOR PREFERENCE AND METABOLISM OF LEUKOTRIENE D_4 IN THE GUINEA PIG TRACHEA

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Cysteinyl-leukotrienes (cysLTs; LTC_4, LTD_4 and LTE_4) are potent airway constrictors and mediators of asthma. In the guinea pig trachea, LTD_4 and LTE_4 activate a $CysLT_1$ receptor whereas LTC_4 activates a $CysLT_2$ receptor (Snyder *et al.* 1987). LTC_4 and LTD_4 are metabolised into LTE_4 (Snyder *et al.* 1984) and we have previously described the use of S-hexyl-glutathione (ShGSH) as an inhibitor of LTC_4 metabolism (Bäck *et al.* 1996). The aim of this study was to characterise the effect of ShGSH on LTD_4 responses in the guinea pig trachea.

Spiral tracheal preparations were prepared from male Dunkin Hartley guinea pigs (300-450 g) and set up at a resting tension of 10 mN in 5-ml organ baths containing Tyrode's solution gassed with 6.5 % CO_2 in O_2 at 37° C. The preparations were incubated for 30 min with either L-cysteine (L-cys, 5mM) or a combination of L-cys (5 mM) and ShGSH (100 μM). CysLT receptor antagonists were administered 15 min before cumulative concentration effect curves for LTD_4 (0.1 nM – 1 μM) were established. Changes in isometric force were recorded with an EMKA data acquisition system. The effects of antagonists are expressed as pA_2 values ± s.e.mean, calculated from the dose-ratio at EC_{50}. For metabolic studies, guinea pig tracheal tissue was incubated for 30 min in 0.5 ml PBS buffer with [^{3}H]LTD_4 (10 μCu, 2 pM). The distribution of radioactivity in the fractions corresponding to authentic cysLT standards was determined by RP-HPLC. Results are expressed as percent ± s.e.mean of total detected [^{3}H]cysLTs.

In the presence of L-cys, the $CysLT_1$ receptor antagonist ICI 198,615 (1 nM – 10 nM; Snyder *et al.* 1987) competitively inhibited LTD_4 contractions with a pA_2 value of 9.3±0.2 (n=5-9). In contrast, in the presence of ShGSH and L-cys, the LTD_4 contractions were resistant to ICI 198,615 (10 nM – 300nM, n=5-11) but competitively inhibited by the combined $CysLT_1$ and $CysLT_2$ receptor antagonist BAY u9773 (0.3-10 μM; Bäck *et al.* 1996) with a pA_2 value of 6.8±0.1 (n=4-16). Tracheal tissue metabolised [^{3}H]LTD_4 into [^{3}H]LTE_4 and this metabolism was inhibited by L-cys (Table 1). After treatment with ShGSH and L-cys, [^{3}H]LTC_4 was detected.

In summary, ShGSH changed the functional response to LTD_4 from $CysLT_1$ into $CysLT_2$ and induced formation of LTC_4 from LTD_4. In conclusion, cysLT function may be regulated by interconversion between the LTC_4 and LTD_4.

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Table 1: Metabolism of [^{3}H]LTD_4 (means±s.e.mean)

	[^{3}H]LTC_4	[^{3}H]LTD_4	[^{3}H]LTE_4
no trachea(n=18)	0±0 %	100±0 %	0±0 %
control (n=7)	0±0 %	51±5 %	49±5 %
L-cys (n=5)	0±0 %	90±4 %	10±5 %
ShGSH+L-cys (n=7)	20±3 %	75±4 %	5±2 %

239P THE EFFECT OF TYROSINE KINASE AND MAP KINASE KINASE INHIBITION ON CONTRACTILE RESPONSES TO U46619 AND 5-HYDROXYTRYPTAMINE IN BOVINE PULMONARY SUPERNUMERARY ARTERIES.

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5-hydroxtryptamine (5-HT) and the thromboxane A2 mimetic U46619 constrict bovine pulmonary supernumerary arteries (Bunton et al., 2000). Recent evidence indicates that vasoconstriction to 5-HT may involve the activation of tyrosine and MAP kinases (Watts, 1996). This study investigated the involvement of PLCβ and tyrisine kinase/ MAP kinase pathwaws in the constrictor response of these arteries to U46619 and 5-HT.

Bovine lungs were obtained fresh from the abattoir. Segments of supernumerary arteries (diameter 0.5 -1 mm) were dissected from the lung, suspended between stainless steel hooks in Krebs-Henseleit buffer (37°C) under a tension of 1 g and gassed with a mixture of O2:CO2 (95%/5% v/v). Concentration response curves for U46619- and 5-HT-induced constriction were constructed. A second curve was then constructed in the presence of an inhibitor. 5-HT2A-mediated responses were examined using the receptor selective agonist DOI. 5-HT1-mediated responses were investigated by examining the ability of 5-HT to reverse forskolin-induced relaxation in the presence of 5-HT2A receptor blockade with ritanserin (100nM). Results are means ± s.e. mean. The significance of differences was determined using Student's t-test.

5-HT (1nM-100μM) and U46619 (0.1nM-1μM) induced a concentration dependent constriction (pEC50 values; 5-HT, 6.19 ± 0.1 n = 7; U46619, 7.07 ± 0.11, n = 4), which were unaffected by the PLCβ inhibitor U73122 (2μM). The tyrosine kinase inhibitor genistein did not alter the concentration response curve to U46619 but produced a 6.3 fold rightward shift of the 5-HT concentration response curve (pEC50 values; control 6.64±0.11; genestien (30 μM), 5.84±0.08, n = 7, $p < 0.001$) without depressing the maximum response. The non-competitive inhibitors of mitogen-activeted protein kinase kinases MEK1/2, PD098059 and U0126 did not alter the contractile response to U46619 or the sensitivity to 5-HT but reduced the maximum response to 5-HT (pEC50 values; PD098059 control, 6.41 ± 0.02; 10μM PD098059, 6.03±0.13, $p>0.05$; maximum response 52 ± 9% of control, $p<0.05$, n=4. ; U0126 control. 6.16±0.28, 10μM U0126, 5.48±0.27, $p>0.05$, maximum response, 55 ± 6% of control, $p<0.05$, n=4). In the presence of the 5-HT2 receptor antagonist ritanserin (100nM) 5-HT produced a concentration dependent constriction of forskolin-relaxed artery rings, supporting the view that 5-HT activates a receptor which is negatively coupled to adenylyl cyclase. This response was unaffected by PD098059 (10μM) or U0126 (10μM). In contrast PD098059 (10μM) reduced the maximum contractile response to the 5-HT2A-receptor-selective agonist DOI (1μM) by approximately 80%.

In bovine pulmonary supernumerary arteries neither U46619 nor 5-HT-induced constriction appears to involve the activation of PLCβ. Constrictor responses to 5-HT, but not U46619, are mediated, in part, by a tyrosine kinase/MAP kinase pathway which is coupled to the 5-HT2A receptor.

