55P UPTAKE OF FLUORESCENTLY LABELLED ANTISENSE TO $\alpha_{2A/D}$-ADRENOCEPTORS INTO RAT BRAIN FOLLOWING i.c.v. INJECTION

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Antisense oligonucleotides (ODN) are now being used to investigate the functions of a diverse range of proteins in the CNS (Robinson *et al.*, 1997a). In previous studies we have shown that antisense to the $\alpha_{2A/D}$-adrenoceptor significantly inhibits α_2-adrenoceptor expression in specific brain areas (Robinson *et al.*, 1997b) and induces behavioural changes (Robinson *et al.*, 1998a,b). In order to fully interpret the binding and functional data we have examined the anatomical distribution of the antisense sequence using a fluorescently labelled ODN (sequence described by Nunes, 1995).

Male Wistar rats (270-310 g) were anaesthetised with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and placed in a stereotaxic frame. Each rat received a single, unilateral injection of the fluorescent ODN (2 nmol in 2µl H_2O, n=4) into the left lateral ventricle (0.92mm caudal to bregma, 1.4mm lateral and 3.5mm below the dura). Injections were made over a 5 min period. The rats were allowed to recover and, 10 hr after injection, were re-anaesthetised (as above) and the brains sectioned (25µm), fixed and mounted for fluorescent microscopy.

The fluorescent signal from the antisense sequence was detected for 2-3mm into the brain tissue surrounding the site of injection. On the left side of the brain a strong signal was seen in the septum, striatum, frontal cortex, corpus callosum, thalamus, hypothalamus and the rostral hippocampus. On the right side of the brain a strong signal was only evident in the septum, thalamus and hypothalamus. The unilateral distribution is evident in fig 1, which shows uptake in the pyramidal and lacunosum moleculare layers of the hippocampus on the left and the right side of the brain. The signal in the hypothalamus was stronger than expected relative to the distance from the injection site and may reflect some other mechanism of uptake from the lateral ventricle. Although a fluorescent signal was detected in the ependymal layer of the third and fourth ventricle the ODN was not evident in the surrounding parenchyma. The results of the present study reveal that, following i.c.v. administration the antisense is detected, predominantly, on the same side as the infusion and in brain structures close to the site of the infusion. Despite the administration of the ODN into the ventricular system of the CNS, the antisense distribution is limited and is not detected in structures of the midbrain and hindbrain.

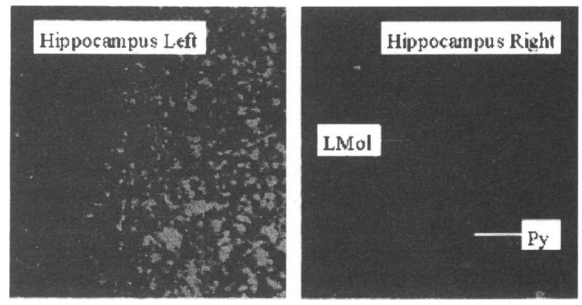

Fig 1: Representative fluorescent images taken from the CA3 region (bregma –2.80mm) of the hippocampus showing the predominantly, unilateral uptake of a fluorescently labelled, 18 base, phosphorothioate antisense ODN. Abb: Py, pyramidal layer, LMol, lacunosum moleculare layer. Scale Bar ——— ~ 100µm.

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56P INVESTIGATION OF THE AFFINITY OF MONOAMINE REUPTAKE INHIBITORS FOR RAT BRAIN IMIDAZOLINE 2 BINDING SITES

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2-(2-Benzofuranyl)-2-imidazoline (2BFI) is a highly selective ligand for the I_2 class of imidazoline binding sites (Hudson *et al.*, 1997). Brain dialysis studies have shown I_2 sites to elevate brain monoamine concentrations through an unknown mechanism (Nutt *et al.*, 1995). Furthermore, 2BFI shows activity in the Porsolt forced swim test which could indicate antidepressant activity (Nutt *et al.*, 1995). Given this profile of activity we examined several monoamine uptake inhibitors for their ability to inhibit [^{3}H]2BFI binding to rat brain membranes.

Brains were removed from male Wistar rats (250-280g), homogenised (10 w/v 50mM Tris-HCl buffer, 1mM $MgCl_2$ buffer, pH 7.4) and pelleted by centrifugation (32,000g). Aliquots of membrane (500µg) were incubated with 1nM [^{3}H]2-BFI, to label I_2 sites (45min, 25°C, final volume 0.5ml). The specific component of binding was defined by 10µM BU224 (2-(4,5-dihydroimidaz-2-yl)quinoline). Bound ligand was separated by filtration and determined by scintillation counting. Results were analysed by Prism 2.01 (GraphPad Software).

Unlabelled 2BFI demonstrated high affinity for I_2 sites (Table 1), which is in agreement with our previous findings. The monoamine uptake inhibitors tested inhibited the binding of [^{3}H]2BFI (Table 1). Paroxetine and fluoxetine showed low affinity for I_2 binding sites displacing 2BFI in a monophasic manner with K_i values in the low µM range (Table 1). Fluvoxamine and sertraline showed very low affinity again displacing with Hill slopes close to unity. Interestingly, nisoxetine exhibited a biphasic profile for the displacement of [^{3}H]2BFI, with nM affinity for 67% of the labelled sites (Table 1).

Compound	I_2 site (K_i, nM)
2BFI	1.74 ± 0.24
Nisoxetine	560 ± 156* 31300 ± 10000
Paroxetine	8287 ± 471
Fluoxetine	1330 ± 149
Fluvoxamine	28000 ± 8000
Sertraline	41000 ± 11000

Table 1. Affinities (K_i) of compounds for I_2 sites in rat brain membranes, data are means ± s.e.mean, from 3 - 5 experiments performed in triplicate. *High affinity site.

Although these uptake inhibitors are not imidazolines, two of them, fluoxetine and nisoxetine, displayed low to moderate affinity for I_2 binding sites. The noradrenaline uptake inhibitor nisoxetine recognised two apparent populations of I_2 binding sites, one with nM affinity. This is unlikely to reflect an association with uptake inhibition, as we have previously shown 2BFI to be a weak inhibitor of monoamine uptake (Lalies *et al.*, 1996). Furthermore, the affinities of these uptake inhibitors for their respective monoamine transporters are in the very low nM range (Cheetham *et al.*, 1993).

MME is a BBSRC CASE student with Knoll Pharmaceuticals.

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57P AFFINITIES OF β-CARBOLINES FOR I_2-BINDING SITES IN RABBIT BRAIN MEMBRANES

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The imidazoline-2 (I_2) binding site is proposed to be a domain on the enzyme monoamine oxidase (MAO) and therefore plays a role in central monoamine turnover (for review see Eglen *et al.*, 1998). Agmatine has been proposed to be the endogenous ligand for I_2 sites (Li *et al.*, 1994) although it has rather low affinity in binding studies (Lione *et al.*, 1996). At a previous meeting of the Society we proposed certain *β*-carbolines be considered as endogenous ligands due to their high affinity in rat brain membranes (Hudson *et al.*, 1999). We have now investigated the affinity of several *β*-carbolines for rabbit brain I_2 sites using the selective I_2 ligand [^{3}H]2BFI (2-(2-benzofuranyl)-2-imidazoline).

Rabbit (New Zealand, either sex, 1.5-3.5 kg) brains were homogenised (10 w/v 50mM Tris-HCl, 1mM $MgCl_2$ buffer, pH 7.4 containing 320mM sucrose) and membranes prepared by the methods of Lione *et al.* (1996). Aliquots of thawed membrane were incubated (24°C, 45min) with 1nM [^{3}H]2BFI to label I_2 binding sites. Specific binding was defined by 10μM BU224 (Lione *et al.*, 1996). The *β*-carbolines were examined for their ability to compete with [^{3}H]2BFI over the range of 0.01nM-1mM. Bound ligand was separated by rapid filtration and determined by liquid scintillation counting. Results were analysed by Prism (GraphPAD Software, 1994).

Agmatine was of low affinity whereas the precursor of many *β*-carbolines, tryptamine, demonstrated reasonable affinity with a K_i of 3μM. Several *β*-carbolines displaced [^{3}H]2BFI in a biphasic manner yielding high affinity (K_iH, approx. 50% of labelled sites) and low affinity (K_iL) components (Table 1). Of these compounds, Harmane showed the highest affinity for I_2 sites with a K_iH of 9.7 nM. In comparison the exogenous *β*-carbolines, harmine and harmaline, also demonstrated very high affinity for around 50% of labelled sites (Table 1). Ethyl *β*-carboline-3-carboxylate (*β*-CCE) displaced from an apparent single site with very low affinity (Table 1).

Compound	K_iH (nM)	*n*	K_iL (nM)	*n*
Tryptamine	3027 ± 219	3		
Agmatine	856,633 ± 4068	3		
Norharmane	52.87 ± 7.5	4	30490 ± 8241	4
Noreleagnine	36.6 ± 3.7	3	30417 ± 8424	3
Harmane	9.7 ± 2.4	4	3592 ± 586	4
Harmine	15.4 ± 5.2	3	1049 ± 343	3
Harmaline	12.5 ± 2.9	3	6651 ± 1105	3
β-CCE	76617 ± 2978	3		

Table 1 Affinities of compounds for I_2 binding sites in rabbit whole brain membranes. Data are mean K_i values ± s.e.mean derived from three to four experiments performed in triplicate.

These data demonstrate particular *β*-carbolines show higher affinity for rabbit I_2 binding sites than agmatine, the proposed endogenous ligand. These results parallel those for rat brain and reinforce our proposal that particular endogenous *β*-carbolines for example noreleagnine may have a neuromodulatory role *via* I_2 sites.

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58P UNILATERAL QUINOLINIC ACID LESION DECREASES IMIDAZOLINE-2 SITE BINDING IN RAT STRIATUM

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Imidazoline-2 (I_2) binding sites are widespread in mammalian brain and particularly dense in human basal ganglia (for review see Eglen *et al.*, 1998). In post-mortem Huntington's disease putamen we have reported a marked decrease in the density of I_2 binding sites, suggesting they are located on the degenerating neurones (Reynolds *et al.*, 1996). To study this in further detail, we have investigated the density of I_2 sites in a rat model of Huntington's, based on a quinolinic acid (QA) lesion of the striatum (Nicholson *et al.*, 1995). The resulting gliosis was examined using [^{3}H]PK11195 which labels peripheral-type benzodiazepine binding sites (PTBBS) found on glial cells (Dubois *et al.*, 1988).

Following pentobarbitone (60 mg kg^{-1} i.p.) anaesthesia of the rats (male Wistars, 250-300g), stereotaxic procedures were employed to inject QA (200 nmoles in 2μl) or vehicle (phosphate-buffered saline) into the right striatum (3.2mm lateral to bregma, depth 5.5mm from the dura). Clonazepam (1 mg kg^{-1} i.p.) was administered post-operatively and the rats allowed to recover for 7 days. Brains were removed under anaesthesia (as above), frozen, sectioned (12μm) and processed for autoradiography according to Lione *et al*, (1998). In adjacent sections, PTBBS were labelled with 1 nM [^{3}H]PK11195 and I_2 binding sites with 1nM [^{3}H]2-BFI (45min, 25°C). Sections of brain were apposed to [^{3}H]-sensitive film for 2 and 4 weeks respectively, and bound ligand determined by quantitative image analysis.

Sham lesions did not alter binding of either [^{3}H]PK11195 or [^{3}H]2BFI (see Table 1; vehicle injections, right *vs* left striatum). QA lesion of the right striatum led to a marked increase in the binding of [^{3}H]PK11195 relative to the unlesioned (left) side, consistent with an increase (by 160%) in the density of PTBBS. Conversely, [^{3}H]2BFI binding was decreased in QA lesioned striatum relative to the unlesioned striatum.

Treatment / ligand	Left Striatum fmol mg^{-1} tissue	Right Striatum fmol mg^{-1} tissue
Vehicle / [^{3}H]PK	16.2 ± 6.8	21.1 ± 8.9
QA / [^{3}H]PK	17.6 ± 3.0	45.8 ± 5.6 *
Vehicle / [^{3}H]2BFI	15.1 ± 1.8	15.3 ± 1.8
QA / [^{3}H]2BFI	18.4 ± 1.4	10.0 ± 2.3*

Table 1. Densities of [^{3}H]PK11195 binding and [^{3}H]2BFI binding in rat striatum following vehicle or QA injection into the right striatum, mean specific ligand bound ± s.e.mean, n=5: *P<0.05 relative to left striatum (unpaired t-test).

The increase in [^{3}H]PK11195 binding seen in the QA lesioned striatum is consistent with neuronal loss and subsequent gliosis (Dubois *et al.*, 1988). The corresponding loss of [^{3}H]2BFI binding would therefore indicate that a significant proportion of I_2 sites are on intrinsic neurones of the striatum which are destroyed due to the excitotoxic lesion. This loss of I_2 sites (54%) is comparable to that seen in Huntington's disease (56% loss, Reynolds *et al.*, 1996).

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59P INDUCTION OF LTP IN THE CA1 AREA OF RAT HIPPOCAMPUS FOLLOWING ATP PERFUSION

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Adenosine 5'-triphosphate (ATP) activates two classes of membrane receptor, a ligand gated ion channel- the P2X receptor, of which seven subtypes have been cloned, and a G-protein coupled receptor- the P2Y receptor. Many of the electrophysiological effects of ATP on brain neurones are a result of degradation to adenosine (Stone, 1989). In this study we report an ATP-induced long-term potentiation in rat hippocampal CA1 neurones.

Slices (450μm thick) of rat hippocampus were prepared as described by O'Kane & Stone (1998). Test stimulation (0.05Hz) was delivered via a concentric bipolar electrode placed in the stratum radiatum. After 45min, recordings of orthodromic extracellular population spikes (PS) were made from the stratum pyramidale and their amplitude measured. In some slices population excitatory postsynaptic potential (EPSP) slopes were also measured from the stratum radiatum. The temperature of the recording chamber was approximately 33 °C. The results are expressed as mean ± s.e. mean.

Application of 10μM ATP for 10min decreased the PS amplitude to 33.9±13.7% of control values (n=5, $p<0.05$) and EPSP slope to 72.3±9.7% of control values (n=3, $p<0.05$). Following 30min washout these potentials increased to levels above their control values resulting in long-term potentiation (LTP). PS amplitude was increased to 142.2±14.8% of control and EPSP slope to 119.7±6.4% of control. 2.5μM ATP also resulted in LTP, increasing PS amplitude to 127.8±3.7% of control (n=3, $p<0.05$). It did not produce any inhibition of spike amplitude during perfusion. 10min application of 40μM ATP (n=3) caused an almost complete inhibition of PS amplitude but no LTP was observed following 30min washout. The stable analogue of ATP, α,β-methylene ATP (10μM, n=3) had no long-term effects on PS amplitude or EPSP slope, nor did it produce a depression of these responses during 10min perfusion. Blocking P2 receptors with suramin (50μM, n=4) had no effect on the depression of responses seen during perfusion of 10μM ATP but resulted in a loss of LTP. Similarly, blocking receptors for N-methyl-D-aspartate (NMDA) with 50μM 2-amino-5-phosphonopentanoic acid (AP-5, n=5) prevents induction of LTP but not the inhibition of responses following ATP perfusion. When adenosine A_1 receptors were blocked using 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 100nM, n=6), or when both adenosine A_1 and A_2 receptors were blocked using a 10μM concentration of 8-phenyltheophylline (8-PT, n=10), no inhibition of responses was seen during perfusion of 10μM ATP, nor was LTP induced. Similarly, when adenosine deaminase was used to remove adenosine from the system (0.2U/ml, n=4), 10μM ATP did not cause a significant depression of response size and did not induce LTP. However adenosine itself (10μM, n=5) did not induce LTP. Interestingly, when A_2 receptors only were blocked with 4-(2-[7-amino-2-{2-furyl}{1,2,4,}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385, 50nM, n=6), perfusion of 10μM ATP still induced LTP, increasing PS amplitude to 124.5±7.0% and EPSP slope to 125.7±5.4% of control values. However ATP no longer caused any inhibition of responses during 10min perfusion.

These results suggest that induction of LTP following ATP perfusion as reported by Wieraszko and Seyfried (1988) requires the stimulation of both P2 and A_1 receptors.

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Stone, T.W. (1989) *Adv. Drug Res.*, 18, 292-429.
Weiraszko, A. & Seyfried, T.N. (1989) *Brain Res.*, 491, 356-359.