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240P CHARACTERIZATION OF BRADYKININ-INDUCED INTRACELLULAR Ca^{2+} INCREASE IN HUMAN COLON SMOOTH MUSCLE CELLS

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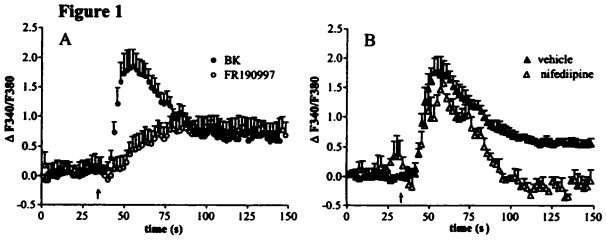
Smooth muscle cells from both vascular and non-vascular tissues, particularly in the airways and in the intestinal tract, have been shown to be highly responsive to bradykinin (BK) (Regoli & Barabé, 1980; Hall, 1992). We have studied the effect of BK on $[Ca^{2+}]i$ mobilization in cultures of human colon smooth muscle cells (SMCs). The receptor type(s) involved in BK response was characterized by the use of both peptide and nonpeptide BK receptor agonists (BK and FR 190997) and antagonists (MEN 11270 and FR173657) (Altamura *et al.*, 1999; Meini *et al.*, 1999).

Specimens of human colon were obtained from patients (n=3) underwent to surgery for colon carcinoma. Colonic SMCs were isolated following the protocol described by Ennes *et al.*, 1992. $[Ca^{2+}]i$ increase was measured by fluorimetry in cell suspensions loaded with Fura-2. The changes of the ratio of Fura-2 fluorescence intensities at 340 and 380 nm, ΔF340/F380, were used as an index of $[Ca^{2+}]i$. Data are expressed as mean±s.e.mean.

BK (1μM) induced a rapid and transient increase in $[Ca^{2+}]i$ from a ratio of 2.1±0.11 (baseline value of 104±5 nM) to a ratio of 3.85±0.3 (peak value of 300±25 nM) ($P<0.01$, Student's *t*-test, n=14) that was followed by a plateau phase reaching 30±9% the initial peak. The non-peptide B_2 receptor selective agonist, FR 190997 (1 μM), produced a slowly developing and persistent $[Ca^{2+}]i$ increase that amounted to 47±4% (n=8) of the BK peak effect (Fig. 1A). BK response (0.1 μM) was inhibited by both peptide (MEN 11270) and nonpeptide (FR 173657) B_2 receptor antagonists (1 μM) (92±8 and 91±9% inhibition, respectively, n=3). B_2 receptor antagonists were ineffective against endothelin-1 (ET-1, 10 nM) which produced an effect comparable to that of BK (1 μM). BK (1 μM) response was abolished by prior administration of BK itself or FR 190997 (1 μM) with 0±0% and 5±4% residual response (n=3 and 5, respectively). Heterologous desensitization of the Ca^{2+} response between BK and ET-1 was partial since BK response was reduced by 60±13% by prior stimulation of the cells with an equieffective concentration of ET-1 (10 nM) and, inversely, ET-1 response was reduced by 31±15% by pretreatment with BK (1 μM). The L-type calcium channel blocker nifedipine (10 μM) did not affect the initial peak of Ca^{2+} increase induced by BK (94±8% of control, n=3) but abolished the late phase (Fig. 1B). The addition of the nonselective cation channel blocker SKF 96365 (30 μM) did not reduce the residual response. Similar results were obtained for FR 190997 (n=3).

Figure 1



In conclusion, human colon SMCs express bradykinin B_2 receptors that are coupled to a biphasic increase in $[Ca^{2+}]i$, with an immediate peak response and a delayed plateau phase which is due to Ca^{2+} influx through L-type Ca^{2+} channels. The BK response was affected by both homologous and heterologous desensitization. FR 190997 is a weak agonist in this model.

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241P A NOVEL RELAXATION RESPONSE TO 5-HT IN THE MOUSE TETRODOTOXIN-PRECONTRACTED ISOLATED COLON

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The ability of 5-hydroxytryptamine (5-HT) to mediate contractile and relaxation responses in the gastrointestinal tract is complex and is mediated by a number of 5-HT receptors (Costall & Naylor, 1990). In the present study we report a novel tetrodotoxin (TTX)-resistant relaxation response to 5-HT in the mouse distal colon.

Segments of distal colon (approximately 2 cm in length and taken 4-5 cm from the ileo-caecal juction) were obtained from BKW mice of either sex (25-35 g) and mounted in 10 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs solution at 37° C under an initial tension of 1 g. The tissues were allowed to equilibrate for at least 30 minutes. Tetrodotoxin (0.3 μM) was added to the organ baths and once the tension increase in the tissues had stabilized (1-2 min following TTX addition) 5-HT was added and the tissues washed after 2 min. A concentration relaxation response curve to 5-HT was obtained in a non-cumulative manner using a 10 min dose cycle. A submaximal concentration of 5-HT (3 μM) was used when examining the effects of 5-HT receptor antagonists on the contractile response to TTX and the relaxation response to 5-HT. The antagonists were equilibrated for at least 30 min before the addition of TTX and 5-HT. Statistical comparisons were made using the unpaired Students' *t* test.

An addition of TTX produced contraction in all segments of the distal colon (0.76 ± 0.09 g, n= 24). 5-HT produced a concentration dependent relaxation on the TTX precontracted tissues with a maximum response of 0.55 ± 0.18 g (n=6) at 10 μM. The 5-HT_3 receptor selective antagonist granisetron (0.1 μM, n= 8) and the 5-HT_4 receptor selective antagonist GR113808 (0.1 μM, n=4) (Gale *et al.*, 1994) had no effect on either the TTX induced contraction or the relaxation response to 5-HT (3 μM). Methysergide (3 μM, n=8), a $5\text{-HT}_{1/2 \text{ or } 7}$ receptor antagonist and clozapine (3 μM, n=4), a $5\text{-HT}_{2/7}$ receptor antagonist, both significantly reduced ($P<0.05$) the contraction due to TTX thus making examination of their effects on the 5-HT response problematic.

The ability of TTX to contract the tissue indicates the existence of a neuronal relaxant tone in the mouse distal colon. The relaxation response to 5-HT observed in the present study is most likely a direct smooth muscle response. The inability of granisetron and GR113808 to antagonise the 5-HT response excludes the involvement of 5-HT_3 and 5-HT_4 receptors in the relaxant response to 5-HT. A further study is required to understand the mechanism by which methysergide and clozapine reduce the contractile response to TTX and identify the receptor mediating the relaxation response to 5-HT.

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Gale, J.D., Grossman, C.J., Whitehead, J.W.F. et al. (1994). Br. J. Pharmacol., **111,** 332-338.

242P COMPARATIVE INHIBITORY EFFECTS OF NIFLUMIC ACID AND NOVEL SYNTHETIC ANALOGUES ON CONTRACTILE RESPONSES OF THE RAT STOMACH FUNDUS

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Activation of rat fundus 5-HT_{2B} receptors expressed in *Xenopus* oocytes elicits activation of calcium-activated chloride ($Cl_{(Ca)}$) channels (Foguet et al., 1992). We have recently shown that niflumic acid (NFA), a blocker of $Cl_{(Ca)}$ channels, selectively inhibits 5-HT-induced contractions of the rat stomach fundus (Scarparo et al., 2000). In the present study we have examined the importance of the carboxylic acid moiety of NFA to its potency and selectivity, by comparing its inhibitory effects with those of two synthetic analogues, NFA-amide (NFA_{am}; 2-[3-(trifluoromethyl)-analino]nicotinamide) and NFA-methyl-ester (NFA_{me}; methyl 2-[3-(trifluoromethyl)-analino]nicotinate) on 5-HT- and KCl-induced contractile responses of the rat fundus.

Longitudinal strips of stomach fundus from male Wistar rats (200-300g) were mounted in aerated Tyrode's solution (37°C, pH 7.4) for isometric tension recording using a computerized data acquisition system. The effects of increasing cumulative concentrations of NFA, NFA_{me} or NFA_{am} (10^{-6}– 10^{-3}M) were assesssed separately on 5-HT- (10μM) and KCl (60mM)-induced contractions, using a contact time of 10 minutes per concentration, and inhibitory curves for each compound constructed. IC_{50} values (expressed as mean with 95% confidence limits in parentheses) were calculated and compared using a non-paired Student's t-test.

5-HT (10 μM) and KCl (60 mM) elicited reproducible, tonic contractions of 1.67±0.16g (n=26) and 2.61±0.34g (n=24), respectively. NFA induced a concentration-dependent inhibition of 5-HT-induced contractions with a mean IC_{50} value of 0.24×10^{-4}M (0.11-0.37, n=9), whilst it was approximately 6-fold weaker at inhibiting KCl-induced reponses; IC_{50} value of 1.49×10^{-4}M (0.76-2.22, n=9; *$p<0.05$). In contrast, NFA_{me} and NFA_{am} were significantly less potent inhibitors of 5-HT-induced contractions than NFA, with respective IC_{50} values of 1.64×10^{-4}M (0.51-2.77, n=8; *$p<0.05$) and 1.87×10^{-4}M (0.56-3.18, n=9; *$p<0.05$). In addition, there was no significant difference between their inhibitory effects on 5-HT-induced and KCl-induced contraction, with respective IC_{50} values of 2.61×10^{-4}M (0.46-4.76, n=8) and 2.55×10^{-4}M (1.30-3.80, n=7) for inhibition of the latter. Moreover, the effects of the analogues on KCl-induced contraction were not significantly different from those of the parent compound NFA.

Our data suggest that derivatization of the carboxylic acid function of NFA results in a loss of inhibitory potency and selectivity of the compound for 5-HT-induced contraction, possibly due to a weaker interaction with $Cl_{(Ca)}$ channels, although other factors such as net charge of the molecule may be important.

Foguet, M. *et al.* (1992). *EMBO J.*, 11, 3481-3487.
Scarparo H.C. *et al.* (2000) *Br J Pharmacol.*, 130, 678-684.

243P RAT GASTRIC ACID SECRETION: FUNCTIONAL EVIDENCE FOR A ROLE OF H_3 HISTAMINE RECEPTORS

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Although in the past decades considerable progress has been made in the understanding of the pathophysiological role of H_3 histamine receptors, so far contradictory findings have been reported dealing with their involvement in the control of gastric acid secretion (Lewin *et al.*, 1992). In the attempt to further elucidate to what extent this histamine receptor subtype is involved in the physiological regulation of gastric secretory function we investigated the effects produced by H_3 agonist/antagonist challenge on gastric acid hypersecretion induced in anaesthetized rats by pharmacological or electrical stimulation.

In urethane (1.25 g/kg ip) anaesthetized male Wistar rats (200-250g), gastric acid output was measured by flushing the gastric lumen with 5 ml boluses of saline every 10 min and titrating the flushed perfusate with 0.02 N NaOH to pH 7.0 (Ishikawa *et al.*, 1988). Basal secretions were collected for 30 min before stimulation. Cervical left vagus nerve was electrically stimulated (50V, 10 Hz, 0.5 ms for 30 min) at 30 min intervals whilst, in a second series of experiments, secretagogues (pentagastrin 40 μg/kg/h iv or betanechol 0.5 mg/kg/h iv) were infused throughout the experiments. The selective H_3 agonist (R)-α-methylhistamine (RαMH) was administered (100 μg/kg iv) 10 min before electrical stimulation or when a stable hypersecretory plateau was reached. Pretreatment with the H_3 antagonist Thioperamide (THIO) (100 μg/kg iv) was performed 20 min before RαMH challenge. Control animals received the vehicle alone. Gastric acid output was expressed as $\Delta\mu EqH^+$/30 min by subtracting the basal value from the maximal hypersecretory responses. Unpaired Student's t test was used for statistical analysis.

RαMH significantly inhibited vagal stimulated gastric acid response, slightly potentiated pentagastrin evoked acid secretion leaving immodified betanechol secretory effect. Thioperamide pretreatment antagonized RαMH inhibitory effect and significantly potentiated pentagastrin-induced secretory response. Data are summarized in Table 1.

Table 1. Gastric acid output expressed as $\Delta\mu EqH^+$/30 min. Values are mean±s.e.mean, n≥8

	Vagal Stimulation	Pentagastrin (40 μg/kg/h)	Betanechol (0.5 mg/kg/h)
Control	49±4	24±3	53±6
RαMH	25±6*	30±6	48±8
RαMH+THIO	55±10	27±8	-
THIO	42±5	34±3*	51±6

*P < 0.05 versus control (t-test)

These results, supporting the data from Yokotani *et al.* (2000), provide *in vivo* functional evidence that gastric H_3 receptors negatively control vagally induced rat gastric acid secretion and that they are tonically activated during pentagastrin infusion.

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Lewin M.J.M *et al.*, (1992) *Yale J.Biol.Med.*, 65, 607-611.
Yokotani K.*et al.*, (2000) *Eur.J.Phrmacol.*, 392. 23-29.

244P INVESTIGATION OF CB_1 CANNABINOID RECEPTORS MEDIATING ANTISECRETORY EFFECTS IN RAT STOMACH

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In the gastrointestinal tract, cannabinoid receptors (CB-R) are involved in the control of intestinal motility (Izzo *et al.*, 2000) and gastric acid secretion (Coruzzi *et al.*, 1999; Adami *et al.*, 2000). The aim of this study was to investigate the mechanism of cannabinoid action on gastric acid secretion in rats *in situ* that was evoked by different secretory stimuli. In addition, the localization of CB-R immunoreactivity in the rat stomach was examined by immunocytochemistry.