60P ATP RESPONSES OF INTERNEURONES IN THE RAT HIPPOCAMPUS

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While there have been reports of purine nucleotide effects on membrane currents in hippocampal neurones, there is less evidence for changes of overall neuronal excitability in individual cell populations. We now report effects of adenine nucleotides on a population of interneurones in the hippocampal slice.

Slices were prepared 450μm thick from adult male rats and preincubated for at least 1 hour before experimentation at 33-35°C. Recordings were made using glass microelectrodes containing 200mM NaCl (extracellular) or 1M Kacetate (intracellular). Stimuli were delivered via a concentric bipolar electrode in the stratum radiatum. Neurones were classified as pyramidal tract cells or interneurones on the basis of their stimulus-dependent responses to stimulation in the stratum radiatum, interneurones showing variable latencies which tended to decrease with increasing stimulus strength. Agonists were applied into the vicinity of the recorded neurones by microiontophoresis (Stone, 1985) from multibarrelled pipettes containing 10mM solutions of the agents.

Of 18 neurones tested with ATP, 3 showed a purely inhibitory response. Ten cells showed an initial inhibition which coincided with the ejection period, followed by an excitation which often lasted for several minutes. Of other agonists tested, α,β-methyleneATP had no effect on 8 cells and adenosine produced only inhibition. Biphasic responses similar to those seen with ATP were produced by β,γ-methyleneATP on 4 of 6 cells.

Intracellular recordings revealed that the initial inhibitory effect of ATP involved a hyperpolarisation of up to 16mV, while the post-ejection excitation was accompanied by depolarisation.

The biphasic responses to ATP showed evidence of desensitisation, with a second and third application eliciting around 70% and 50% respectively of the depolarisation produced by the first application. The application of 1,3-dipropyl-8-cyclopentylxanthine (50nM) prevented the hyperpolarisation produced by ATP (5.5% ± 3.4, n = 4) and reduced the excitatory component to 22% ± 6.3 (n = 4). Suramin (100 μM) had no effect on the inhibitory phase but reduced excitation to 37.7% ± 9.8 (n = 4).

The results indicate that a population of interneurones respond to adenine nucleotides with a biphasic pattern including a slow but long-lasting excitatory component. The effects of antagonists suggest that P1 receptors may be involved, while the reduction by suramin implies a role for P2 receptors. One possible interpretation is that co-activation of P1 and P2 receptors may be required for these late excitatory responses. The existence of ATP-sensitive interneurones may account for reports of ATP effects in the hippocampus, including long-term potentiation.

Stone, T.W. (1985) *Microiontophoresis and Pressure Ejection.* Wiley, Chichester.

61P LOCALISATION OF P2 RECEPTORS ON CEREBRAL CORTICAL CELL CULTURES FROM EMBRYONIC RATS

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We recently reported effects of stimulating G protein-coupled P2Y receptors in cerebral cortical cells from embryonic rats (Bennett *et al.* 1999). The responses were consistent with predominantly $P2Y_1$ receptor activation. These brain cultures are mainly neurones and glial cells. The aim of this study was to determine which cell type is involved in the purinergic profile observed. Three approaches were pursued: comparison of agonist dose/response curves in mixed and glial cultures, differential effect of agonists on neurones and glia using single cell imaging and use of BAY K8644 and subsequent quenching by manganese as a neuronally selective indicator.

E19 Sprague Dawley embryonic rat cerebral cortices were dispersed by enzymatic digestion. Cells were cultured in DMEM containing either the neuronal survival supplement, B27 (mixed population) or 10% HIBS (glial culture). Cells were loaded with Fluo-3AM and agonist dose/response curves constructed using a fluorescence imaging plate reader. 20μM BAY K8644 was co-added with 18 mM KCl and the effect of manganese on a subsequent addition of agonist monitored. For single cell imaging the cells were loaded with Fura-2AM. The purines shown in Table 1 produced dose dependant increases in $[Ca^{2+}]_i$ giving the pEC_{50} values in Table 1.

Table 1. Agonist pEC_{50} values for mixed and glial cultures

Agonist	2MeSADP	2MeSATP	ADP	ATP	ATPγS
pEC_{50} (mixed)	8.2	7.4	6.9	6.7	6.2
pEC_{50} (glial)	8.7	8.3	7.7	6.9	7.2

In the glial cultures, the response to 100 mM KCl was reduced by two thirds in comparison to the mixed culture indicating that the neuronal subpopulation was greatly reduced. The glial cultures gave a maximal response to nucleotides which was approximately twice that of the mixed cell culture. The rank order of agonist potency for the glial cell culture was similar to the mixed population (Table 1). The similar profiles in the mixed culture and the glial culture suggests that the neurones in the mixed culture were not playing a large part in the observed response.

When L-type voltage sensitive Ca^{2+} channels were opened by co-addition of 20 μM BAY K8644 and 18 mM KCl, manganese added to the extracellular environment should selectively enter neurones and quench the dye. When ADP, ATP and 2MeSADP were then added, the magnitude of their response was identical to control levels (no manganese). This would suggest that the responses were not neuronal.

Single cell fluorescence studies showed that, in a mixed culture, 82% of neurones and 50% of glial cells respond to ATP. 15% of neurones as opposed to close to 100% of glial cells responded to 2MeSATP. 16% of neurones responded to α,β-meATP and no cells displayed activity to UTP.

From the three pieces of evidence presented, it would appear that there is purinergic activity in both neurones and glia. Glial cells exhibit behaviour consistent with $P2Y_1$ activation and neurones respond largely to ATP but also to a small degree to α,β-meATP.

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Bennett, G.C., Ford, A.P.D.W., Boarder, M.R. (1999). Brighton BPS.

62P TONIC ADENOSINE NEUROMODULATION IS UNALTERED IN MOTOR NERVE ENDINGS OF AGED RATS

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Adenosine can either inhibit, via A_1 receptors, or facilitate, via A_{2A} receptors, [^{3}H]acetylcholine (ACh) release from rat motor nerve endings (Correia-de-Sá *et al.*, 1991). Since modulation of ACh release by adenosine in the hippocampus is altered by aging (Cunha *et al.*, 1996), we now compared the tonic modulation of [^{3}H]ACh release by endogenous adenosine in the innervated hemidiaphragm of young adult (6 weeks) and aged (2 years) male Wistar rats.

Phrenic nerve-hemidiaphragm preparations from male Wistar rats were mounted in 2 ml superfusion chambers and loaded with 40 nM [^{3}H]choline (specific activity: 85 Ci/mmol). After washing, the preparations were stimulated through the nerve trunk (15 V, 40 μs, 5 Hz for 2 min) twice (S_1 and S_2) and the superfusate collected for tritium quantification. Drugs were added before S_2 and their effect quantified as the modification of tritium release in S_2/S_1 compared to control (absence of added drugs) (see Correia-de-Sá *et al.*, 1991). Results are mean ± SEM and significance was assessed by the Student's *t* test.

Both the basal outflow of tritium (507±63 Bq/g tissue in young adult *vs* 165±15 Bq/g tissue in aged rats) and the evoked release of [^{3}H]ACh (213±32 Bq/g tissue in young adult *vs* 109±16 Bq/g tissue in aged rats) were depressed in aged animals, but the weight of tissue was lower in young adult (57±2 mg) than aged rats (168±7 mg). Data not normalized by weight of preparation sustains the hypothesis that no difference in [^{3}H] outflow is observable between aged and young animals. The ratio of the evoked release of [^{3}H]ACh in the two different stimulation periods was similar in the two aged groups (1.15±0.03 in young adults *vs* 1.08±0.05 in aged rats) which allowed the comparison of drug effects in both age groups. The A_1 antagonist, 1,3-dipropyl-8-cyclo-pentyl-xanthine (DPCPX, 50 nM) increased [^{3}H]ACh release by 74±13% (n=7) in young rats (6 weeks), and by 91±20% (n=5) in aged rats (2 years). The A_{2A} antagonist, 4-(2-[7-amino-2-(2-furyl-{1,2,4}-triazolo-{2,3-a{1,3,5}triazin-5-yl-amino-ethyl)-phenol (ZM 241835, 50 nM) decreased [^{3}H]ACh release by 77±9% (n=4) in young rats and by 73±8% (n=5) in aged rats.

The present results provide a direct pharmacological demonstration of tonic facilitatory effects mediated by adenosine A_{2A} receptors at the rat innervated hemidiaphragm of young adult rats, together with the well known tonic inhibition via A_1 receptor activation. The results also show that, in aged rat innervated hemidiaphragms, endogenous adenosine also exerts a modulatory tonus via both A_1 and A_{2A} receptors, which is not significantly different (P>0.05) from that found in young adult rats.

Correia-de-Sá *et al.* (1991) *Br.J.Pharmacol.* 103, 1614
Cunha *et al.* (1996) *Drug Dev.Res.* 37, 179

63P GLIAL AND NEURONAL CHANGES IN A POTENTIAL MODEL OF STROKE USING *IN VITRO* SPHEROID CULTURES: A COMPARATIVE STUDY OF ANOXIA/ISCHAEMIA

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Mechanisms causing ischaemic damage following a stroke are still poorly understood, although excitatory amino acids, free radicals and cytokines have all been implicated. The development of organotypic *in vitro* models may allow analysis of more discrete cellular events associated with anoxic and ischaemic cell damage. Whole brain spheroids are an *in vitro* model containing differentiated neurones and glia, thus providing an organotypic representation of the brain (Atterwill; 1987).

Foetal Wistar rat whole brain spheroids were cultured for 12 days in Dulbecco's Modified Eagle's Medium and 10% foetal calf serum at 37°C, 9% CO_2. Cultures were made anoxic or ischaemic for a period of 2 hours. Anoxia was achieved by suspending the spheroid culture in Tris-buffered Krebs medium supplemented with glucose and ischaemia was achieved by suspending cultures in glucose-free Tris-buffered Krebs medium. Oxygen was removed from cultures by gassing with N_2 in sealable tissue culture chambers. Following treatments, the spheroids were immediately harvested and homogenised. Homogenates were then assayed for protein content with a colorimeter, for the glial marker glial fibrillary acidic protein (GFAP) by Elisa, and for the neuronal marker acetylcholinesterase (AChE) by a kinetic assay.

Table 1: Effects of 2 h anoxia or ischaemia on GFAP or AChE

	GFAP (μg/mg protein)		AChE (IU/mg protein)	
	mean	% control	mean	% control
Control	0.90+/-0.29		0.029+/-0.005	
Anoxia	1.12 +/-0.38	124+/-36	0.03 +/-0.006	105+/-25
Ischaemia	2.22+/-0.75	247+/-73**	0.052 +/-0.0.01	181+/-43*

*=P<0.05 **= P <0.01 multiple t test (n=6)

Significant increases in AChE activity and GFAP levels were detected with an ischaemic insult. No significant changes were detected with anoxic treatments (Table 1). The ischaemia data indicated concomitant alterations in glial and neuronal function. The GFAP increases may reflect reactive gliosis (hypertrophy of glial cells) an effect known to be associated with neurotoxic insult (Dell'Anna et al., 1995). Similarly, increased AChE activity may reflect an increase in acetylcholine release, an event that has been demonstrated in previous ischaemic studies utilising *in vivo* microdialysis (Kumagae & Matsui, 1991). These data suggest, that in contrast to ischaemia, anoxia does not cause damage within the parameters measured, and ischaemia produces more substantial effects upon glial cells. In conclusion, neuronal and glial ischaemic changes have been demonstrated with this novel *in vitro* spheroid model for stroke which can be investigated further with pharmacological intervention.

Acknowledgments to Knoll Pharmaceuticals and the BBSRC for the funding of this research.

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64P ACUTE TREATMENT WITH CLOMIPRAMINE AND THE NMDA ION CHANNEL ANTAGONIST AMANTADINE INCREASES EXTRACELLULAR SEROTONIN IN THE FRONTAL CORTEX OF THE RAT

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For over a quarter of a century the 'monoamine theory' has dominated research into depressive illness. It has been postulated that a deficiency in synaptic 5-hydroxytryptamine (5-HT) plays a role in the aetiology of depression. However, more recently it has been suggested that dysfunction of N-methyl-D-aspartate (NMDA)-glutamatergic receptors may also play a role in depression (Cappello, *et al.*, 1997). Activation of NMDA receptors in the raphé has been observed to alter 5-HT release in the raphé and frontal cortex (FC) of the same rat (Pallotta, *et al.*, 1998) We have recently observed that the antidepressant clomipramine (CIM) decreases NMDA-evoked changes in serotonergic transmission between the raphé nuclei and FC of the rat (Whitton, *et al.*, 1998). This in turn suggests that modifying NMDA receptor function may be significant in the treatment of depression.

In the present study we have investigated the effects of altering NMDA receptor activity using amantadine, a clinically used, low affinity NMDA ion channel antagonist on CIM-induced changes in 5-HT and glutamate (Glu) in the raphé and FC of freely moving rats. Male Wistar rats (225-275g) were anaesthetised with halothane and concentric dialysis probes implanted into the raphé and FC of the same rats as previously described (Pallotta, *et al.*, 1998). The following day the rats were dialysed with artificial cerebrospinal fluid (composition in mM; KCL 2.5; NaCl 125; $MgCl_2$ 1.18; $CaCl_2$ 1.26). After four 30 minute samples had been collected, CIM (20mg/kg i.p.), amantadine (40mg/kg i.p.) or vehicle were administered. All data are represented as mean ± s.e.mean, n= 5.

Acute CIM increased 5-HT to 410 ± 70% above basal in the raphé, which was associated with a fall in 5-HT release in the FC to 47 ± 8% of basal. Following a slight increase, extracellular Glu in the FC fell to 29 ± 10% of basal, while in the raphé no effect was seen. Amantadine treatment caused a small but significant decrease in raphé levels of 5-HT, while in the FC a significant increase to 173 ± 59% of basal levels was seen. No effects of the drug on levels of extracellular Glu were seen in either brain region.

When amantadine was given thirty minutes prior to CIM, the increase in raphé 5-HT release seen with CIM alone was abolished and an increase in FC 5-HT release was observed. The abolition by amantadine of CIM-induced increases in raphé 5-HT would be expected to prevent activation of somatodendritic 5-HT_{1A} autoreceptors. This in turn might be expected to prevent a resultant decrease in serotonergic transmission (Artigas, *et.al.* 1996). While neither amantadine nor CIM alone altered raphé Glu release, the combination of the two drugs led to a steady increase in Glu levels (upto 460 ± 63% of basal). In the FC, amantadine and CIM altered glutamate release in a manner similar to that seen with either drug alone.

The present findings support an interaction between serotonergic and glutamatergic transmitter systems regulating 5-HT transmission. The principal observation of the present study is that prior treatment with amantadine altered the effect of CIM on 5-HT release in both brain regions and also that of Glu in the raphé. The combination of these drugs seems to abolish the latent period typically required before increased cortical 5-HT release is observed following chronic antidepressant therapy. This may suggest that amantadine, in some way, removes the requirement for desensitisation of 5-HT_{1A} receptors in the raphé (Artigas, *et al.*, 1996). Since latency of onset is probably the major drawback in antidepressant therapy, the present findings may be of future clinical benefit.

JCES holds an MRC studentship.

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65P EFFECT OF REPEATED EXPOSURE TO *d*-AMPHETAMINE ON ENDOGENOUS ANTIOXIDANTS IN THE RAT BRAIN

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d-Amphetamine has been shown to be a potential brain neurotoxic agent, particularly to dopaminergic neurons. Reactive oxygen species generated by amphetamine have been indicated as an important factor in the appearance of neuronal damage (Huang et al, 1997). However, little is known about the adaptations of brain antioxidant systems to its chronic administration. As a first step towards assessing the variation of antioxidant defenses against repeated administration of *d*-amphetamine we measured the activities of the antioxidant enzymes glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), catalase, and superoxide dismutase (SOD) in rats treated with this drug.

Male Wistar rats (160-180g) were randomly divided into 3 groups of 4 animals each and housed individually in metabolic cages at 22±2°C. One group of animals was dosed with *d*-amphetamine sulphate (20 mg/kg s.c., once daily, for 14 days). Another group was the pair-fed control (i.e. on each day it was given the same amount of food consumed by the amphetamine treated group in the previous day) and a third group was used as the *ad libitum* control. At the end of the experiment, animals were anaesthetized with diethyl ether, and their brains were rapidly removed and dissected over a cold plate into discrete brain areas including hypothalamus, hippocampus, striatum, nucleus accumbens, and medial prefrontal cortex. These brain areas were frozen on dry ice and stored at -70°C until enzyme kinetic analysis according to standardized methodology.

Statistical comparisons were made with one-way ANOVA followed by Fisher's test.

When compared to the pair-fed group, *d*-amphetamine treatment enhanced the activity of GST in hypothalamus (167%; $p<0.05$), GPx in striatum (127%; $p<0.01$), nucleus accumbens (192%; $p<0.01$), and medial prefrontal cortex (139%; $p<0.05$), GR in hypothalamus (139%; $p<0.05$), as well as catalase in medial prefrontal cortex (153%; $p<0.05$). However, the same comparison revealed a decrease in the activity of GR in medial pre-frontal cortex (35%; $p<0.05$) and SOD in striatum (23%; $p<0.05$). Food restriction itself reduced GPx activity (49%; $p<0.01$) and increased catalase activity (271%; $p<0.05$) in nucleus accumbens.

To our knowledge, this is the first report of antioxidant enzyme adaptation of the studied brain areas to a repeated administration of *d*-amphetamine. The observed adaptations of the antioxidant enzymes in the studied brain areas imply that after repeated administration of *d*-amphetamine they became generally more prepared to fight oxidative burst.

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66P GAMMA-HYDROXYBUTYRATE IS NOT POTENTIATED BY NIPECOTIC ACID

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Gamma-hydroxybutyrate (GHB) is a naturally occurring analogue of GABA in the mammalian brain. Systemic administration produces a behavioural depression and anaesthesia in animals and humans. It has uses in the treatment of narcolepsy but is also used unofficially to promote bodybuilding and is considered a drug of abuse. In the brain GHB inhibits the firing of midbrain dopamine neurones and hyperpolarises hippocampal CA1 neurones, which can be blocked by $GABA_B$ receptor antagonists. (Harris et al 1989; Xie & Smart 1992). It has also been suggested to be a possible neurotransmitter acting at specific GHB receptors (Vayer et al. 1987). However, the effects of GHB in the brain occur only at high concentrations. One possibility is that the low potency of GHB is due to a rapid uptake process, so to see effects, high concentrations of GHB are required to saturate the uptake system. The aim of this study was therefore to see if blockade of the GABA uptake system could potentiate the effects of GHB in brain slice preparations
Intracellular recordings were made from guinea pig brain slices containing the substantia nigra as previously described (Harris and Constanti 1995). Recordings of the paroxysmal activity in rat cortical wedge preparations were conducted as described by Horne et al. (1986).
GHB (0.3 - 30 mM) was applied to 39 neurones in the substantia nigra pars compacta (SNC) inhibiting spontaneous firing and causing a dose dependent hyperpolarisation of the membrane potential (EC_{50} = 1.7mM; n=15). GABA (0.1 – 3mM) was also applied to 15 cells and produced both hyperpolarising and depolarising responses such that it was not possible to produce dose dependent responses. The addition of 100 – 500 μM (±) nipecotic acid potentiated the responses to GABA but had little effect on the potency of GHB (EC_{50} = 1.9 mM; n=4). The effect of GHB was antagonised by the $GABA_B$ antagonist CGP 36742 (20 –100 μM), but was not by bicuculline (100 μM).
In cortical wedge preparations, both GHB and GABA inhibited the paroxysmal activity. At 100 μM GABA had a 48±9% (n=4) reduction in the paroxysmal events. In the presence of 100 μM nipecotic acid, 100 μM GABA reduced the events by 90±6% (n=4). While GHB at 10 mM inhibited the paroxysms by 53±4% (n=8) and in the presence of 100 μM nipecotic acid there was a reduction of 57±3% (n=6), which was not significantly different from GHB alone (ANOVA with post hoc Dunn's test).
Thus it is concluded that blocking the GABA uptake system does not potentiate the effects of GHB in brain slices.

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67P PRESYNAPTIC $GABA_B$ RECEPTORS CONTROL SPONTANEOUS GABA RELEASE IN ENTORHINAL CORTICAL NEURONES

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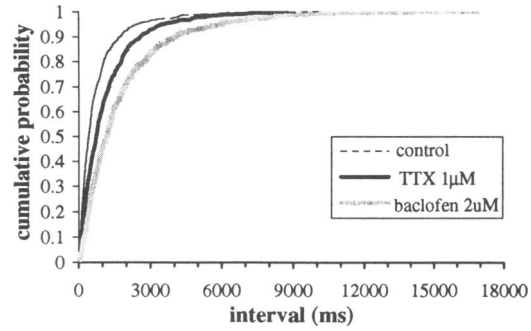
Spontaneous inhibition, mediated by GABA, is evident in both deep and superficial layers of the entorhinal cortex (EC) (Wood & Jones, 1999). However, the frequency of GABA mediated inhibitory postsynaptic currents (IPSCs) in layer II is over twice that recorded in layer V neurones and this may contribute to the latter being relatively "seizure-susceptible" (Jones & Lambert, 1990). We are currently investigating the mechanisms by which GABA release may be controlled in the EC, and which may be implicated in layer specific differences in release. In this study we report the presence of presynaptic $GABA_B$ autoreceptors controlling spontaneous release in layer V neurones.

Whole-cell patch-clamp recordings were made from neurones in EC slices prepared from young adult Wistar rats. Layer V neurones were visually identified using a microscope fitted with an infra red camera and DIC optics. Recording electrodes were filled with a solution containing (mM): CsCl 135, HEPES 10, $MgCl_2$ 2, QX-314 5, $CaCl_2$ 0.5, EGTA 5 (pH 7.3, 285 mosmol). DL-AP5 (50 μM) and NBQX (10 μM) were perfused to block spontaneous excitatory events mediated via NMDA and AMPA receptors respectively. At a holding potential of -60 mV, the remaining currents were abolished by bicuculline (5 μM) indicating that they were spontaneous IPSCs mediated by $GABA_A$ receptors. Drugs were applied by bath perfusion. Statistical comparisons were performed on cumulative probability distributions using the Kolmogorov-Smirnov test.

In pooled data from 7 neurones (minimum of 100 events per neurone), spontaneous IPSCs had a mean (± s.e.mean) amplitude of 26.37±0.69 pA and an inter-event interval of 0.74±0.04 s. To isolate activity-independent miniature IPSCs (mIPSCs), tetrodotoxin (TTX, 1μM) was bath applied. Under these conditions, mIPSC inter-event interval increased to 1.14±0.05 s ($P<0.0001$) while there was no change in IPSC amplitude (25.89±0.73 pA, $P>0.10$). Addition of the $GABA_B$ receptor agonist baclofen (1-2 μM, n=5) significantly increased mIPSC inter-event interval to 1.85±0.10 s ($P<0.0001$) without altering the amplitude (25.19±0.83 pA, $P>0.10$).

Figure 1. Effects of TTX and baclofen on spontaneous IPSC inter-event interval.



Control of spontaneous inhibition by presynaptic $GABA_B$ receptors has often proved difficult to demonstrate in cortical areas (e.g. Scanziani *et al.* 1992). The decrease in frequency of mIPSCs with no change in amplitude suggests that such an effect occurs in layer V of the EC. We are now investigating whether this autoreceptor control of release differs in layer II.

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68P L-AP4-INDUCED FACILITATION OF GLUTAMATE RELEASE IN LAYER V OF THE RAT ENTORHINAL CORTEX *IN VITRO*

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Recent work (Berretta and Jones, 1996) has shown that glutamate release in the entorhinal cortex (EC) is facilitated by presynaptic NMDA autoreceptors. Evidence from other brain areas suggests presynaptic metabotropic glutamate receptors (mGLURs) have an inhibitory effect on glutamate release (e.g. Baskys & Malenka, 1991; Takahashi *et al*, 1996). We are interested in how the two types of autoreceptor may interact in to control glutamate release. In the present study we report results of our initial studies of the effect of mGLURs on glutamate release in the EC.

Experiments were performed using slices of EC prepared from male Wistar rats (70-110g). Whole-cell patch-clamp recordings were made from neurones in layer V, visually identified using a microscope fitted with an infra-red camera and DIC optics. Recording electrodes were filled with a solution containing (in mM): 130 Cs-methanesulphonate, 10 HEPES, 5 QX-314, 0.5 EGTA, 1 NaCl, 0.34 $CaCl_2$, 1 $MgCl_2$, 1 MK801, (290 mOsmol, pH adjusted to 7.3 with CsOH). Neurones were voltage-clamped at –60 mV and drugs were applied by bath perfusion

With MK801 in the patch pipettes to block postsynaptic NMDA receptors we recorded spontaneous AMPA receptor mediated excitatory post-synaptic currents (EPSCs). In pooled data from 5 neurones (minimum of 100 events per neurone). EPSCs had a mean (±s.e.mean) frequency and amplitude of 1.63±0.11Hz and 8.63±0.16 pA respectively. Application of the group III mGLUR agonist L-AP4 (500μM) caused an increase in EPSC frequency to 2.95±0.21 Hz, and an increase in amplitude to 12.42±0.29 pA. Comparison of cumulative probability distributions using the Kolmogorov-Smirnov (KS) test showed that the increases in both frequency and amplitude were highly significant ($P<0.0001$ in both cases). Changes in EPSC amplitude distribution could reflect a postsynaptic effect so the Na-channel blocker tetrodotoxin (TTX, 1μM) was used to isolate activity-independent miniature EPSCs. Under these conditions (n=3), pooled data again showed an increase in EPSC frequency with L-AP4 (from 0.98± 0.08Hz to 1.72±0.16Hz) but without an increase in amplitude (11.76±0.41 *v* 9.73±0.22 pA). KS tests confirmed that the increased frequency was significant, but the amplitude change was not.

Previous reports have indicated that activation of group III mGLURs by L-AP4 results in a reduction in EPSP amplitude (Baskys & Malenka, 1991) via inhibition of calcium channels (Sahara and Westbrook, 1993) at the presynapse (Takahashi et al, 1996). Our observations in EC suggest that input to layer V may be modulated by group III mGLURs, and that in contrast to other systems, a *facilitation* of presynaptic release is effected by L-AP4. This unusual effect of L-AP4 in EC suggests that the group III mGLUR agonist may be activating a different receptor subtype than that described in previous reports. Alternatively, it may be that the physiological correlates of mGLUR activation depend not only upon specific receptor subtypes but also on their location within the CNS.

D.I.P.E. is a BBSRC research student. We also thank the Wellcome Trust for financial support.

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69P SIMILAR BRAIN CONCENTRATIONS OF MDMA HAVE DIFFERENT NEUROTOXIC EFFECTS FOLLOWING CENTRAL OR PERIPHERAL DRUG ADMINISTRATION

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Systemic administration of 3,4-methylenedioxymethamphetamine (MDMA or 'ecstasy') to rats produces degeneration of 5-HT nerve endings in the brain (Green *et al.*, 1995). Intraraphe microinjection of MDMA does not produce neurotoxicity to 5-HT terminals (Paris & Cunningham, 1991) which is not surprising since systemic administration of MDMA leaves intact the 5-HT cell bodies in the raphe nucleus (Battaglia *et al.*, 1991; Lew *et al.*, 1996). We have now investigated the long-term effect induced by perfusion of MDMA on the hippocampal 5-HT terminals in a range of concentrations higher than the extracellular concentration reached after a systemic administration of a neurotoxic dose of MDMA.

Male Dark Agouti rats (170-200g) were anaesthetized with sodium pentobarbitone and implanted with a microdialysis probe in the right hippocampus (Colado *et al.*, 1997). The next day probes were perfused at a rate of 1 μl min^{-1} with artificial csf. After collection of 3 x 30 min baseline samples, MDMA (100, 200 and 400 μM) was administered through the probe during 2.5 h or injected i.p. at a dose of 15 mg kg^{-1}. Dialysate was collected for measurement of MDMA. One week later, rats were sacrificed and the concentration of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in the right and left hippocampi determined by h.p.l.c. coupled with electrochemical detection (Colado *et al.*, 1997).

Rats perfused with MDMA (100, 200 and 400 μM) did not show any change in the concentrations of 5-HT (Figure 1) and 5-HIAA in either right and left hippocampi compared with those seen in animals perfused with csf. Using the difference method, the percent of delivery *in vivo* for MDMA 400 μM to the extracellular fluid was 20.0±1.0 % (5). Following systemic injection of a neurotoxic dose of MDMA (15 mg kg^{-1}) there was a rapid increase in the extracellular concentration of MDMA in the hippocampus, reaching a peak of 4.35 μM 1 h after and declining rapidly thereafter (Figure 2).

After this correction for *in vivo* delivery it was revealed that the extracellular concentration of MDMA obtained after perfusion with MDMA 400 μM seems to be 4-fold higher than that detected after i.p. injection of a neurotoxic dose of MDMA. These data demonstrate that the local application of relatively high concentrations of MDMA does not produce neurotoxicity to hippocampal 5-HT terminals in rats which are normothermic.

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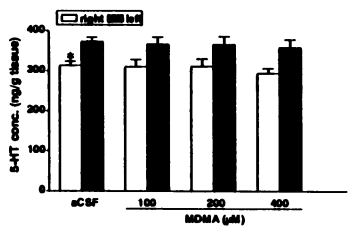

Figure 1. 5-HT concentrations in the right and left hippocampi 7 days after perfusion with MDMA (100, 200 and 400 μM) through a microdialysis probe. Results shown as means ± s.e.mean, n= 8-13. Different from left side: *$P<0.05$.

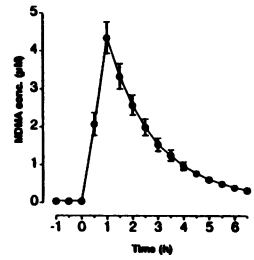

Figure 2. MDMA concentration in the hippocampal dialysate following MDMA (15 mg kg^{-1}, i.p.). MDMA was injected at time zero. Each value is the mean ± s.e.mean, n=9.

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70P EFFECT OF 7-NITROINDAZOLE ON THE HYPOTHERMIA AND THE STRIATAL DOPAMINE LOSS INDUCED BY MDMA IN MICE

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Several previous studies have demonstrated that the neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole (7-NI), protects against the neurodegeneration of dopaminergic nerve terminals induced by methamphetamine in mice. However the mechanism by which 7-NI exerts this effect is controversial. While some workers (Di Monte *et al.*, 1996; Itzhak & Ali, 1996) reported that 7-NI did not alter methamphetamine-induced hyperthermia, others have suggested that the neuroprotective effect of 7-NI was due to its ability to decrease body temperature (Callaghan & Ricaurte, 1998). We have now examined the effect of 7-NI on both the neurotoxicity and the hyperthermia induced by 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') in mice.

Adult male Swiss-Webster mice (30-35 g) were injected with MDMA (20 mg kg^{-1}, i.p.) or saline at 3 h intervals for a total of 3 injections. 7-NI (50 mg kg^{-1}, s.c.) or peanut oil was given 30 min before each dose of MDMA. Rectal temperature was measured during the 8 h following the first MDMA administration. Seven days later the mice were killed and the striatal concentrations of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined by h.p.l.c. coupled with electrochemical detection.

MDMA increased rectal temperature by approx. 1.7°C and 1°C above saline-injected controls after the first and second injections (Figure 1). Seven days later, rats injected with MDMA showed a substantial loss in the striatal concentrations of DA (40%), DOPAC (43%) and HVA (30%). Co-administration of 7-NI with MDMA provided neuroprotection but abolished the hyperthermia induced by MDMA, rectal temperature being below 35°C at some time points recorded. When given alone 7-NI produced a sustained hypothermia (Figure 1).

MDMA-induced neurodegeneration of 5-HT nerve endings in rat brain is prevented by compounds either producing hypothermia or preventing the MDMA-induced hyperthermia (Colado *et al.*, 1998; 1999a,b). The present results suggest that the neuroprotective effect of 7-NI against MDMA-induced neurotoxicity on striatal DA terminals could be due to its ability to decrease rectal temperature and agree with those found with methamphetamine (Callaghan & Ricaurte, 1998).

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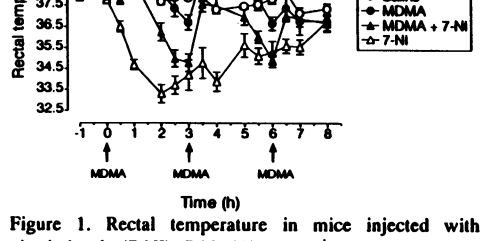

Figure 1. Rectal temperature in mice injected with MDMA and 7-nitroindazole (7-NI). 7-NI (50 mg kg^{-1}, s.c.) or peanut oil was administered 30 min before each dose of MDMA (20 mg kg^{-1}, i.p.) or saline. Results shown as mean ± s.e.mean, n=6-15. MDMA produced a significant rise (F (1,22)= 8.18, $P<0.01$) in body temperature during 5 h post-injection compared with the saline-injected group. 7-NI administered to MDMA-treated mice produced a significant hypothermia (F (1,25)= 14.44, $P<0.001$)

M.I.C. thanks CICYT (SAF98-0074), CAM (08.8/003/1998) and Astra Arcus for support.