Acid secretion was measured in anaesthetised (urethane, 1.25 g kg^{-1} i.p.) Wistar rats with lumen-perfused stomachs (Bertaccini *et al.*, 1968). Drugs were administered by intravenous infusion (6 ml h^{-1}) or rapid injection through the tail vein, or by i.c.v. injection (5 μl rat^{-1} i.c.v.). Sections of fundus, corpus and antrum isolated from Sprague-Dawley rat stomachs were incubated with rabbit anti-human CB_1-R or CB_2-R, and an antibody against choline acetyltransferase (ChAT). Cy3- or FITC-conjugated secondary antibodies were used. Receptor immunoreactivity was visualized by confocal laser microscopy.

The selective CB_1-R agonist HU-210 (0.1 and 0.5 mg kg^{-1} i.v.) significantly inhibited (51.47±12.66% and 71.04±10%, respectively) the acid secretion induced by the indirectly acting cholinergic agent 2-deoxy-D-glucose (2-DG, 200 mg kg^{-1} i.v), but not by histamine (HA, 40 mg kg^{-1} h^{-1}). Moreover, HU-210 (0.1 mg kg^{-1} i.v.) reduced by 83.71±7.43% acid secretion evoked by pentagastrin (20 μg kg^{-1} h^{-1}). The non-selective CB agonist WIN 55,212-2 (20 μg rat^{-1} i.c.v.) did not affect the gastric acid secretion stimulated by pentagastrin (PGAS, 10 μg kg^{-1} i.v.). CB_1-R, but not CB_2-R immunoreactivity was often colocalised with ChAT immunoreactivity in neuronal elements innervating smooth muscle, mucosa and submucosal blood vessels in fundus, corpus and antrum of rat stomach.

HU-210 appears to inhibit 2-DG- and pentagastrin-, but not HA-induced acid secretion, suggesting that its effects are mediated by CB_1-R located on neurones, but not on parietal cells. The distribution of CB_1-R immunoreactivity in gastric cholinergic neurones supports the functional results.

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Coruzzi, G., Adami, M., Coppelli, G., *et al.* (1999) *Naunyn Schmiedeberg's Arch. Pharmacol.* **360**, 715-718.
Izzo, A.A., Pinto, L., Borrelli, F., *et al.* (2000) *Br. J. Pharmacol.*, **29**, 1627-1632.

245P MODULATION OF COLONY STIMULATING FACTOR RELEASE AND APOPTOSIS IN HUMAN COLON CANCER CELLS BY ANTICANCER DRUGS

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Treatment of cancer widely relies on the use of cytotoxic drugs. These drugs interfere with cell division and damage the cell, which in many instances would result in apoptosis. However, there is now an increasing interest in manipulating the immune response against the cancer cells. Some studies have shown that secretion of colony stimulating factors (CSF) by cancer cells enhances the immune response against them by promoting the activity of antigen presenting cells (Colombo *et al.*, 1991; Dranoff *et al.*, 1993). We have observed that human colon cancer cells are able to secrete granulocyte-macrophage-CSF (GMCSF), granulocyte-CSF (GCSF) and macrophage-CSF (MCSF). Thus, the aim of this study was to ascertain the effects of anticancer drugs on CSF release and cell death in the human colon cancer cell line HT29. HT29 cells were cultured to confluency. After 24h of serum deprivation, cells were incubated for a further 24h period with a combination of interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α) (10ng/ml both) in the presence or absence of anticancer drugs. A purine analog (6-mercaptopurine, 6-MP), a DNA-damaging agent (cisplatin) and a drug interfering with the microtubule function (taxol) were used. GMCSF, GCSF or MCSF in conditioned medium was measured by ELISA. Apoptosis was evaluated by detection of mono- and oligo-nucleosomes in the cytoplasmatic fraction of cell lysates (ELISA). Under control culture conditions, HT29 cells released low levels of either GMCSF, GCSF or MCSF, however, significant increases in the release of all were seen after stimulation with IL-1β and TNF-α. 6-MP and cisplatin induced significant increases in GMCSF, GCSF and MCSF secretion by cytokine treated HT29 cells (Figures 1A and 1B). Taxol increased MCSF, reduced GCSF and did not modify GMCSF secretion (Figure 1C).

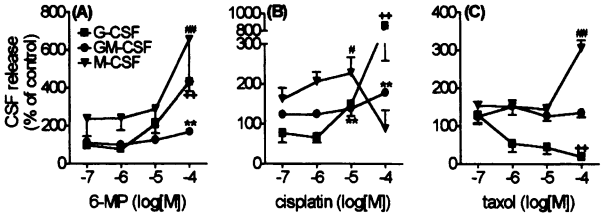

Fig 1.- Effects of anticancer drugs on GMCSF, GCSF and MCSF release. Mean ± s.e.m. of 3-5 experiments, each performed in triplicate. (# $p<0.05$, ## $p<0.01$, ** $p<0.01$, ++ $p<0.01$ vs respective value in vehicle treated cells, ANOVA + Dunnet test).

HT29 cell apoptosis was stimulated by cisplatin (10^{-4} M, 53% increase) and taxol (10^{-4} M, 22% increase) while unaffected by 6-MP. No correlation between cell apoptosis and secretion of CSFs was found for any of the anticancer drugs used.

The present results indicate that anticancer drugs with different mechanisms of action are able to modify the secretion of CSFs from colon cancer cells and these effects are independent of their action on cell apoptosis. Given the reported beneficial effect of CSFs secretion by cancer cells to the immune host response against them, the present results suggest that immune modulation may be part of the action of anticancer drugs.