71P INHIBITION OF CALCIUM CHANNEL CURRENTS BY SOMATOSTATIN AND L362855 IN NG108-15 CELLS

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NG108-15 neuroblastoma-glioma hybrid cells are known to express somatostatin receptors (Vanetti *et al.*, 1992) predominantly of the sst_{2B} subtype, but the presence of other somatostatin receptors is uncertain. We have previously reported that in these cells the inhibition of the calcium channel current (I_{Ca}) by somatostatin shows marked desensitisation (Beaumont *et al.*, 1998). Here we report studies with the somatostatin analogue L362855 (Raynor *et al.*, 1993).

The inhibition of N type I_{Ca} was studied in differentiated NG108-15 cells, using the amphotericin-perforated patch clamp recording technique (Beaumont *et al.*, 1998). Cells were held at -98mV and I_{Ca} evoked every 20s by stepping to +2mV Cells were superfused with a solution containing (mM): tetra-ethylammonium chloride 121; CsCl 5.4; $BaCl_2$ 10; $MgCl_2$ 1; HEPES 10; glucose 10 and sucrose 50 plus nimodipine (3μM) to block L type currents. The pipette solution was composed of (mM) CsCl 20; Cs methyl sulphonate 140; HEPES 20 and amphotericin B 240 $\mu g.ml^{-1}$.

The concentration-response curve for I_{Ca} inhibition by somatostatin had an IC_{50} of 3nM (95% confidence limits 1nM to 10nM) a Hill coefficient of 0.61 and a maximum of 49% inhibition (95% confidence limits 36 to 62) while for L362855 the IC_{50} was 44nM (confidence limits 15nM to 130nM), the Hill coefficient was 0.76 and the maximum was 38% inhibition (95% confidence limits 29 to 48). During a 5 min superfusion with somatostatin (300nM) the inhibition desensitised by approximately 50% within 3-4 mins. In contrast during a 5 min superfusion with L362855 (300nM-1μM) the response showed less than 10% desensitisation.

We next sought to determine whether somatostatin and L362855 were acting at different somatostatin receptors in NG108-15 cells. The I_{Ca} inhibition by both somatostatin and L362855 was abolished by pretreatment with Pertussis toxin (200ng/ml; for 24 hrs). The response to L362855 (1μM) was abolished following a 5 min application of a maximally desensitising concentration, 300nM, of somatostatin. L362855 did not appear to be a partial agonist at the somatostatin receptor because in the presence of L362855 (1μM), the concentration-response curve to somatostatin was shifted to the left. However, there was no additive effect on coapplication of a maximal concentration of both drugs. The sst_2 receptor antagonist cyanamid 154806 (100nM) (Bass *et al.*, 1996) fully reversed the I_{Ca} inhibition by L362855 (100nM), but only partially reversed (by 35%) the inhibition by somatostatin (10nM).

These data suggest that in NG108-15 cells, somatostatin elicits its response through both cyanamid-sensitive and cyanamid-insensitive receptors, while L362855 acts solely through cyanamid-sensitive receptors.

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72P TRYPTOPHAN: A DISTINCT BUT BIOLOGICALLY INACTIVE COMPONENT OF CLONIDINE-DISPLACING SUBSTANCE

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The structure of the endogenous ligand for imidazoline binding sites known as clonidine-displacing substance (CDS) has not been identified to date. We have previously reported the presence of "classical" CDS (orginally defined by Atlas *et al.*, 1984) in the bovine lung, adrenal gland and brain, by utilising the technique of reverse phase-HPLC (RP-HPLC; Parker *et al.*, 1999, in press). This study now reports the existence of high concentrations of the amino acid, tryptophan, co-eluting with biologically active CDS.

CDS was extracted from bovine lungs by the method of Singh and coworkers (1995). The crude CDS extracts were fractionated by C-18 RP-HPLC eluting with a linear gradient of aqueous methanol (5-65%; 1 $ml.min^{-1}$ flow rate) over 50 minutes. Previously we have shown the biological activity of CDS to elute with the second peak of the "classical" three peak profile exhibited by CDS (Parker *et al.*, 1999). Subsequently, the active peak was collected and concentrated via lyophilisation. This active peak was then analysed by spectrophotometry, electrospray mass spectroscopy (ESMS) and radioligand binding.

Analyses revealed the presence of tryptophan in the active extract, with ESMS exhibiting masses of 187.99 and 204.97 Daltons. These correspond to anhydrous and hydrous tryptophan, respectively. Authentic L-tryptophan (1 $mg.ml^{-1}$) exhibited masses of 188.52 and 205.29 Daltons, that agree with the masses exhibited by the purified CDS.

The full absorption spectrum of CDS yielded absorbance peaks at 217.5 and 278.5 nm, with the L-tryptophan standard (1 $mg.ml^{-1}$) yielding absorbances at 218.0 and 278.5 nm. This again matched the absorption spectra exhibited by CDS.

To assess whether tryptophan was an active component of "classical" CDS, competition radioligand binding studies were performed, to determine the ability of L-tryptophan to displace specific [^{3}H]clonidine (3 nM) binding to α_2-adrenoceptors in rat brain membranes. The binding was performed according to the methods described by Hudson and coworkers (1992), rauwolscine was used to define non-specific binding. L-Tryptophan failed to displace any [^{3}H]clonidine binding to α_2-adrenoceptors, even at concentrations up to 100 μM. This suggests that L-tryptophan does not represent the active component of "classical" CDS.

Collectively, these data show high concentrations of tryptophan are present in CDS eluting from the RP-HPLC column. However, this amino acid does not represent the active component of CDS. This finding may explain some of the problems in isolating the structure of CDS, due to a high masking level of tryptophan in the active component.

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73P ESTIMATING THE INHIBITORY POTENCY OF PROSTANOID EP RECEPTOR AGONISTS IN HUMAN NON-PREGNANT MYOMETRIUM *IN VITRO*

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Rigorous pharmacological classification of prostanoid EP receptors in human non-pregnant myometrium (HNPM) is difficult because of the lack of selective antagonists, the promiscuity of many agonists, and the apparent complexity of EP receptor expression in this tissue (Popat & Crankshaw, 1997). Valuable information about the EP receptors that mediate relaxation in HNPM might be obtained if one could compare the relative molar potencies of inhibitory agonists in HNPM (Brown & Crankshaw, 1995) with those in less complex tissues. The major impediment to making such comparisons is uncertainty about the accuracy of estimates of the inhibitory potency of compounds that simultaneously inhibit and excite HNPM (mixed agonists). In this study we have created an artificial mixed agonist (MA) by combining equipotent concentrations of the purely excitatory agonist sulprostone (SULP) and the purely inhibitory agonist butaprost (BUTA). We have attempted to compare the excitatory potency of MA with SULP and the inhibitory potency of MA with BUTA using assay systems appropriate for the two types of responses.

Strips of HNPM were set up for isometric recording and cumulative concentration-effect (c/e) curves were obtained. Excitatory effects were investigated using spontaneously active tissues, inhibitory responses were studied using tissues stimulated with 2 μM cloprostenol for 2h. All methods were as described by Fernandes & Crankshaw (1995) except that excitatory responses were expressed as a percentage of the tissue's contractile response to hypotonic shock (Crankshaw & Popat, 1997). Results are means ± s.e.mean.

Over the concentration-range tested, SULP produced monophasic excitatory c/e curves (pEC_{50} = 8.2 ± 0.2, max = 65 ± 12, n=3) whereas MA produced biphasic c/e curves typical of the response to mixed agonists in this tissue (Popat & Crankshaw, 1997), from which a single excitatory pEC_{50} could not be determined (Figure 1).

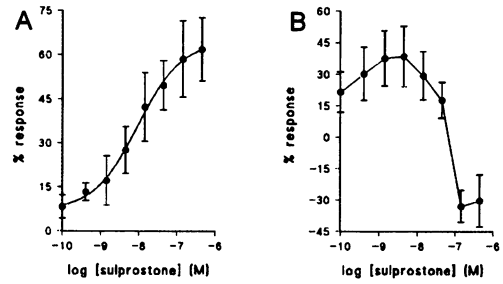

Figure 1 *Effects of sulprostone (A) and a synthetic mixed agonist (B) on the contractility of matched strips of HNPM. The concentration of mixed agonist is plotted according to its sulprostone content.*

In cloprostenol-stimulated tissues both BUTA and MA produced monophasic inhibitory c/e curves that were analysed according to the BUTA content of each agonist. There were no significant differences between the BUTA (pEC_{50} = 5.7 ± 0.2, max = 114 ± 7) and MA curves (pEC_{50} = 5.7 ± 0.2, max = 105 ± 6), Student's t-test, $p<0.05$, n = 5.

We conclude that the cloprostenol-stimulated technique can accurately assess the inhibitory potency of mixed agonists in HNPM when the potencies of the two components are approximately equal. The failure of the excitatory component to influence the potency of the inhibitory component is probably explained by heterologous desensitization produced by a 2h exposure to cloprostenol.

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74P BELL-SHAPED CONCENTRATION-EFFECT CURVES TO EXCITATORY EP RECEPTOR AGONISTS IN HUMAN NON-PREGNANT MYOMETRIUM *IN VITRO*

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We have previously reported bell-shaped cumulative concentration-effect (c/e) curves to sulprostone in human non-pregnant myometrium (HNPM) (Popat & Crankshaw, 1997). The downturn in the response at high agonist concentrations may result from activation of an inhibitory receptor, coupling of EP_3 and/or EP_1 receptors to an inhibitory G-protein, or receptor desensitization. In the present study we have attempted to elucidate the mechanism behind this phenomenon.

Strips of HNPM were obtained and set-up for isometric recording as described by Fernandes and Crankshaw (1995). In experiment 1, tissue strips were exposed to two consecutive concentrations of sulprostone: [A] = the concentration which elicited the maximum response in a c/e curve constructed in a matched strip from the same tissue (44 or 144 nM); and [B] = the final concentration in the c/e curve (the end of the downturn, 4.4 μM). Strips were treated with [A], washed for 2 hours, and then treated with [B]. Paired strips were treated in the reverse order. In experiment 2, strips were first challenged with EC_{50} concentrations of either the TP receptor agonist U46619 or arginine vasopressin (AVP). Strips were then washed and left for 2 hours. Half the strips served as controls and half were treated with sulprostone (4.4 μM). After another 2 hours, all strips were re-challenged with U46619 or AVP. Responses were expressed as a percentage of the response induced by hypotonic shock (Crankshaw & Popat, 1997). Values are means ± SEM from n=5 donors. Significant differences were identified by a paired *t* test ($p<0.05$).

Experiment 1:
The contractile responses to initial treatment with [A] (74 ± 19) and [B] (118 ± 7) were not significantly different. Strips treated initially with [A] were still able to respond to [B] after the two-hour washout period (61 ± 5). However, strips treated initially with [B] showed a significantly reduced response to treatment with [A] (16 ± 4).

Experiment 2:
Sulprostone-treated strips had significantly reduced fractional responses to second treatments with U46619 (0.0 ± 0.2) and AVP (0.1 ± 0.2) than did untreated strips (2.7 ± 0.9 and 1.5 ± 0.3, respectively).

Since treatment with [A] and [B] produced the same response in naive tissues, the decreased response to 4.4 μM sulprostone during a cumulative c/e experiment is unlikely to be due to activation of inhibitory receptors or G proteins. The significant reduction in the response to [A] following exposure to [B] suggests desensitization is occurring. The significant reduction in the responses to U46619 and AVP following prolonged exposure to sulprostone suggest that the desensitization is heterologous.

We conclude that the downturn phase of the c/e curve to sulprostone is most likely mediated by a heterologous desensitization mechanism resulting from prolonged exposure to high agonist concentrations. It is unlikely to result from the EP_3 receptor-mediated activation of inhibitory G-proteins that has been observed in recombinant receptor expression systems (Kotani *et al*, 1997).

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75P EFFECT OF PROLONGED TREATMENT WITH SULPROSTONE ON Gα PROTEIN LEVELS IN HUMAN NON-PREGNANT MYOMETRIUM *IN VITRO*

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Functional studies suggest that prolonged exposure of human non-pregnant myometrium (HNPM) to high sulprostone concentrations produces heterologous desensitization (Popat & Crankshaw, 1999). This form of desensitization may be generated by down-regulation of G proteins that are activated by agonist-occupied receptors or up-regulation of G proteins that activate an opposing signal-transduction pathway (Milligan, 1993). In the present study we investigated whether the mechanism of sulprostone-induced heterologous desensitization involves regulation of Gα proteins found in HNPM.

Strips of human myometrium from non-pregnant donors were set up as previously described (Fernandes & Crankshaw, 1995). Half the strips served as controls and half were treated with sulprostone (4.4 μM) for 2 hours. Strips were then prepared into membrane fractions as described by Senchyna & Crankshaw (1996) and the total protein concentration determined using the method of Bradford (1976). Membrane proteins were resolved for 2h by electrophoresis in 12% SDS-polyacrylamide gels, transferred to polyvinyl-difluoride sheets, and immunoblotted using antibodies QL ($G\alpha_{q/11}$), AS/7 ($G\alpha_{i1/2}$), EC/2 ($G\alpha_{i3}$), RM/1 ($G\alpha_s$) (Mandel-NEN, Boston, MA), and anti-α-actinin (Sigma, St. Louis, MO). Antibody complexes were detected by enhanced chemiluminescence (ECL) using Kodak X-Omat AR film (exposure time 30 sec to 5 min). Densitometry analysis was performed using the Northern Exposure, *Empix Imaging®* programme (Mississauga, ON). Data were obtained under conditions in which a linear relationship existed between ECL signal intensity and protein amount. To account for any potential errors in protein loading, Gα protein levels were expressed as a percent of α-actinin within each sample. All immunoblots were performed in triplicate.

Similar levels of $G\alpha_{q/11}$, $G\alpha_{i1/2}$, $G\alpha_{i3}$, and both isoforms of $G\alpha_s$ proteins were detected in myometrial membranes from control and sulprostone-treated tissues (Table 1).

Table 1. Levels of Gα proteins in human myometrial membranes prepared from control and sulprostone-treated tissues. Gα protein levels expressed as a percent of α-actinin.

	Control	Sulprostone-Treated
$G\alpha_{q/11}$	33 ± 1	36 ± 1
$G\alpha_{i1/2}$	38 ± 2	39 ± 1
$G\alpha_{i3}$	24 ± 3	26 ± 0
$G\alpha_s$ (52 kDa)	45 ± 1	44 ± 4
$G\alpha_s$ (45 kDa)	45 ± 3	51 ± 3

No significant difference between control and treated preparations, paired *t* test, $p<0.05$. Values are means ± SEM from three donor tissues.

In conclusion, the heterologous desensitization resulting from a 2 hr exposure of HNPM to sulprostone does not appear to result from changes in the levels of Gα proteins. Instead, the mechanism of desensitization may involve phosphorylation of a variety of proteins by second messenger-dependent kinases.

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76P MECHANISM OF AIRWAY HYPERREACTIVITY TO ADENOSINE INDUCED BY ALLERGEN CHALLENGE IN ACTIVELY SENSITITSED BROWN NORWAY RATS

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Airway hyperreactivity (AHR) is a critical component of bronchial asthma. The phenomenon is particularly well illustrated by the bronchoconstrictor response to adenosine which is prominent in asthmatic patients and generally not present in control subjects (Phillips & Holgate, 1995). The present studies were carried out to characterise a potential new experimental animal model of AHR, based on changes in the bronchoconstrictor response to adenosine following allergen challenge in actively sensitised Brown Norway (BN) rats (Mazzoni *et al.*, 1998).

BN rats weighing approximately 200g were used. For sensitisation, ovalbumin (OA; 0.02mgml^{-1}) was mixed with aluminium hydroxide (20mgml^{-1}) and injected (0.5ml per animal s.c.) coincidentally with *Acullulare pertussis adsorbat vaccine* (Acel-P Lederle; 0.2ml per animal i.p., diluted 1:4 with saline 0.9%) on days 1, 15 and 22. On day 29 animals were anaesthetised (pentothal 70mgkg^{-1}, i.p., a dose sufficient to maintain full anaesthesia throughout the experiment), ventilated, immobilised (vecuronium bromide (12mgkg^{-1})) and cannulated for measurement of cardiovascular parameters (carotid artery) and drug administration (jugular vein). Airway resistance (R_L) was calculated from measurements of airflow and transpulmonary pressure by use of a digital electronic respiratory analyser (PMS 8000, Mumed Ltd., U.K.) (Hannon *et al.*, 1995). In separate groups of animals (n=10), total leukocyte numbers and differential cell counts in bronchoalveolar lavage (BAL) fluid were obtained using an automated cell analysing system (Cobas Helios, Axon Lab, Switzerland). Eosinophil peroxidase (EPO) activity and protein concentrations in BAL fluid were determined using standard photometric assays.