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246P OPPOSITE ROLE OF OPIOID AND CHOLECYSTOKININ SYSTEMS ON THE WITHDRAWAL RESPONSE TO ADENOSINE A_1-RECEPTOR AGONIST IN THE GUINEA-PIG ISOLATED ILEUM

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In the isolated guinea-pig ileum (GPI), exposure to adenosine agonists induces a state of tissue dependence, shown by the tissue contraction upon agonist withdrawal (Chahl, 1990). In the GPI the adenosine system exerts an inhibitory control on μ-opioid withdrawal response and this is reversed by cholecystokinin octapeptide (CCK8) (Morrone et al., 2000). Here we now report original data demonstrating that the opioid system exerts an inhibitory control on the adenosine withdrawal response. In addition we have tested whether CCK8 reverses the inhibition elicited by the opioid system on the adenosine withdrawal response. The experimental procedure was essentially that used by Romanelli and co-workers (1999). GPI segments from males (300–400 g) were set up under 1 g tension in an organ bath containing Tyrode's solution. The contractile responses were recorded by an isotonic force transducer and expressed as percent of the maximal ACh response. To obtain the adenosine withdrawal response, tissue preparations were exposed for 5 min to the selective adenosine A_1 agonist, N6-cyclopentyladenosine (CPA; 0.3 and $3x10^{-8}$ M) and then challenged with the selective adenosine A_1 antagonist, 8-cyclopentyl-1-3 dimethylxantine (CPT, $4x10^{-7}$ M). In subsequent CPA/CPT tests, naloxone (NL, $5.4x10^{-7}$ M) was added to the bath 30 sec before CPT. Tissues were then tested with CPA/CCK8/CPT (CCK8 $1.1x10^{-9}$ M was added 2 min after CPA; CPT was added 3 min after CCK8). NL significantly increased the contractile response to CPT when the lower CPA concentration was used. CCK8 significantly increased the CPT-induced contraction with both CPA concentrations (Table 1). CPA caused a decrease of the amplitude of CCK8-induced contractions; this CPA-induced decrease was prevented by NL, added 2 min before CPA (data not shown).

Table 1. Responses to CPT after a 5 min exposure to CPA, with or without NL or CCK8. Values are means; s.e. mean is shown in brackets (N=10)

	CPT	NL/CPT	CCK8/CPT
CPA $0.3x10^{-8}$ M	34(7.5)	80(13.5) *	73(6.0)*
CPA $3x10^{-8}$ M	13(7.8)	42(12.5)	71(6.4)

*: $p<0.05$ *vs.* the responses to CPT only (ANOVA followed by Student-Newman-Keuls test).

In conclusion, these results demonstrate that the adenosine withdrawal response is under the inhibitory control of the opioid system and that the latter is counteracted by CCK-8.

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247P PURINORECEPTORS ARE INVOLVED IN THE CONTROL OF ACUTE MORPHINE WITHDRAWAL

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Dependence can be induced and measured *in vitro* by using guinea-pig ileum: the characteristics of dependence development and the precipitation of withdrawal by naloxone in the guinea-pig ileum are very similar to those of acute dependence in experimental animals and man.

There are no data available, to our knowledge, on the effect exerted by P1 and P2 purinoceptors agonists and antagonists on the acute opiate dependence. Two subtypes of purinoceptors, the P1 and P2 purinoceptor, have been identified by pharmacological studies. The availability of agonists and antagonists for P1 and P2 provides powerful tools that can be used to determine the roles of these receptor types in mediating some of physiological and pharmacological effects of purinoceptors in the central nervous system (CNS).

The experiments described here were undertaken to provide insight into the role of specific purinoceptor subtypes in mediating opioid withdrawal. Adenosine was used as P1 adenosine receptor agonist; α,β-methylene ATP (APCPP) was used as P2 adenosine receptor agonist. Caffeine was used as P1 purinoceptor antagonist and quinidine as P2 purinoceptor antagonist. Results were tested for statistical significance using the Student's t-test for paired data. EC50 values were calculated accorting to Litchfield and Wilcoxon. 6-9 animals were used for each experiment..

Following a 4 min *in vitro* exposure to morphine, the guinea-pig isolated ileum exhibited a strong contracture after the addition of naloxone (80% of contraction *vs* acetylcholine control). The addition of adenosine, P1 purinoceptor agonist, ($1x10^{-9}$-$5x10^{-9}$-$1x10^{-8}$ M) 10 min before morphine produced a concentration-dependent reduction of the morphine withdrawal: ED50 = $1.5x10^{-9}$ M ($2.3x10^{-9}$-$1.03x10^{-9}$). By contrast, the P2 purinoceptor agonist APCPP ($1x10^{-9}$-$5x10^{-9}$-$1x10^{-8}$ M) was also able to increase significantly and dose-dependently morphine withdrawal: ED50= $2.2x10^{-9}$ M ($3.5x10^{-9}$-$1.3x10^{-9}$). The same effects were obtained when the drugs were injected 10 min after morphine.

The addition of P1 purinoceptor antagonist caffeine ($1x10^{-5}$-$5x10^{-5}$-$1x10^{-4}$ M) 10 min before morphine induced a significant increase of morphine withdrawal: ED50= $1.13x10^{-4}$ M ($1.22x10^{-5}$-$1.05x10^{-3}$) for caffeine. By contrast, the P2 purinoceptor antagonist quinidine ($1x10^{-10}$-$5x10^{-10}$-$1x10^{-9}$ M) was also able to reduce significantly and dose-dependently the morphine withdrawal: ED50= $1.01x10^{-9}$ M ($1.22x10^{-11}$-$1.05x10^{-8}$). The same results were obtained when the drugs were injected 10 min after morphine. After washout, the ACH response was not affected by the treatments with adenosine receptor agonists or antagonists whereas the final opioid withdrawal responses were still reduced or increased respectively (Data not shown).

The results of our experiments indicate that both P1 and P2 purinoceptor agonists and antagonists are able to influence opiate withdrawal *in vitro*, suggesting an important functional interaction between the purinergic system and opioid withdrawal.

248P PREJUNCTIONAL ACTIONS OF MDMA IN MOUSE VAS DEFERENS

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We have recently shown that methylene-dioxymethamphetamine (MDMA, 'ecstasy') has major agonist actions at prejunctional α_2-adrenoceptors (Lavelle et al., 1999), which are mainly of the $\alpha_{2A/D}$-subtype. We now investigate the effects of MDMA and α_2-adrenoceptor agonists in vas deferens from wildtype and knockout mice lacking the $\alpha_{2A/D}$-adrenoceptor.

C57BL/6 mice (18-28g, male, wildtype and $\alpha_{2A/D}$-knockout: Jackson Laboratories) were killed by CO2 overdose, and vas deferens was set up between platinum electrodes under 0.5g tension for recording of isometric tension. Tissues were stimulated every 5 min with a single stimulus or trains of 40 pulses at 10Hz.

Single pulse stimulation produced a contraction of 0.120±0.040g (mean±s.e.m., n=16) and 0.090±0.016g (n=12) in wild type and knockout mice, respectively in the presence of cocaine 3 μM (no significant difference). The potency of the α_2-adrenoceptor agonist xylazine was significantly reduced in knockout (pIC50 of 8.27±0.07, -log M, n=4) as compared with wildtype mice (8.69±0.08, n=4; Student's t-test, P<0.05), whereas potency of MDMA was unchanged (5.39±0.06, n=4 versus 5.38±0.06, n=6). Stimulation with 40 pulses at 10Hz produced a contraction of 0.40±0.05g (n=32) and 0.75±0.08g (n=23) in wildtype and knockout mice, respectively (P<0.001). This was reduced to 0.12±0.03g (n=9) and 0.20±0.05g (n=8) following exposure to nifedipine (10μM) (P<0.05). In the presence of nifedipine, the potency of the α_2-adrenoceptor agonist xylazine was significantly reduced in knockout (4.54±0.17, n=4) as compared with wild type mice (7.01±0.48, n=4), whereas potency of MDMA was unchanged (4.26±0.24, n=4 versus 4.32±0.14, n=4). Likewise, potency of the α_2-adrenoceptor agonist oxymetazoline was significantly reduced in knockout (6.72±0.49, n=5; P<0.05) as compared with wildtype (8.56±0.48, n=4). In ligand binding studies of α_2-adrenoceptor sites, MDMA showed approximately equal affinity for all subtypes (Lavelle et al., 1999).

It is concluded that MDMA is a potent agonist at more than one subtype of α_2-adrenoceptor: its potency at inhibiting nerve-stimulation-evoked contractions is similar in wild-type and $\alpha_{2A/D}$-adrenoceptor knock-out mice. The prejunctional $\alpha_{2A/D}$-adrenoceptor is replaced by the α_{2B}-adrenoceptor or α_{2C}-adrenoceptor in this knockout mouse (Altman et al., 1999).

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249P INTRACELLULAR Ca^{2+} RESPONSES TO CARBACHOL IN HUMAN CULTURED DETRUSOR SMOOTH MUSCLE CELLS: EVIDENCE FOR AN INVOLVEMENT OF BOTH M_2 AND M_3 MUSCARINIC RECEPTORS.

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Contraction of human detrusor smooth muscle (HDSM) can be elicited by M3 muscarinic receptor stimulation (Hegde & Eglen 1999). We have previously shown that M3 muscarinic receptors mediate carbachol-stimulated hydrolysis of inositol phospholipids in cultured cells derived from HDSM (Harriss et al., 1995). The aim of this study was to determine which muscarinic receptor subtype is responsible for the increases in $[Ca^{2+}]_i$ in response to carbachol in HDSM cells.

HDSM cell lines were established from explants of human bladder biopsies as previously described (Harriss et al., 1995) Total ^{3}H-inositol phosphate (IP) accumulation and changes in $[Ca^{2+}]_i$ using Fura-2AM were measured as previously described (Harriss et al. 1995, Dickenson et al., 1994). The M3-receptor antagonist 4-diphenylacetoxy-N-methyl-piperidine-methobromide (4-DAMP) acted as a potent competitive antagonist of the carbachol-induced ^{3}H-inositol phosphate response (pA_2 of 9.2 ± 0.3; n=3) while, 1μM methoctramine (M2 selective antagonist) was without significant effect on the response to carbachol (n=3).

Carbachol produced a significant and concentration-dependent rise in $[Ca^{2+}]_i$ in HDSM cells (log EC_{50} = -4.3 ± 0.3; n=6). This response was competitively antagonised by both atropine (pA_2=8.1 ± 0.5; n=10) and 4-DAMP (pA_2 of 7.3 ± 0.3; n=12). In contrast, methoctramine (1μM) caused no significant change to the $[Ca^{2+}]_i$ response to carbachol (n=10). It is notable, however, that the PA_2 determined for 4-DAMP from antagonism of carbachol-stimulated calcium mobilization is two orders of magintude lower than that obtained from measurement of ^{3}H-inositol phosphate accumulation.

In the presence of the M2-antagonist methoctramine (1μM), however, 4-DAMP (100nM) produced a larger shift in the concentration-response curve to carbachol (pA_2 = 9.3 ± 0.4; n=3). Similarly, the potency of 4-DAMP was increased following treatment with pertussis toxin (PTX; 100ng/ml; 18h) to uncouple $G_{i/o}$-mediated responses (pA_2 = 8.7 ± 0.3; n=3). These pA_2 values are much closer to that calculated from measurement of inositol phospate accumulation.

The above experiments were repeated using the M3-agonist Oxotremorine-M (Oxo-M; log EC_{50} = -4.8 ± 0.2; n=6) with similar results. Thus, the apparent pA_2 values obtained for 4-DAMP from antagonism of Oxo-M-stimulated calcium responses, under the different conditions, were: 7.7 ± 0.6 (control; n=5), 9.0 ± 0.4 (in the presence of 1μM methoctramine; n=3) and 9.2 ± 0.2 (PTX-treated; n=3). To investigate the origin of the Ca^{2+} mediating the M2 response, the effect of 4-DAMP (100nM) on carbachol-stimulated $[Ca^{2+}]_i$ responses were measured in Ca^{2+}-free medium containing 0.1mM EGTA (pA_2= 8.3 ± 0.2; n=6). These data suggest that although the M3 muscarinic receptor subtype is primarily involved in the $[Ca^{2+}]_i$ response to carbachol and Oxo-M, $G_{i/o}$-coupled M2 receptors also contribute to the response to these two agonists.

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250P TAURINE AMELIORATES *IN VITRO* AND *IN VIVO* THE ELECTRICAL AND CONTRACTILE PROPERTIES OF SKELETAL MUSCLE FIBRES OF DYSTROPHIC MDX MOUSE

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An important task in Duchenne muscular dystrophy (DMD), a life-threatening inherited muscle disorder, is to find effective drugs with less side effects with respect to the currently used glucocorticoid therapy. The findings that in the mdx mouse, the animal model of DMD, muscle degeneration is paralleled by a reduction of taurine muscle content (McIntosh *et al.*, 1998), prompted us to investigate the therapeutic potential of this side effect-free amino acid. In fact, taurine works in skeletal muscle by exerting both short- and long-term control on membrane chloride conductance (gCl) and cytosolic calcium levels (De Luca *et al.*, 1996), possibly involved in the progression of muscular dystrophy (Rüegg & Gillis, 1999).

We first evaluated the effect of *in vitro* application of taurine on the mechanical threshold of extensor digitorum longus (EDL) muscle fibres of 6-month-old male mdx mouse, by means of "point" voltage clamp method (De Luca *et al.*, 1996). As expected by an increase of cytosolic calcium levels (Rüegg & Gillis, 1999), the rheobase voltage for contraction in mdx muscle fibres was significantly more negative (-70.1 ± 1.3 mV; value calculated from the fit of the data points as in De Luca *et al.*, 1996) than that of controls (-62 ± 0.9 mV). The *in vitro* application of 60 mM taurine (dissolved in normal physiological solution) significantly shifted the rheobase voltage toward more positive potentials (-65 ± 1.3 mV). An *in vivo* taurine treatment was performed by feeding 4-week-old mdx mice with a taurine-rich diet (10% w.w.), throughout a 4-8 week period of exercise on treadmill, a protocol to worsen the dystrophic condition during the active regeneration period typical of this animal model.