In actively sensitised, non-challenged animals adenosine (0.3 & 1mgkg^{-1} i.v.) was only a weak bronchoconstrictor (incremental increase in R_L (mean± s.e.mean): 20.0±3.0 and 78.8±15.9 cmH$_2$O/l/s n=5, respectively). However, intratracheal (i.t.) administration of OA (0.3mgkg^{-1}, -3h), which did not *per se* affect R_L, induced a marked increase in R_L to adenosine (251.2±50.5 and 1232.9±144.2 cmH$_2$O/l/s n=10, respectively). Similar results were obtained when adenosine (3-30mgkg^{-1}) was administered i.t. In contrast, bronchoconstrictor responses to both methacholine (3-10μgkg^{-1}, i.v.) and 5-HT (3-30μgkg^{-1}, i.v.) were similar and those to bradykinin (30-100μgkg^{-1}, i.v.) only marginally enhanced, following allergen challenge. The increased responsiveness to adenosine was associated with a significant ($p<0.05$) 1.8-fold increase in neutrophils, and 1.4-fold decrease in eosinophils recovered from BAL fluid obtained 3h post OA exposure. The initiation of inflammatory events was indicated by increases in EPO (a marker of eosinophil activation) and protein concentration (indicating plasma extravasation) in BAL fluid (5.4 and 1.5-fold, respectively).

The muscarine receptor antagonist, atropine, at a dose (10μgkg^{-1} i.v.) which fully antagonised the bronchoconstrictor response to methacholine (3-30μgkg^{-1} i.v.), was without effect on the response to adenosine (1mgkg^{-1} i.v.), thus excluding the involvement of a cholinergic muscarinic mechanism in the response. An indication of mast cell involvement was demonstrated by the dose-dependent attenuation of the response to adenosine by pretreatment (-5 min) with disodium cromoglycate (20-40mgkg^{-1} i.v.) and complete inhibition by the 5-HT receptor antagonist, methysergide (10μgkg^{-1} i.v.).

These data demonstrate a marked and selective augmentation of the response to adenosine 3h post 'low-level' allergen challenge in actively sensitised BN rats. The response occurs against a background of pulmonary inflammation is primarily, if not exclusively, due to activation of mast cells and thus bears similarity to the response to inhaled adenosine in asthmatic patients (Phillips & Holgate, 1995).

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77P THE RECEPTOR MEDIATING THE AUGMENTED BRONCHOCONSTRICTOR RESPONSE TO ADENOSINE INDUCED BY ALLERGEN CHALLENGE IN ACTIVELY SENSITISED BROWN NORWAY RATS

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A critical component of asthma is airway hyperreactivity (AHR). This phenomenon is particularly well illustrated by the bronchoconstrictor response to adenosine which is prominent in asthmatic patients and generally not present in control subjects (Phillips & Holgate, 1995). A new model of AHR, based on changes in the bronchoconstrictor response to adenosine following allergen challenge in actively sensitised Brown Norway (BN) rats, has recently been described (Mazzoni *et al.*, 1998; Hannon *et al.*, 1999). The present studies were carried out to characterise the receptor(s) mediating the augmented response to adenosine.

BN rats weighing approximately 200g were used. For sensitisation, ovalbumin (OA; 0.02mgml^{-1}) was mixed with aluminium hydroxide (20mgml^{-1}) and injected (0.5ml per animal s.c.) coincidentally with *Acullulare pertussis adsorbat vaccine* (Acel-P Lederle; 0.2ml per animal i.p., diluted 1:4 with saline 0.9%) on days 1, 15 and 22. On day 29 animals were anaesthetised (pentothal 70mgkg^{-1}, i.p., a dose sufficient to maintain full anaesthesia throughout the experiment), ventilated, immobilised (vecuronium bromide (12mgkg^{-1})) and cannulated for measurement of cardiovascular parameters (carotid artery) and drug administration (jugular vein). Airway resistance (R_L) was calculated from measurements of airflow and transpulmonary pressure by use of a digital electronic respiratory analyser (PMS 8000, Mumed Ltd., U.K.) (Hannon *et al.*, 1995). Responses to selective adenosine receptor agonists were established by bolus i.v. injection (n=4-5 per group). Only one agonist response was generated per animal. For antagonist studies, animals (n=4-5 per group) were injected i.v. with antagonist 5min prior to adenosine (1mgkg^{-1}). Fifteen min later a dose-response curve to 5-HT (3-30μgkg^{-1}) was performed. Finally, a dose-response curve to an adenosine receptor agonist corresponding to the selectivity of the antagonist was established.

Sensitivities of the airways to i.v. injection of selective adenosine receptor agonists were determined in sensitised BN rats challenged either with vehicle or with OA 3h previously. The selective A_1 receptor agonist CPA (10-30μgkg^{-1}) and the selective A_3 receptor agonist 2-Cl-IB-MECA (40-1000μgkg^{-1}) induced only very weak bronchoconstrictor responses (expressed as cmH_2O/l/s) following allergen challenge (incremental increase in R_L (mean±s.e.mean): 34.3±3.5 (CPA-30μgkg^{-1}) and 25.2±4.3 (2-Cl-IB-MECA-1mgkg^{-1}) for vehicle-treated and 134.2±34.3 (CPA-30μgkg^{-1}) and 103.2±6.6 cmH_2O/l/s (2-Cl-IB-MECA-1mgkg^{-1}) for antigen challenged animals). The selective A_{2A} receptor agonist CGS 21680 (30μgkg^{-1}) was devoid of effect on the airways. The suitability of the doses selected was established as they were sufficient to induce the expected cardiovascular effects from the corresponding receptor profile. In contrast both adenosine (0.1-10mgkg^{-1}) and the non-selective adenosine receptor agonist NECA (3-100μgkg^{-1}) showed a marked and dose-dependent increase in response following allergen challenge (incremental increases in R_L 1232.9±144.2 (adenosine 1mgkg^{-1}, n=10), and 1031.1±93.4 cmH_2O/l/s (NECA 100μgkg^{-1}, n=5). The selective adenosine receptor antagonists DPCPX (A_1, 100μgkg^{-1}) and ZM 241385 (A_{2A}, 30μgkg^{-1}) were without effect on the augmented response to adenosine despite blocking the A_1 and A_{2A} receptor mediated components of the cardiovascular response to the corresponding selective agonists respectively. In contrast the broad spectrum adenosine receptor antagonists 8-SPT (20-40mgkg^{-1}) and theophylline (3-10mgkg^{-1}) induced selective and dose-dependent inhibition of the response on the airways to adenosine (mean inhibiton: 55% (20mgkg^{-1}) and 82% (40mgkg^{-1}) for 8-SPT; and 37% (3mgkg^{-1}) and 64% (10mgkg^{-1}) for theophylline).

The fact that bronchoconstrictor responses to NECA, like those to adenosine, were augmented by allergen challenge whereas those to selective A_1, A_{2A} or A_3 receptor agonists were minimally affected is consistent with an effect mediated through the A_{2B} receptor. The conclusion is strengthened by the fact that responses to adenosine can be suppressed by 8-SPT and theophylline, which block A_1, A_{2A} and A_{2B} receptors, but not by DPCPX or ZM 241385 which are selective antagonists at A_1 or A_{2A} receptors, respectively.

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78P THE EFFECT OF SUBCUTANEOUS CHRONIC INFUSION WITH R-, S- AND RS-SALBUTAMOL ON AIRWAYS RESPONSIVENESS TO HISTAMINE, BRADYKININ AND LEUKOTRIENE C4 IN THE GUINEA-PIG

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Previous studies have demonstrated that chronic treatment with racemic salbutamol increases bronchial hyperresponsiveness to methacholine and histamine in guinea pigs (Buchheit *et al* 1995). The aim of our study was to investigate the effects of racemic salbutamol and its enantiomers (R and S) on airways responsiveness to histamine (H), bradykinin (BK) and leukotriene (LT)C4.

Male Dunkin-Hartley guinea pigs (350 - 500g) were implanted with osmotic mini pumps under anaesthesia (inhaled halothane). The pumps contained either RS-, R- or S-salbutamol (1mg/kg/day) or vehicle (0.9% saline). The pumps remained implanted for 10 days and were removed 1 hour prior to the measurement of lung function. Guinea pigs were anaesthetised (1.5g/kg urethane) and artificially ventilated (10ml/kg). Total lung resistance (RL) was recorded using a Pulmonary Monitoring System. Drugs were administered by iv. injection. Bronchoconstriction was expressed as the percentage increase in RL and values represented as mean ± s.e.m. The dose-response curve to the agonists was analysed with ANOVA and differences in mean values with a Student's non-paired t-test with Bonferroni correction. Values were considered significant if $P < 0.05$ (Table 1; n =4-6).

Airways hyperresponsiveness to racemic, R- and S-salbutamol was observed with LTC4 ($P < 0.05$) and to a lesser extent bradykinin ($P < 0.05$) but not histamine. This supports the heterogeneous nature of airway responsiveness (O'Connor *et al* 1999). Only the racemic and S-salbutamol enhanced hyperresponsiveness to LTC4 and BK. The ability of the S enantiomer alone to induce the hyperresponsiveness observed, suggests that the effect of chronic RS-salbutamol on airways hyperresponsiveness is not solely related to the occupancy and activation of beta-adrenoceptors.

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Table 1. % increase in RL with corresponding saline control values to the left of RS-, R-, and S-salbutamol respectively.

(μg/kg)	saline	RS-salbutamol	saline	R-salbutamol	saline	S-salbutamol
H 1	36.4 ± 14.3	66.3 ± 11.9	20.6 ± 4.2	59.2 ± 31.6	93.6 ± 39.1	102.0 ± 49.6
H 2	94.6 ± 68.3	143.0 ± 92.9	66.2 ± 19.7	119.9 ± 56.5	129.1 ± 60.5	182.2 ± 63.0
H 5	187.7 ± 63.7	248.5 ± 84.8	295.4 ± 55.1	260.1 ± 44.1	316.2 ± 78.4	466.6 ± 58.6
BK 2	20.0 ± 5.0	57.7 ± 17.4	24.6 ± 9.6	44.7 ± 5.6	9.7 ± 2.9	26.4 ± 2.9
BK 5	60.4 ± 10.7	154.8 ± 42.9 *	67.7 ± 21.4	89.3 ± 11.9	32.1 ± 5.8	85.5 ± 21.6 *
LTC4 1	21.3 ± 11.7	128.6 ± 60.6 *	16.8 ± 9.0	45.9 ± 20.9	16.8 ± 9.0	149.7 ± 43.1 *
LTC4 2	16.9 ± 9.0	158.8 ± 61.4 *	59.7 ± 16.9	100.3 ± 31.4	59.7 ± 16.9	248.8 ± 22.8 *
LTC4 5	42.95± 11.5	233.6 ± 39.1*	167.1 ± 57.2	168.8 ± 28.3	167.1 ± 57.2	352.5 ± 18.1 *

* $P < 0.05$ (non paired t-test)

79P DOES *HELICOBACTER PYLORI* ACCOUNT FOR INCREASE IN SERUM INTERLEUKIN-6 AND TUMOUR NECROSIS FACTOR-α WITH AGE, AND INTERLEUKIN-6 AND CORTISOL WITH PARKINSONISM?

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Sufferers from idiopathic parkinsonism and their sibings, who share facets of it, are three times more likely than controls to be seropositive for *Helicobacter pylori* (Charlett *et al*, 1999). Familial transmission of chronic infection plus part of syndrome links *Helicobacter* with causality. Like *H pylori* antibody titre, serum interleukin-6 (IL-6) (Purkiss *et al*, 1997) and tumour necrosis factor-α (TNF-α) (Charlett *et al*, 1997), but not cortisol (Charlett *et al*, 1998) increase with age. Parkinsonism had an effect on IL-6, equivalent to that of >10 y of ageing , and increased cortisol by 17 %. We, therefore, ask whether the effect of age and parkinsonism on immune/inflammatory markers can be explained by the *H. pylori* antibody titre in subjects with and without idiopathic parkinsonism. None had been treated for *H. pylori* infection.

Enzyme-linked immunosorbent assay was used to measure serum concentrations of IL-6 (Cytoscreen ultrasensitive kit, Biosource International, Camarillo, US) and TNF-α (Medgenix Diagnostics SA, Fleurus, Belgium). For IgG antibody titre against a known fraction of *H pylori* urease (SIA *Helicobacter pylori* (HM-CAP), Sigma-Aldrich Ltd, Poole), a calibration curve converts absorbence to an "ELISA value" (EV). Serum cortisol concentration was measured by a solid-phase radioimmunoassay, using ^{125}I-labelled cortisol (Cort-A-Count, Diagnostic Products Corporation, Los Angeles). The between assay coefficients of variation, for samples assayed in duplicate, were 13.0 & 9.0 %, at IL-6 concentrations of 5.4 & 7.8 pg.ml^{-1}, 11.7 & 13.6 %, at TNF-α concentrations of 17 & 143 pg.ml^{-1}, 5.9, 4.8 and 2.9 %, at cortisol concentrations of 77, 339 and 1086 nmol.l^{-1}, and 13.0, 8.0 & 6.0%, at EV's of 0.8, 2.4 & 5.9. (An EV of >2.2 is the recommended cut-point for seropositivity.) Analysis of covariance was performed, with $\log_e$ transformation of concentrations and EV's to ensure validity of assumptions.

In 210 controls of 30-90 y, EV rose by 1.55 (95 % C.I.: 0.72, 2.39) %.y^{-1} (P<0.001): the expected birth-cohort effect. The effect of age on IL-6 of 2.84 (1.74, 4.00) %.y^{-1} (n=128, P<0.001) was unaffected (3.06 (1.93, 4.19), P<0.001) by adding EV into the analysis as a covariate (P=0.1). Its effect on TNF-α of 1.41 (0.67, 2.15) %.y^{-1} (n=143, P<0.001) was also unaffected (1.41 (0.66, 2.17), P<0.001) by introduction of EV (P=0.9). In 105 sufferers from parkinsonism of 40-90 y, a raised *H. pylori* antibody titre obliterated the birth cohort effect (disease status.age interaction, P<0.05): sufferers were more likely to be seropositive (odds ratio 2.04 (1.04, 4.22), P<0.04) before 72.5 years; less, on average, after (0.69 (0.34, 1.41). The effect of the presence/absence of parkinsonism on IL-6 of 30.8 (0.1, 71.1) % (n=76 P & 128 C, P=0.05, after adjusting for age) was unaffected (34.1 (0.9, 78.3) %, P=0.04) by adding EV into the analysis (P=0.3). Similarly, the effect of group on cortisol of 17.1 (7.7, 27.5) % (n=96 P & 207 C, P=0.001, after adjusting for gender and sampling time (10.30-17.00 h)) was unaffected (15.8 (5.9, 26.5) %, P<0.001) by introducing EV (P=0.2).

The immune/inflammatory response in ageing and in parkinsonism is not in direct response to the urease fraction measured by the ELISA. However, other antigens produced by the "pathogenicity island" of the *H.pylori* genome are known to stimulate cytokine production (Yamaoka, Y *et al*).

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80P IS *HELICOBACTER PYLORI* ANTIGEN RESPONSIBLE FOR CHANGES IN CIRCULATING IMMUNOGLOBULIN ASSOCIATED WITH AGEING?

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Serum IgA concentration increases with age, IgM decreases (Purkiss *et al*, 1995). Is the effect of age on immunoglobin classes explained by a ubiquitous infection? The ratio of subclasses, IgA_2 to IgA_1, increases to a turning point at approximately 60 years (Purkiss *et al*, 1998), suggesting that a gut mucosal response may be suppressing the systemic IgA response. *Helicobacter pylori* infection is ubiquitous, its antibody titre increasing with age, due to a birth cohort effect (Banatvala *et al*, 1993). Consequent atrophic gastritis may lighten the microbial load in the elderly individuals, as gastric pH rises (Mathialogan *et al*, 1994). We report the relationship between serum IgA, IgM & IgG and antibodies against *H. pylori* in 205 subjects, of ages from 30 to 90 years. Unfitness and chronic or intercurrent illness led to exclusion. None had been treated for *H. pylori* infection.