Taurine administration significantly counteracted *in vivo* the exercise-dependent 40-50% loss of fore limb strength. At the end of the treatment, the effect of taurine was also evaluated *in vitro* by means of intracellular microelectrode recordings on gCl of diaphragm (DIA) and EDL muscle fibres, since gCl is an index of dystrophic progression (De Luca *et al.*, 1997). A dramatic decrease of gCl was caused by exercise in mdx EDL muscle fibres (mean ± s.e.m.: 1660 ± 78 μS/cm^2; n = 5 mice/41 fibres). Taurine significantly counteracted such a decrease, gCl being 2351 ± 80 μS/cm^2 (n = 6/56; $p<0.001$), a value overlapping that recorded in regenerating EDL muscle of sedentary mdx mice. The further decrease of gCl observed in DIA muscle fibres of exercised mdx mice (1256 ± 74 μS/cm^2; n = 5/32) was also, albeit not significantly, ameliorated by taurine (1414 ± 85 μS/cm^2; n = 5/33).

The results corroborate the potential usefulness of this safe amino acid for treating muscular dystrophy

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251P CYTOTOXICITY OF THE THIAZOLIDINEDIONE COMPOUNDS TROGLITAZONE AND PIOGLITAZONE IN HUMAN ISOLATED HEPATOCYTES

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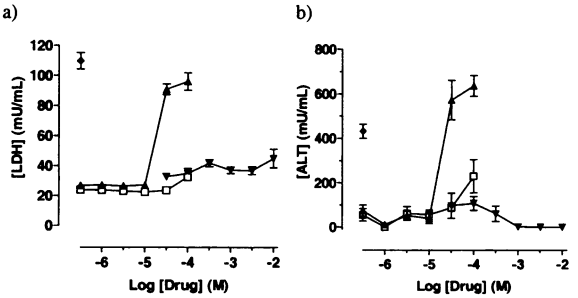
The thiazolidinedione compounds troglitazone and pioglitazone represent a new class of drugs used in the treatment of type II (non-insulin dependent) diabetes mellitus (Subramaniam, 1999). Recently, there have been several reports of hepatic injury associated with troglitazone, leading to its withdrawal from world markets (Kohlroser *et al.*, 2000; Warner-Lambert Press Release, 2000). In the present study, we evaluated the effect of troglitazone and pioglitazone upon release of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as markers of acute cytotoxicity in human freshly isolated hepatocytes. Acetylsalicylic acid was also studied, as it has been previously shown to cause cytotoxicity in rat hepatocytes (Shrivastava *et al.*, 1992).

All tissues were obtained through medically qualified intermediates, with the full consent of the donor, and with the approval of the local ethics committee. Hepatocytes were isolated from macroscopically normal liver wedges by two-step collagenase digestion (Strom *et al.*, 1982), re-suspended in William's E medium and cultured on collagen-coated 96-well plates for 10-12 h. Hepatocytes were treated with troglitazone, pioglitazone and acetylsalicylic acid in serum-free, basic Williams E medium for 24 h. Control and solvent (DMSO) treated cultures were also prepared. Cell supernatants were removed and assayed for LDH, ALT and AST release using standard kits. Statistical analysis of the data was performed using a one-way ANOVA and Dunnett's post-test.

Troglitazone demonstrated significant release of LDH, ALT and AST at 100 μM in all donors when compared to control activity values (Figure 1, $P < 0.01$; AST graph not shown). Maximum release was similar to that observed with 1% Triton X-100, suggesting complete lysis of the cells. Pioglitazone caused release in only one donor at 100 μM and acetylsalicylic acid produced significant release of LDH and AST, but not ALT in one donor at 10 mM ($P < 0.01$).

The rank order of potency of enzyme release was troglitazone > pioglitazone >> acetylsalicylic acid.

Figure 1. Effect of troglitazone (▲), pioglitazone (□), acetylsalicylic acid (▼) and 1% Triton X-100 (◆) upon a) LDH release and b) ALT release. Data are the means ± SEM from one donor and representative of data from three donors.



These data demonstrate for the first time that the glitazone compounds cause differential acute toxicity in human isolated hepatocytes on the basis of the markers studied. The concentrations of troglitazone required to produce this enzyme release are high and it is not clear whether this response relates to the hepatotoxicity associated with this compound. Further studies will be required to characterise the observed phenomena.

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252P GASTROINTESTINAL ABSORPTION OF TOBRAMYCIN INCORPORATED IN SOLID LIPID NANOPARTICLES

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Tobramycin is an antibiotic that, as other aminoglycosides, is practically not absorbed by oral route and is normally administered parenterally. Solid lipid nanoparticles (SLN) have been proposed by various researchers as alternative drug carriers. SLN are constituted of solid biodegradable lipids and may be obtained by several preparation methods. It is possible to incorporate several lipophilic and hydrophilic drugs, provided that different approaches are used during the preparation process.

Recently we have shown that unloaded SLN administred into rat duodenum are absorbed and transported to lymph and bood. The aim of the present research was to study the plasma concentrations and tissue distribution of tobramycin-loaded SLN (Tobra-SLN) and free tobramycin (Tobra-sol) in solution after duodenal and intravenous (i.v.) administration to rats. 228 male albino rats (Wistar derived strain) weighing 350-450 g. were used. The nanospheres were prepared as a warm oil-in water (o/w) microemulsion consisting of stearic acid (0.7 mmol) as internal phase, Epikuron 200 (0.14 mmol) as surfactant, taurocholate (0.66 mmol) as cosurfactant and filtered water (111.11 mmol) as continuous phase. Epikuron 200 and warm filtered water were added to melted stearic acid at about 70° C; an amount of tobramycin complex containing 0.05 mmol tobramycin was added. Tobramycin plasma and tissue concentrations were determined by using an HPLC system with UV detector

The Tobra-SLN dispersion was administered directly into the duodenal lumen through a duodenal cannula in fed awake rats, surgically implanted under anesthesia 24 h before. Blood samples were collected through an implanted cannula into the jugular vein; blood (300 μl) was collected in heparinized tubes at the designed times until 24 h after the administration. Tobra-sol and Tobra-SLN were also injected in vein (i.v.).

In a separate set of experiments tissue samples were collected from brain, lung, heart, liver and spleen, The peak plasma concentration after tobra-sol i.v was about 10 μg ml^{-1}, while the peak plasma concentration after Tobra-SLN i.v. administration was about 37 μg ml^{-1} ($P<0.001$). After duodenal administration peak plasma concentration of Tobra-SLN was about 28 μg ml^{-1} statistically significant lower ($P<0.001$) than after Tobra-SLN i.v. Pharmacokinetic analysis showed that the AUC of Tobra-SLN after duodenal administration is higher than that of i.v. Tobra-sol ($P<0.001$) and that of i.v. Tobra-SLN ($P<0.01$). 30 min after duodenal administration of Tobra-SLN no Tobramycin was found in the different tissues; the concentration increased over time: in liver Tobramycin was higher after 24 h than after i.v. In the kidney the drug concentration was lower than after i.v. Tobra-SLN; instead the amounts in spleen after 24 h were higher after duodenal than after i.v. administration of SLN preparation. .In the brain 4 h. after duodenal administration of Tobra-SLN, tissue concentrations were similar to that observed after i.v. administration.

In conclusion, Toba-SLN was present in plasma after duodenal administration. Pharmacokinetic parameters are different after i.v. or duodenal administration. Tobra-SLN i.v. or duodenally administered can cross the blood brain barrier.

253P ACUTE CYCLOSPORINE A NEPHROTOXICITY: PROTECTIVE EFFECT OF L-PROPIONYLCARNITINE ON LIPID PEROXIDATION IN ISOLATED AND PERFUSED RAT KIDNEY (IPRK).

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Nephrotoxicity is the most important side effect of Cyclosporine A (CyA; Calne *et al.*, 1979). CyA may induce an impairment of endothelial cell function, leading to a reduced production of nitric oxide (NO; Bobadilla *et al.*, 1998) and enhanced release of endothelin (Meyer-Lehnert *et al*, 1997). However, the exact mechanism remains unclear. Recently, CyA-induced fall in GFR and RBF has been associated with a dose-related increase in renal lipid peroxidation (Parra *et al.*, 1998). L-propionylcarnitine (LPC), is able to prevent CyA-Induced nephrotoxicity in IPRK (Giovannini *et al*, 1998). Moreover its antioxidant activity has been demonstrated in various experimental models (Packer *et al*, 1991).

In order to evaluate whether LPC is able to prevent CyA-induced renal lipid peroxidation in an IPRK (cell free) model, we administered LPC (200mM/20 minutes) before CyA (1μg/ml/ 20minutes) and measured renal content of malondialdehyde (MDA) and 4-hydroxyalckenales (4-HDA). We also assayed NO and ET-1 release in the eluate.

The IPRK model was performed on 24 male Wistar rats (250-300 g body wt.), anaesthetised with thiopental (5mg/1000g b.w. i.p.) as previously reported (Giovannini *et al*, 1998). CyA and LPC were infused by two side arms connected with two infusion pumps. The kidney was perfused by an oxygenated Krebs-Henseleit solution. NO_2-NO_3 in eluate, were evaluated colorimetrically (Granger's method). ET-1 was assayed by a RIA method. Renal cortical samples were assayed for MDA+4-HAD content by a colorimetric kit (Calbiochem, Italy).

Results are expressed as mean ± standard error; the differences between groups were studied by ANOVA and the S.N.K. test was used to study statistical significance ($p<0.05$).

CyA enhanced renal lipid peroxidation (MDA+4-HDA = 6.1+0.8 and 1.8±0.3 mM/mg prot., respectively, $p<0.01$), NO_2+NO_3 generation (2.61±0.5 *vs* 0.98±0.12 mM, $p<0.01$) versus the controls. No alterations were observed in ET-1 secretion (3.3±1.6 *vs* 2.9±0.5 fmol/ml) LPC pretreatment significantly reduced cortical levels of MDA+4-HDA (3.9+0.6 mM/mg prot, $p<0.05$) and R.V.R., but did not affect NO_2+NO_3 levels (2.27±1.4 pM) and ET-1 secretion (2.6±1.0 fmol/ml).

We conclude that LPC is able to prevent CyA-induced acute nephrotoxicity by reducing lipid peroxidation without affecting NO_2+NO_3 generation. The increased level of NO_2+NO_3 may be related to enhanced shear stress subsequent to CyA-induced renal vasoconstriction. The constant level of ET-1 may be related to the short time of CyA administration.

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254P THE PROTECTIVE EFFECT OF NO-ASPIRIN ON HAEMODYNAMICS FOLLOWING ISCHAEMIA-REPERFUSION INJURY TO THE KIDNEYS OF ANAESTHETISED RATS

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Acute renal failure arising from hypoxic damage to the kidney has a significant morbidity and high mortality in man. The mechanisms brought into play by the period of hypoperfusion which lead to renal vascular and tubular damage are multifactoral and include superoxide free radicals, endothelins, nitric oxide and arachidonic acid metabolites.

This study aimed to examine how a novel compound, NO-aspirin, NCX4016 (Minuz *et al,* 1998), a NO donor plus non-selective cyclo-oxygenase (COX) blocker, altered the regional vascular changes to a short period of renal ischaemia and to compare its action with that of a selective COX-2 blocker, celecoxib.

Groups of male Wistar rats (250-230 g) received either polyethylene glycerol/saline vehicle (n=7), NO-aspirin (100 mg/kg, n=7) or celecoxib (10 mg/kg, n=6) once per day by gavagein 2 ml/kg, for 14 days. The animals were anaesthetised with sodium pentobarbitone, 60 mg/kg i.p. and cannulae placed in the femoral artery to measure blood pressure and femoral vein to infuse saline (150 mmol NaCl) at 3 ml/h and sodium pentobarbitone at 15 mg/kg/h. The left kidney was exposed via a flank incision, cleared of attached tissue, the artery separated from the vein and then placed securely into a holding cup. A small area of capsule was removed and two single fibreglass probes (0.5 mm diameter) inserted into the kidney to depths of 1.5 mm (cortex) and 5 mm (medulla) which were attached to a laser-Doppler flowmeter to measure blood perfusion in these regions.

After a 1 h stabilisation period, 5 min baseline readings were taken, the renal artery clamped for 30 min and, on release, further measurements were taken every 10 min for the subsequent 90 min. Means ± sem were compared using ANOVA and significance taken at $P<0.05$.

In the vehicle group, baseline blood pressure, at 102±3 mmHg, rose during the ischaemic period to 131±7 mmHg, but after 40 min reperfusion had normalised. Cordcal perfusion, at 159±29 perfusion units (PU) and medullary perfusion, at 66±10 PU, in the baseline period, but were reduced by 37% (both $P<0.001$) during the whole of the reperfusion period. The blood pressure responses to the renal ischaemia in the NO-aspirin treated rats were comparable to the vehicle treated group, but in the reperfusion period both cortical and medullary perfusions returned towards or even above the baseline values at 80 min, which was significantly different from the vehicle treated group (both $P<0.001$). By contrast, in the rats treated with celecoxib, the period of renal ischaemia elicited a similar pattern of change in blood pressure and reductions in cortical and medullary perfusions in the reperfusion period comparable to that observed in the vehicle treated rats.

These data show that the cortical and medullary vasoconstrictions to renal ischaemia were largely prevented by the NO-aspirin, but not by the selective COX-2 inhibitor. Whether this reflects an action of the NO generation alone, the COX-inhibition or a combination of both remains to be clarified.

Minuz, P., Lecki, C., Zuliana, V. *et al.* Cardiovasc. Drug Rev. 16: 31-47, 1998