Immunoglobulins were measured by immunoturbidity. Enzyme-linked immunosorbent assay measured IgG antibody against a known fraction of *H pylori* urease (SIA Helicobacter pylori (HM-CAP), Sigma-Aldrich Ltd, Poole), a calibration curve converting absorbence to an "ELISA value" (EV). The between assay coefficients of variation, for samples assayed in duplicate, were 6.5 & 4.9 %, at 7.5 & 11.6 g.l^{-1} IgG, respectively, 5.9 & 4.4 %, at 1.3 & 2.5 g.l^{-1} IgA, 4.5 & 3.4 % at 0.7 & 1.1 g.l^{-1} IgM, and 6.0, 8.0 & 13.0 %. at EV's of 5.9, 2.4 & 0.8. (An EV of >2.2 is the recommended cut-point for seropositivity.) For analysis of covariance, $\log_e$ transformation ensured normality and equality, between groups, of residual variance. IgA and IgG are adjusted for the covariate, gender.

Serum IgM concentration decreased by: 1.04 (95 % C.I.: 0.50, 1.58) %.y^{-1}, P<0.001). IgM was 19.75 (3.80, 33.05) % (P=0.02) lower in the *H. pylori* seropositive (39 %) than in the seronegative, inclusion of antibody status diminishing the effect of age to 0.88 (0.33, 1.42) %.y^{-1}. Thus, seropositivity had an effect on IgM equivalent to >20 y of ageing. The magnitude of the effect of *H. pylori* status on IgM was irrespective of age (P=0.8 for status.age interaction). IgA increased by 0.50 (0.10, 0.91) %.y^{-1}, P=0.01), with no interaction between the effect of *H. pylori* status and age on IgA (P=0.7), nor any effect of status alone (P=0.5). No significant effect of age, *H. pylori* status or their interaction was seen on IgG (P=0.1, >0.9, & =0.8, respectively).

Serum IgA was not directly related to seropositivity for *H. pylori*, but secretory IgA might be. Secretory IgA, when complexed with antigen, is known to suppress synthesis of immunoglobulin by plasma cell classes other than IgA producing (Laissue & Gebbers, 1992). Complexing with *H. pylori* antigen could explain the suppression of IgM. The additional effect of age on IgM was small. Lack of suppression of IgG, of which the main antibody response to *H. pylori* comprises, could be the result of overriding autoimmunity (Appelmelk *et al*, 1997).

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81P BLOOD-RETINA BARRIER PERMEABILITY AND INTERLEUKIN-1β LEVEL IN RETINAS FROM STREPTOZOTOCIN MODEL OF DIABETIC RATS

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Breakdown of the blood-retina barrier (BRB) occurs in diabetes mellitus and is one of the causes that leads to diabetic retinopathy. However, the mechanisms by which BRB is altered in diabetes is unclear (Vinores et al.). Interleukin 1β (IL-1β) and nitric oxide (NO) have been implicated in the development of a variety of pathological conditions, namely diabetes. In this work, we evaluated the level of IL-1β and the activity of NO synthase (NOS) in retinas from streptozotocin model of diabetic rats and we correlated these results with the BRB permeability.

Diabetes was induced by an injection of streptozotocin (STZ) (60mg/ml/kg) in Wistar rats. Control rats were injected with an equal volume of citrate buffer. All animals were assessed for weight, blood glucose and glycosuria and were maintained for eight days. For each experiment it was used five control and five diabetic animals. Ocular fluorophotometry was performed using a Fluorotron Master with the small animal adapter according the method described in Carmo et al. 1998b before and after the induction of diabetes. NOS activity was determined by measuring the L-[^{3}H]-citrulline formation from L-[^{3}H]-arginine in retinal lysates from control and diabetic rats. The level of IL-1β was determined by ELISA in retinal lysates from control and diabetic rats.

Vitreous fluorofotometry indicated that 30 min after the injection of fluorescein, the penetration ratio value for the free fluorescein in control rats was 374.97±22.48 min^{-1} and in diabetic rats was 425.37±33.59 min^{-1}. The NOS activity in retinal lysates from diabetic rats was significantly increased, $p<0.05$ (270±21 pmol L-[^{3}H]-citrulline/mg protein/30 min) as compared to the NOS activity in retinal lysates from control rats (125±19 pmol L-[^{3}H]-citrulline/mg protein/30 min). We also found a significant increase ($p<0.05$) in the levels of IL-1β in retinal lysates from diabetic rats (0.31±0.08 pg/mg protein) as compared to that in control rats (0.093±0.013 pg/mg protein).

Our results indicate that in retinas from STZ model of diabetic rats the BRB permeability was increased as compared to that in control rats. This increase was followed by an increase in the level of IL-1β and NOS activity. Since it has been reported that the permeability of BRB increases after exposure to IL-1β (Claudio et al. 1994) and that IL-1β induces NO production in large amount (Sjöholm et al. 1998), our results shows that both inflammatory mediators IL-1β and NO are involved in the development of diabetic retinopathy.

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82P N-ACETYLCYSTEINE, DIPHENYLENEIODONIUM AND N-MONOMETHYLARGININE INHIBIT IL-1β-INDUCED NO PRODUCTION IN ARTICULAR CHONDROCYTES: A STUDY OF POTENCY

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There is evidence that nitric oxide (NO) plays a significant role in the pathogenesis of arthritis (Farrell *et al.*, 1992). Interleukin-1β (IL-1β) is one of the major stimuli for NO production by chondrocytes (Grabowski *et al.*, 1996). Moreover, L-arginine analogues, which inhibit NO synthase, suppressed the development of arthritis in animal models (Stefanovic-Racic *et al.*, 1994).

The objectives of this work were to study and compare the ability of N-Acetylcysteine (NAC), Diphenyleneiodonium chloride (DPI) and N-Monomethylarginine (NMMA) to inhibit IL-1β-induced iNOS expression and/or NO production in articular chondrocytes and, thus, to assess their pharmacological potential as anti-arthritic agents.

In vitro primary cultures of bovine articular chondrocytes were treated with NAC, DPI or NMMA for 2 hours before addition of IL-1β, 20 ng/ml. iNOS, GAPDH and β-actin mRNA levels were determined by Northern blot analysis, as described by Lo et al. (1996). NO production was assessed as the amount of nitrites produced, which was measured by a colorimetric reaction using the Griess reagent (Green *et al.*, 1982).

IL-1β induced iNOS expression and NO production in a time- and dose-dependent manner. All the three compounds, NAC, DPI and NMMA, were effective in inhibiting NO production, although their potency was significantly different, as indicated by the respective half-maximal inhibitory concentrations (IC_{50}):
NAC= 37 ± 0.5 mM; DPI= 0.03 ± 0.004μM, NMMA, 80 ± 1.1 μM. DPI and NMMA, but not NAC, were also effective in inhibiting NO production when added to the cultures after the treatment with IL-1β. NAC completely inhibited IL-1β-induced iNOS expression at a concentration of 60 mM, whereas DPI, 4 μM reduced it by only 40%.

These results indicate that NAC inhibits iNOS expression without affecting the enzyme activity. The identification of the mechanism by which NAC prevents iNOS expression may have pharmacological significance, despite the high concentrations required. On the contrary, DPI seems to act more effectively at the level of enzyme activity, without significantly interfering with the signal transduction mechanisms involved in the induction of iNOS expression. These results also show that DPI is an inhibitor of NO synthase more potent than the L-arginine analogue tested. Thus, DPI deserves to be further investigated to assess its pharmacological potential as an anti-arthritic agent.

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83P LEUKOCYTE ACTIVATION AND OXIDATIVE STRESS IN PSORIASIS

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Psoriasis is a common chronic and recurrent inflammatory skin disorder. Histopathological examination of clinically active lesions reveals infiltration of leukocytes, predominantly neutrophils. Several studies suggest that these neutrophils are activated in the peripheral blood of psoriatic patients. As activated neutrophils are important sources of oxygen metabolites and proteases, they will favour the development of an oxidative and proteolytic environment, which may impose oxidative and proteolytic changes on neighbouring cells and plasma constituents. The aim of this study was to test the hypothesis that neutrophils may play an important role in the pathogenesis of psoriasis.

The study was performed in active and inactive psoriatic patients, and in a healthy control population. The protocol was approved by the Committee on Ethics of the University Hospital of Coimbra. Patients and controls were aged between 18 and 70 years old and the duration of the disease ranged from 0.5 to 50 years. None of the patients had received any systemic medication or any phototherapy treatment for at least one month. As neutrophil activation products we studied elastase (μg/l) and lactoferrin (μg/ml) by immunoassay. To evaluate oxidative damage we studied total RBC membrane and plasma lipid peroxidation products (μM) by TBA assay (Yagi, 1976); to evaluate RBC membrane sensitivity and resistance to lipid peroxidation we used the parinaric acid assay (Kuypers *et al*, 1987); RBC resistance was evaluated by the quantification of plasma hemoglobin by spectrophotometry and haptoglobin by nephelometry (mg/dl). As inflammatory markers and acute phase reactants we studied transferrin, α_1-antitrypsin, α_2-macroglobulin, C-reactive protein (mg/dl) by nephelometry, γ-GT (U/l) by spectrophotometry and erythrocyte sedimentation rate (ESR; mm/h) by the Westergreen method. All the results were expressed as mean ± SD and compared using the Student *t* test.

All the inflammatory markers presented higher values in psoriatic patients (n=27) than in control population (n=20; $p<0.001$); this was accompanied by neutrophil activation. Patients presented a significant rise in elastase (145±79 *vs* 56±21, $p<0.001$) and lactoferrin (1.5±0.9 *vs* 0.8±0.3, $p<0.01$) concentrations. Oxidative damage was evident either in plasma (6.1±1.3 *vs* 2.0±0.7, $p<0.001$) and in RBC membrane lipids (30±8 *vs* 9.8±2.3, $p<0.001$); or in RBC membrane sensitivity ($p < 0.001$). Considering the extension of lesions (active *vs* inactive), we found that the changes in inflammatory markers are enhanced with its enlargement. We found higher values in active psoriatic patients, though only transferrin (245±11 *vs* 221±12), C reactive protein (1.02± 0.03 *vs* 0.59±0.02) and ESR (42±12 *vs* 22±7) presented significant differences ($p<0.001$). Neutrophil activation also seems to rise with the enlargement of the lesions, as shown by the significantly higher values ($p<0.001$) of lactoferrin (2.11±0.92 *vs* 0.98±0.36) and elastase (209 ±80 *vs* 97±26) in active patients. Plasma (6.79±0.96 *vs* 5.50±1.20) and RBC membrane (34.6±7.8 *vs* 26.4±7.0) lipid peroxidation prod-ucts also presented significantly higher values in active patients ($p<0.01$)..

In conclusion, psoriasis is an inflammatory state associated with an increase in neutrophil activation, and we believe that it may be through neutrophil activation and the release of proteases as well as reactive oxygen species underly the oxidative changes presented by the neighbouring RBC and by plasma constituents. We must emphasize that these changes seem to correlate with the extension of the psoriatic lesions and, therefore, elastase and lactoferrin concentration may provide the earliest and the most sensitive inflammatory markers of the development of a psoriatic crisis.

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84P ANTI-OEDEMA AND ANTINOCICEPTIVE EFFECT OF NITROPREDNISONE AND NITROFLURBIPROFEN

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Nitro-nonsteroidal anti-inflammatory drugs (NO-NSAIDs) exhibit anti-inflammatory activity with reduced incidence of gastrointestinal side effects (Wallace et al., 1994). However, the effect of nitro-steroidal anti-inflammatory drugs (NO-SAID) has not previously been evaluated in animal models of inflammation or nociception. Accordingly, we have now examined the ability of nitroprednisone (NOP) and nitroflurbiprofen (NOF) to inhibit carrageenan-induced hindpaw oedema in the rat and to exhibit antinociceptive activity in the mouse.

Carrageenan (100 μl, 2% w v^{-1}) was injected intraplantar and the increase in hindpaw volume determined by plethysmography as described previously (Handy & Moore, 1998). Carrageenan injection caused a time-dependent increase in hindpaw oedema (e.g. 0.71 ± 0.02 ml at 6 h, n=6, $P<0.05$). Pretreatment (15 min) with NOP or NOF (2.5-50 mg kg^{-1}, i.p.) produced dose related inhibition of hindpaw oedema formation. NOF was the more potent (e.g. 2.5 mg kg^{-1}, 68.0 ± 1.4% inhibition at 6 h, $P<0.05$) with significant anti-oedema activity first apparent at 60 min (80.8 ± 3.0% inhibition, n=6, $P<0.05$). In contrast, NOP was less effective (e.g. 50 mg kg^{-1}, 38.0 ± 4.0% inhibition at 6 h, n=6, $P<0.05$) with a delayed onset of response such that significant anti-oedema activity was only apparent 180 min after carrageenan injection.

Acetic acid induced abdominal constrictions in the mouse were measured as described previously (Moore et al., 1991). Briefly, animals were injected i.p. with acetic acid (0.6 % w v^{-1} in saline, 0.1 ml $10g^{-1}$) and the number of abdominal constrictions monitored over the following 15 min. Pretreatment of mice with either NOP or NOF (2.5-50 mg kg^{-1}, p.o.) produced dose related inhibition of acetic acid induced abdominal constrictions (e.g. NOP; 50 mg kg^{-1}, p.o., 24.9 ± 1.5 constrictions $15\ min^{-1}$, c.f. 51.7 ± 2.4 constrictions $15\ min^{-1}$ in vehicle-injected animals, n=10, $P<0.05$).

The present data suggest that both nitro-SAID (i.e. NOP) and NO-NSAID (i.e. NOF) exhibit significant anti-inflammatory and antinociceptive activity in animal models. Although not evaluated in the present study the mechanism of action of these compounds most likely involves cleavage of the NO moiety and inhibition of the formation of pro-inflammatory prostanoids by released flurbiprofen or prednisone. Assuming that administration of NO-SAID (like NO-NSAID) is associated with reduced gastrointestinal side effects then these data suggest that nitro-SAID may be clinically useful for the treatment of inflammatory disease.

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85P EFFECT OF GRAPEFRUIT JUICE ON URINARY EXCRETION OF SALICYLATES

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Inhibition of Cytochrome P450 isozyme (CYP 3A4) by grapefruit juice (GFJ) is considered as the mechanism of increased systemic bioavailability of a number of drugs. It is however not known if GFJ can affect the urinary excretion of drugs although it can retard the appearance of coumarin metabolites in urine (Runkel et al., 1996). We have studied the effect of two varieties of grapefruit juice (Yellow and Ruby) on urinary excretion of salicylates following an oral intake of aspirin and compared salicylate excretion after the intake of aspirin with water (W) or diet coke (DK).

Following informed consent 17 non-smoker volunteers (8 male, 9 female, all Caucasians) with a median age of 22 (range 20 to 27) years and not taking any medication were recruited. The study was carried out about 90 minutes after the mid-day meal of two standard sandwiches and a cup of tea or coffee. Each subject was advised to empty bladder into a container and take two aspirin tablets (300 mg each) orally with 250 ml of a liquid. Urine samples were collected at 90 and 180 min, following the ingestion of aspirin and volume accurately measured. Salicylic acid and its metabolites were measured by spectroflurometric method and confirmed using HPLC methods.

The results in table 1 indicate that urinary excretion of salicylates is reduced when taken with GFJ . The effect is seen with both varieties of GFJ and is evident in both genders. There is also a reduction in urinary salicylate excretion at 90 min following the intake of aspirin with DK but this fails to reach a significant level. At 180 minute however the amount of urinary salicylates excreted following the intake of aspirin with GFJ , although still lower, was not significantly different from urinary values after the intake of aspirin with water. Extrahepatic CYP 3A4 is now recognised as a significant contributory factor to drug metabolism but its role in the intestinal metabolism of aspirin is largely unknown . At present we do not know if the inhibition of this enzyme by GFJ contributed to a reduced excretion of salicylates following the intake of aspirin.

Table 1 Urinary excretion of salicylates^ in males and females

	Male		Female	
Liquid	Mean ± SD	p*	Mean ± SD	p*
Water	37.56 ± 19.06	-	30.81 ± 0.86	-
Coke	32.52 ± 18.92	> 0.05	25.08 ± 17.77	> 0.05
YGF	22.48 ± 15.93	< 0.01	18.43 ± 13.06	< 0.01
RGF	25.36 ± 18.54	< 0.01	22.07 ± 16.13	< 0.01

*Compared with water ; paired 't' test : ^mg salicylic acid equivalents

Hydrolysis of aspirin to salicylic acid by non-specific esterases occurs in the liver, the gut and a number of other body cells. A substantial amount of salicylic acid is formed during the absorption of aspirin from the gut but it seems unlikely that GFJ related reduction in urinary salicylates was the result of its inhibitory action on tissue esterases. Metabolism of salicylic acid occurs through glucuronide and glycine conjugate formations but it also seems unlikely that GFJ caused inhibition of these processes. It is however possible that a reduction in urinary salicylates following aspirin intake with GFJ was the result of an altered gastric emptying rate since aspirin absorption from the intestine is slower than the stomach (Mason, 1984) There is also a possibility that aspirin binds to some of the ingredients in GFJ and this retards its absorption from the gut.

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86P KINETIC PROFILE OF THEOPHYLLINE IN CRITICALLY ILL PATIENTS: ASSESSMENT OF THEIR INTER- AND INTRA-INDIVIDUAL VARIABILITY

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Theophylline is often administered to ICU (Intensive Care Unit) patients with respiratory disorders due to its bronchodilator activity. Several previous studies performed with this drug in critically ill adult patients demonstrated a significant inter and intra-individual variability of individual pharmacokinetic parameters in the course of time (Zarowitz et al., 1988). This type of unpredictable pharmacokinetic behaviour has several implications, because changes in theophylline clearance and/or volume of distribution in critically ill patients could potentially result in underdosing or overdosing when a reliable therapeutic effect is most important (Mungall et al., 1983; Emerman et al., 1990). For this reason, in the present work we intend to analyse the kinetic profile of theophylline, especially concerning the sources of variability over time.

The study involved 20 patients, 11 of whom were male and 9 female, with a mean age of 59.9±15.6 years and a mean weight of 56.9±8.9 kg, who were taking theophylline as part of routine clinical care in our ICU (Coimbra University Hospital). Theophylline (aminophylline) was administered by continuous perfusion and the kinetic analysis assumed a one-compartmental open model with zero-order absorption and first-order elimination. The data analysis was made using a nonlinear mixed-effects modelling implemented with the NONMEM software package (Beal & Sheiner, 1992), according to the following pharmacostatistical model:

$$CL_j = \theta_1 * WT\ (\eta^{CL_j}\ \text{proportional})$$

$$V_j = \theta_2 * WT\ (\eta^{V_j}\ \text{proportional})$$

$$C_{ij} = C^*_{ij} * (1+\varepsilon_{ij})$$

where CL_j (L/h) and V_j (L) represent typical values of clearance and volume of distribution predicted by the regression, WT is Lean Body Weight (kg), C_{ij} and C^*_{ij} are, respectively, the observed and predicted concentrations, η^{CL_j} and η^{V_j} are individual random variability from the population mean parameters that are independent and identically distributed with mean zero and variances equal to ω^2_{CL} and ω^2_V, respectively, and ε_{ij} is the residual variability with mean zero and variance σ^2_ε for serum concentrations.

The obtained results for both the fixed and random effect parameters can be observed in Table 1.

Table1 – Final parameters estimates.

Parameters	Estimate	SE(%)	95% CI
CL	0.0291	7.25%	0.0248 - 0.0332
V	0.483	7.56%	0.410 - 0.556
ω_{CL}	35.78%	27.66%	23.92% - 44.59%
ω_V	45.83%	32.90%	26.79% - 59.00%
σ_ε	22.29%	22.74%	16.46% - 26.89%

In conclusion, we observed a marked inter and intra-individual variability in the kinetic profile of the studied population and it will be necessary to do further investigation in order to identify physiopathological covariates that allow us to individualize the therapy in this kind of patient. In the meantime, due to the magnitude of the random effect parameters, the low values for clearance and the instability usually observed in ICU patients, we recommend periodic serum theophylline determinations at at least three day intervals to allow eventual posological readjustments in time.

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87P EFFECT OF P-GLYCOPROTEIN MODULATORS ON ALKALINE PHOSPHATASE ACTIVITY IN RAT CULTURED HEPATOCYTES

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Alkaline phosphatase (ALP) is a membrane-bound metalloenzyme consisting of a group of isoenzymes, all glycoproteins, encoded for by at least four different gene loci: tissue non-specific (liver/bone/kidney), intestinal, placental and germ-cell ALP. We recently demonstrated that ALP concentration in different tissues is positively correlated with the extent of exchange surface per unit volume of the tissue (Calhau et al., 1999), suggesting an association between ALP and transport systems. Multidrug resistance (MDR), caused by overexpression of either P-glycoprotein or the multidrug resistance protein (MRP), is characterized by a decreased cellular drug accumulation due to an enhanced drug efflux. MDR is a serious impediment to successful chemotherapy of cancer. In previous work, we had obtained evidence for a putative involvement of ALP in the modulation of P-glycoprotein activity in hepatocytes (Martel et al., 1996,1998a,b). The aim of the present study was to determine the putative influence of compounds known to modulate MDR-mediated transport on hepatic ALP activity in primary cultured rat hepatocytes.

Male Wistar rats weighing 200-300 g were used. Isolated hepatocytes were prepared by a collagenase-perfusion technique as described previously (Martel et al., 1996). Hepatocytes were finally suspended in L_{15} Leibovitz medium supplemented with 10% fetal calf serum, 5 μg ml^{-1} insulin, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B and seeded on plastic culture dishes (21 cm^2) coated with collagen. After incubation for 3 h at 37°C in a humidified atmosphere (5%CO_2), the culture medium was replaced by DMEM:F-12 (1:1) medium supplemented with 5% fetal calf serum, 0.1 μM dexamethasone, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B. After 48-72 h the cells formed a confluent monolayer and each culture dish contained about 2 mg cell protein. In preliminary experiments, the time course of *p*-nitrophenol formation was determined, using a substrate (p-nitrophenyl-phosphate) concentration of 2.86 mM. Then homogenates were incubated with increasing concentrations of substrate (10 μM-10 mM) for 6 min.

We found the formation of *p*-nitrophenol to increase linearly with time for at least 40 minutes. The ALP activity was found to be dependent on the concentration used (and to be nearly saturated at 2.86 mM). The kinetic parameters K_m and V_{max} values obtained by non-linear analysis of the saturation curves were found to be 657.2 μM (306.8-933.1) and 32.0±1.5 nmol mg $protein^{-1}$ min^{-1} (n=4), respectively. Vanadate and corticosterone concentration-dependently reduced ALP activity, producing maximal reductions of 79 and 71%, respectively. The IC_{50}'s were found to be 7.9 μM (2.1-29.5 μM; n=5) and 2.4 μM (0.2-35.2 μM; n=3), respectively. Cyclosporin A, verapamil, octreotide, kaempferol and genistein produced a concentration-dependent increase in ALP activity. ALP activity was maximally increased to 253%, 390%, 286%, 487% and 193% of control in the presence of 100 μM cyclosporin A, 50 μM verapamil, 100 μM octreotide, 100 μM kaempferol and 1 μM genistein, respectively.

The results show that all P-glycoprotein modulators tested were able to significantly affect the activity of ALP. These effects on ALP may contribute to the modulation of P-glycoprotein activity by these drugs.

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88P CHARACTERIZATION OF THE EFFLUX OF THE ORGANIC CATION MPP IN RAT CULTURED HEPATOCYTES

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The aim of this work was to characterize the mechanisms involved in the hepatobiliar excretion of organic cations. For that purpose, we studied the efflux of ^{3}H-1-methyl-4-phenylpyridinium (^{3}H-MPP$^+$) from primary cultured rat hepatocytes incubated with 200 nM ^{3}H-MPP$^+$ for 15 min.

Male Wistar rats weighing 200-300 g were used. Animals were kept under controlled environmental conditions and food and tap water were allowed *ad libitum*. Isolated hepatocytes were prepared by a collagenase-perfusion technique as described previously (Martel et al., 1996). Hepatocytes were finally suspended in L_{15} Leibovitz medium supplemented with 10% fetal calf serum, 5 μg ml^{-1} insulin, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B and seeded on plastic culture dishes (21 cm^2) coated with collagen. After incubation for 3 h at 37°C in a humidified atmosphere (5%CO_2), the culture medium was replaced by DMEM:F-12 (1:1) medium supplemented with 5% fetal calf serum, 0.1 μM dexamethasone, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B. After 48-72 h the cells formed a confluent monolayer and each culture dish contained about 2 mg cell protein. For efflux studies, cells were preincubated at 37°C for 20 min. Subsequently, they were incubated at 37°C for 15 min in buffer containing 200 nM ^{3}H-MPP$^+$. At the end of this incubation, the buffer was replaced by buffer at 37°C without ^{3}H-MPP$^+$ for 5, 15, 30, 60 or 90 min. For uptake studies, cells were preincubated at 37°C for 20 min, and subsequently incubated at 37°C for 2 min with buffer containing 200 nM ^{3}H-MPP$^+$. Radioactivity present in the cells was measured by liquid scintillation counting.

Efflux of ^{3}H-MPP$^+$ from hepatocytes was rapid, as ^{3}H-MPP$^+$ present in the cells represented only 44 and 17% of the initial cell content after 5 and 90 min of washout, respectively. Efflux of ^{3}H-MPP$^+$ was temperature-dependent, and pH- and metabolic inhibition-independent. It was either strongly reduced (verapamil 100 μM, vinblastine 100 μM and rhodamine123 10 μM), moderately reduced (daunomycin 100 μM) or not affected (cyclosporine A 25 μM) by substrates/inhibitors of P-glycoprotein. The anti-P-glycoprotein antibody UIC2 (20 μg/ml) and vanadate (100 μM) had no effect on ^{3}H-MPP$^+$ efflux. Decynium22 (2 μM) and corticosterone (100 μM), known inhibitors of rOCT1, markedly reduced ^{3}H-MPP$^+$ efflux. Uptake of ^{3}H-MPP$^+$ into hepatocytes, known to be mediated by rOCT1, was inhibited by verapamil and vinblastine (IC_{50}'s of 2.6 (1.5-4.5) and 34.4 (7.2-165.0) μM, respectively; geometric means with 95% confidence limits).

In conclusion, our results do not support the involvement of P-glycoprotein or of an organic cation/proton antiporter in the efflux of ^{3}H-MPP$^+$ from cultured hepatocytes. Instead, we conclude that ^{3}H-MPP$^+$ efflux appears to be mediated by rOCT1, a polyspecific organic cation transporter. It is important to note that the overlapping substrate specificity of P-glycoprotein and rOCT1 makes a comparison betwen these transporters based on a substrate/inhibitor specificity only very difficult.

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89P THE pharma-CAL-ogy PROJECT: IS THERE LIFE AFTER DEATH?

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The pharma-CAL-ogy project started in 1993 with funding (650k GBP) from the Teaching and Learning Technology Programme (TLTP), an initiative of the Higher Education Funding Councils in the UK. The aim was to stimulate the production and use of technology-based teaching and learning materials in undergraduate courses in which pharmacology is a major component. The project has developed some 45 technology-based resources (mainly computer-based learning (CBL) programs and videos) over its 3-year period and distribution of the materials is now a function of the British Pharmacological Society. Some provide tutorial support, others provide support for practical/laboratory teaching while the majority provide learning materials to support lectures.

There has been considerable uptake of materials, particularly in the UK, (over 600 programs) but the integration of the materials into the curriculum has been variable. A survey of teachers suggested that the main reason was a lack of time to develop those additional support materials necessary to optimise the use of CBL resources. We also have some preliminary student usage data from one university. First year BSc Pharmacology students (n=200) were divided into quartiles according to their module performance grade (Q1 (higher grades) to Q4 (lower)). Their use of different CBL was monitored in relation to how they were asked to use it. If they were simply told it was available on the network then use was relatively low Q1=37%; Q2=9%; Q3 = 2%; Q4=0%) whereas if assessed tasks were set and marks recorded there was 100% usage from all quartiles. These data, although preliminary, also support the need to stimulate use by setting assessed exercises rather than simply telling students to "go away and look at the materials".

Our response has been to adopt a strategy of producing support materials (Teaching and Learning Resource Packs: TLRPs) for the teachers to use. These will provide a flexible, off-the-shelf resource (a collection of student tasks, exercises, problems, self-assessment questions and formative and summative assignments with mark schemes) which may be used as they come or individually tailored, with minimal effort and time commitment by the teacher, to suit the needs of different courses, different universities and different student groups.

In conclusion, the pharma-CAL-ogy project was highly successful in producing CAL programs and the new strategy of developing task-led support materials should enable the programs to be used more effectively within curricula.

90P PLATELET AGGREGATION, CALCIUM AND SEROTONIN IN CYCLOSPORIN A-INDUCED HYPERTENSIVE RATS

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Despite the recognized importance of cyclosporin A (CsA) in the prevention of rejection in allograft transplant recipients, its therapeutic use is associated with drug-related hypertension. The platelets may be implicated in this hypertension. Therefore, the purpose of this study was to determine the effects of CsA on platelet Ca^{2+} and activation.

Three groups of male Wistar rats (~300g) were formed: one receiving only orange juice (control) and the others receiving 5 (CsA5) and 30 (CsA30) mg/kg/day (p.o.) of CsA (Sandimmun Neoral®), dissolved in orange juice, daily, for seven weeks. Systolic, diastolic and mean blood pressures were measured by the "tail-cuff" method. Blood collection was carried out by venipuncture (jugular vein) under ketamine anesthesia. Basal and evoked (5-HT, 50 μM; thrombin, 0.1 U/mL) $[Ca^{2+}]i$ (by fluorometry using fura-2/AM - Johnson & Dufault, 1993), platelet [5-HT] (extracted by $HClO_4$ and quantified by HPLC-ED) and whole blood platelet aggregation induced by 5μg/ml of collagen (by the impedance method – Shaw *et al*, 1997) were determined at week 0, 2 and 7 of treatment, for the three rat groups (n=10 for each time, group and experiment) (Table 1). ANOVA and Student's t-test were used, and results are means ± s.e.m. (* - $p < 0.05$ was considered significant: CsA *vs* Control).

SBP, DBP and MBP (mmHg) increased in the CsA-treated groups, after 2 and 7 weeks, when compared with the control (Ctrl) group: Week 2 (n=20): SBP – 163 ± 1 (Ctrl); 190 ± 2 * (CsA5) and 179 ± 1* (CsA30). DBP– 96 ± 1 (Ctrl); 119 ±2 * (CsA5) and 114 ± 1* (CsA30). MBP– 118 ±1 (Ctrl); 142 ±2 * (CsA5) and 136 ± 1* (CsA30). Week 7 (n=20): SBP– 164 ± 1 (Ctrl); 189 ±2 * (CsA5) and 178 ±1 * (CsA30). DBP– 103 ± 1 (Ctrl); 114 ±2 * (CsA5) and 112 ±1 * (CsA30). MBP– 120 ± 3 (Ctrl); 136 ±4 * (CsA5) and 134 ±1 * (CsA30).

Table 1 - Effects of CsA on platelet $[Ca^{2+}]i$, 5-HT content and aggregation after 2 and 7 weeks of administration.

week	$[Ca^{2+}]i$ Basal (nM)	5-HT (ΔnM)	Thrombin (ΔnM)	[5-HT] (ng/ml)	Aggreg. (Ohms)
2					
Ctrl	122±2	149±4	1016±33	1105±41	10.1±0.4
CsA5	138±3*	188±6*	553±67*	899±25*	13.4±1.0*
CsA30	173±3*	208±4*	501±42*	698±21*	16.5±1.1*
7					
Ctrl	131±3	160±5	1072±50	999±41	12.0±1.5
CsA5	139±4	198±8*	1569±77*	877±17*	13.3±0.8*
CsA30	184±5*	198±6*	573±53*	672±19*	17.3±0.9*

Our results demonstrate that both CsA concentrations alter platelet calcium, 5-HT and aggregation. The possibility that platelets contribute to CsA-induced hypertension requires further studies.

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91P DOXAZOSIN, A SELECTIVE α_1-ADRENOCEPTOR ANTAGONIST, ALSO INHIBITS SEROTONIN-INDUCED SHAPE CHANGE IN HUMAN PLATELETS

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Doxazosin (DOX) is a selective α_1-adrenoceptor antagonist, used mainly for the treatment of essential hypertension. Patients with peripheral vascular disease or diabetes mellitus tend to have elevated circulating levels of naturally occurring platelet agonists like serotonin (5hydroxytryptamine; 5-HT, Barradas *et al.*, 1994). This bioamine can induce platelet shape change (PSC), an early phase of platelet activation, which is essentially aspirin resistant. In addition, 5-HT exerts other harmful effects (e.g. stimulating vascular smooth muscle proliferation and inducing vasoconstriction in atheromatous coronary vessels). The aim of this study was to determine whether doxazosin inhibits 5-HT-induced PSC, and whether this action is specific to 5-HT, in human platelets.

Platelet rich plasma (PRP) was prepared from healthy volunteers (n=6, 4 males). PSC was assessed by measuring median platelet volume (MPV) using a high resolution (0.07 fl) channelyzer (Barradas *et al.*, 1992). PSC was induced with 5-HT (0.5-1.0 μM) and the MPV measured at various time points (30 s, 1 min and 2 min). This was repeated after incubation with 0.33 μM of DOX, a concentration similar to peak therapeutic plasma levels (Elliott *et al.*, 1987). To establish whether the effect of DOX was specific to 5HT, we also assessed PSC after adding ADP (0.1-0.2 μM) or U46619, a TXA_2 analogue (0.025-0.05 μM). Statistical analysis is by paired t-test (two-tailed). Platelet count was monitored throughout the experiment to exclude samples where significant (>5%) aggregation occurred in PRP.

Following the addition of 5-HT, the MPV increased significantly at 30 s and 1 min (p=0.03 and p=0.001). This increase was significantly inhibited (p=0.001 and p=0.007) by DOX. There were also significant increases in PSC with both ADP and U46619 at all time points (30s, p=0.03; 1 min, p=0.006; 2 min, p=0.006 and 30 s, p=0.04; 1 min, p=0.003; 2 min, p=0.002, respectively) However, these increases were not inhibited by DOX.

DOX inhibits 5-HT-induced PSC in human platelets. This effect seems to be specific, since there was no effect on ADP- or U46619-induced PSC. These results should be interpreted after considering that in humans the circulating levels of 5-HT are in the nM range. Nevertheless, approximate therapeutic levels of DOX blocked the action of 5-HT that was added at concentrations in the pM range.

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92P THE EFFECT OF IDAZOXAN ON CORTICOSTERONE LEVELS IN CONTROL AND RESTRAINT-STRESSED RATS

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Central catecholamines play an important role in maintaining basal homeostasis and in mediating the response to stress. The α_2-adrenoceptor antagonist idazoxan has been shown to modulate the hypothalamo-pituitary-adrenal (HPA) axis response to a number of stressors in rats (Grino *et al.*, 1994). The present study investigated the effect of idazoxan on plasma corticosterone levels, an index of HPA axis activation and secondly, investigated the effect of idazoxan administration on changes in plasma corticosterone induced by the acute psychological stress of restraint.

Male Sprague-Dawley rats (200-220g) were cannulated through the right jugular vein under sodium pentobarbital anaesthesia (48 mg kg^{-1}). Following surgery, animals were housed singly. The experiment began between 09.00h and 10.00h, 3 days after surgery. At time 0 min a pre-injection blood sample (0.4 ml) was taken from each animal. Rats were then given an i.p. injection of saline vehicle with or without restraint, or idazoxan (10 mg kg^{-1}) with or without restraint. Animals were restrained in an acrylic restrainer for a period of 60 min. Further blood samples (0.4 ml) were taken 30 min and 60 min after injection. A further trunk blood sample was collected after decapitation at 240 min. Total plasma corticosterone was measured directly by radioimmunoassay (Harbuz *et al.*, 1994). Data were analysed using Fisher's PLSD test following one-way analysis of variance.

Idazoxan administration resulted in a significant elevation in plasma corticosterone levels at 30 min and 60 min compared to time-point saline treated controls. Restraint stress also significantly elevated corticosterone at both 30 and 60 min. Idazoxan further increased corticosterone levels in the restrained animals compared to the saline treated restrained group with the increase being significant at 60 min. At 240 min corticosterone levels in all groups had returned to basal.

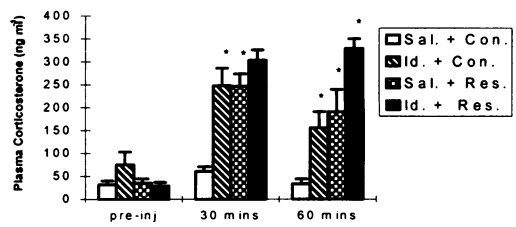

Figure 1. Effects of idazoxan and restraint stress on plasma corticosterone levels in rats. Results are expressed as mean ± s.e.mean (n = 5-8). $p < 0.01$ compared to appropriate time-point controls.

In conclusion, peripheral administration of idazoxan elevates plasma corticosterone in rats. Furthermore at 60 min idazoxan potentiates the stress-induced elevation in plasma corticosterone levels. These effects may be α_2-adrenoceptor and/or imidazoline$_2$ (I_2) binding site mediated. Further studies are needed to clarify the precise mode of action underlying idazoxan's ability to effect the stress response.

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93P THE BINDING AFFINITY AND FUNCTIONAL ACTIVITY OF ELETRIPTAN AND OTHER 5-$HT_{1B/1D}$ AGONISTS AT THE HUMAN RECOMBINANT 5-HT_{1B} AND 5-HT_{1D} RECEPTORS

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Eletriptan is a 5-$HT_{1B/1D}$ agonist, currently being developed for the treatment of migraine. Previous studies have shown that eletriptan is a potent constrictor of isolated canine vascular tissues *in vitro* (Gupta *et al.*, 1999) and it is currently accepted that the contractile effects of 5-$HT_{1B/1D}$ agonists are mediated through smooth muscle 5-HT_{1B} receptors (Hamel *et al.*, 1993; Longmore *et al.*, 1997). In this study the functional activity and binding affinity at the human 5-HT_{1B} and 5-HT_{1D} receptors of eletriptan and other 5-$HT_{1B/1D}$ agonists in use, or under development for treatment of migraine, was studied.

CHO-K1 cells stably expressing the human 5-HT_{1B} (B_{max} 2-4 pmol/mg) and 5-HT_{1D} (B_{max} 5-7 pmol/mg) receptors were seeded into Cytosensor cups at 2-3x10^5/ml 20 h before use. Cells were perfused at 100 µl/min with bicarbonate-free DMEM (pH 7.4) containing 4.5 g/litre of glucose. A pump cycle of 1 min 30 sec was employed during which acidification rate was measured for 12 sec while perfusion was stopped. Agonists (10^{-11} - 10^{-5} M) were added to the perfusion fluid for 2 min 20 sec with 15 min between each of the 6-8 concentrations. A standard addition of 10 µM 5-HT was made at the start and end of the experiment. pEC_{50} values were derived by plotting peak acidification rates against agonist concentration. pK_i values were determined by displacement of [^{3}H]-eletriptan (1 nM, 22°C for 30 min) with 12 concentrations of agonist (3x10^{-11} - 10^{-5}M) from membrane homogenates of cells expressing 5-HT_{1B} (30 µg protein) and 5-HT_{1D} (60 µg protein) receptor. Non-specific binding was defined with 10 µM 5-HT. All experiments were repeated at least 4-6 times.

Basal acidification rates for the 5-HT_{1B} and 5-HT_{1D} receptor were 0.103 and 0.076 pH units /sec (103 and 76 µV /sec) respectively. In response to 5-HT this was increased by 43±2% and 123±5% (mean ± s.e.) respectively. All compounds tested had high affinity for the 5-HT_{1B} and 5-HT_{1D} receptors and were potent agonists producing concentration-dependent increases in the rate of acidification at both receptor subtypes (Table 1). Maximum responses were not different from that produced by 10 µM 5-HT. While there was close agreement between the functional potency and binding affinity at the 5-HT_{1D} receptor, binding affinity tended to be greater than functional potency at the 5-HT_{1B} receptor. Eletriptan was the most potent compound at the 5-HT_{1D} receptor and had similar high potency to frovatriptan, zolmitriptan and naratriptan at the 5-HT_{1B} receptor. This is the first study to show that eletriptan is a potent agonist at the human recombinant 5-HT_{1B} and 5-HT_{1D} receptors a finding which is consistent with its potent constrictor activity in isolated canine vascular tissues *in vitro* (Gupta et al., 1999).

Table 1. Functional potency (pEC_{50}) and binding affinity (pK_i) at the human 5-HT_{1B} and 5-HT_{1D} receptor subtypes (mean ± s.e., n=4-6).

	5-HT_{1B}		5-HT_{1D}	
AGONIST	**pEC_{50}**	**pK_i**	**pEC_{50}**	**pK_i**
5-HT	7.4 ± 0.04	8.4 ± 0.07	8.3 ± 0.07	8.4 ± 0.08
Eletriptan	7.7 ± 0.02	8.6 ± 0.14	9.2 ± 0.02	8.8 ± 0.09
Sumatriptan	7.0 ± 0.03	7.8 ± 0.09	8.3 ± 0.05	8.1 ± 0.06
Zolmitriptan	7.6 ± 0.05	8.6 ± 0.06	8.9 ± 0.06	8.6 ± 0.07
Naratriptan	7.6 ± 0.08	8.6 ± 0.08	8.7 ± 0.06	8.5 ± 0.09
Almotriptan	7.2 ± 0.08	8.3 ± 0.03	7.8 ± 0.08	7.6 ± 0.02
Rizatriptan	7.1 ± 0.04	7.8 ± 0.08	8.4 ± 0.05	7.8 ± 0.04
Frovatriptan	8.2 ± 0.02	7.9 ± 0.08	8.7 ± 0.02	8.0 ± 0.14

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94P INVOLVEMENT OF PROTEIN KINASE C (PKC) IN 5-$HT_{1B/1D}$ RECEPTOR SIGNALLING IN THE RABBIT ISOLATED RENAL ARTERY

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5-hydroxytryptamine (5-HT) causes vasoconstriction of the rabbit isolated renal artey via the 5-$HT_{1B/1D}$ receptor subtype following partial depolarisation (Choppin & O'Conner, 1994). We have previously used the reverse transcription-polymerase chain reaction (RT-PCR) to demonstrate the presence of mRNAs for both the 5-HT_{1B} and 5-HT_{1D} receptor in vascular smooth muscle cells (VSMCs) from the rabbit isolated renal artery (Hill *et al.*, 1998) and primary cultures of VSMCs from this artery (Hinton *et al.*, 1998). In this study we investigated the involvement of protein kinase C (PKC) in the 5-$HT_{1B/1D}$ receptor mediated contractile pathway using Western blotting to determine the expression of specfic PKC isoforms. In primary cultures of VSMCs from the renal artery, we also investigated the subcellular distribution of PKC isoforms using immunohistochemistry

Isolated segments of endothelium denuded artery were mounted in a Mulvany-Halpern myograph. Cummulative concentration-effect curves were constructed to 5-HT in the presence of ketanserin and prazosin (each 1 µM) following prestimulation with 20 mM K^+ and in the presence of the PKC inhibitor Ro 13-8220 (3 µM and 10 µM). Individual PKC isoforms were identified by Western blotting with isoform-specific antibodies in homogenates of endothelium denuded artery (Ohanian *et al.*, 1996). Primary cultures of renal artery VSMCs were fixed, incubated with isoform specific primary antibodies and a fluorescently conjugated secondary antibody, and images then captured using a confocal microscope.

Inclusion of the PKC inhibitor Ro 31-8220 significantly inhibited 5-HT-evoked contraction in arterial segments prestimulated with 20 mM K^+ in the presence of ketanserin and prazosin (each 1 µM) (n=4; P>0.05). Maximum contraction was reduced from 64.6 ± 2.2% to 25.7 ± 3.9% and 12.1 ± 3.2% by Ro 31-8220, 3 and 10 µM respectively. In tissue homogenates of the renal artery Western blotting identified six specific isoforms of PKC; α, δ, γ, ι, λ and µ. No signals were observed for β, ε, ο or ζ. Immunohistochemistry of VSMCs from primary cultures of the renal artery using PKC isoform specific antibodies identified only four isoforms; α, δ, γ and µ. The α isoform was evenly distributed throughout the nucleus and the cytoplasm and the δ isoform was aligned with cytoplasmic actin filaments. The γ isoform was discretely distributed throughout the cytoplasm, while the µ isoform formed a perinuclear halo.

In conclusion, these data demonstrate a role for PKC in 5-$HT_{1B/1D}$ mediated contraction of the rabbit isolated renal artery and provide evidence for at least six PKC isoforms in the native artery. In primary cultures of renal artery VSMCs, we have further demonstrated that four of these isoforms have a distinct subcellular distribution which may indicate a specific cellular function for each isoform.

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95P MODULATION OF FORSKOLIN-EVOKED RELAXATION BY AGENTS WHICH SPECIFICALLY RAISE cGMP IN RAT ISOLATED MESENTERIC ARTERY

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Previous studies have shown cyclic AMP and cyclic GMP-mediated smooth muscle cell relaxation can interact synergistically via cross-talk between the signalling pathways (Jiang *et al.*, 1992) or by both the pathways acting separately and in concert (Murthy & Makhlouf, 1995). Previous studies have shown an ability of forskolin, an activator of adenylyl cyclase, to evoke concentration dependent relaxation in rat isolated mesenteric arteries (Heeson & De Mey, 1990). In the present study, we have investigated whether pharmacological agents which can specifically mimic or increase intracellular [cGMP], 8-Br-cGMP and 4-[[3,4-(Methylenedioxy)benzyl]amino]-6-chloroquinazoline (CQZ), a type V phosphodiesterase inhibitor (Takase *et al.*, 1994), can modify forskolin-evoked relaxation in endothelium-intact and denuded rat isolated mesenteric arteries.

Male Sprague-Dawley rats (250-300 g) were stunned and killed by cervical dislocation. Segments of third order mesenteric artery (ID_{100} = 226.9 ± 13.0 μM; n=14) were mounted in a Mulvany-Halpern myograph under a normalised tension for isometric recording. The tissues were maintained at 37°C in oxygenated Krebs buffer. All data are expressed as mean ± s.e. mean. Differences between mean values were calculated using Students t-test.

Forskolin (3 nM-0.3 μM) evoked concentration-dependent relaxation in phenylephrine (1-3 μM) stimulated endothelium-intact arterial segments (n=4; pEC_{80}=7.27±0.1). However, forskolin-evoked relaxation was significantly attenuated, at concentrations between 3-30 nM, in endothelium-denuded segments (n=6; pEC_{80}=6.99±0.12; $P<0.05$).

In tissues with an intact endothelial cell layer, forskolin-evoked relaxation was not significantly affected by pre-incubation with either 8-Br-cGMP (1 μM; 10 mins; n=4; R_{max}=101.9±3.7%) or further addition of the selective type V phosphodiesterase inhibitor, CQZ (3 μM; 10 mins; n=4; R_{max}=97.9±.9%; $P>0.05$). In contrast, in denuded segments 8-Br-cGMP caused a leftward shift in the dose response curve (n=6; R_{max}=94.8±1.3%) which was further potentiated by incubation with CQZ (n=5; R_{max}=95.8±3.7%).

Pre-incubation of endothelium-intact arteries with the nitric oxide synthase inhibitors, N^G-nitro-L-arginine methyl ester (100 μM; 30 mins) and Nω-nitro-L-arginine (100 μM; 30 mins), did not significantly attenuate forskolin-evoked relaxation (n=4).

In conclusion, these data indicate that forskolin-evoked relaxation can be enhanced by 8-Br-cGMP and CQZ, agents which specifically raise intracellular cyclic GMP levels, but only in endothelium-denuded rat isolated mesenteric artery. The modulatory influence of the endothelium on the latter mechanism remains to be clarified. However, the failure of nitric oxide synthase inhibitors significantly to attenuate forskolin-evoked relaxation in endothelium-intact arteries suggests that basal NO release does not play a significant modulatory role in the cAMP-mediated relaxation of this artery.

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96P INVESTIGATION OF THE α_1 ADRENOCEPTOR SUBTYPE(S) MEDIATING CONTRACTION IN THE HUMAN INTERNAL MAMMARY ARTERY

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Tissue α_1-adrenoceptors can be divided into the α_{1A}, α_{1B} and α_{1D} subtypes, all members of the α_{1H}-adrenoceptor subgroup, as they have a high affinity for prazosin (which does not discriminate between them). In addition there may be an α_{1L}-subtype with a low affinity for prazosin (Muramatsu *et al.*, 1990). The subtypes present in human blood vessels remains unclear. The human internal mammary artery contains α_1-adrenoceptors (Weinstein *et al.*, 1989) and the aim of the present experiments was to characterise these receptors using functional studies with α_1-adrenoceptor subtype selective antagonists.

Human internal mammary arteries were obtained (with local Ethics Committee approval) as surplus from patients (male and female, aged 53-73) undergoing coronary artery bypass graft operations. Rings were suspended in Krebs buffer containing rauwolscine (10^{-7}M), propanolol (10^{-7}M) and cocaine (10^{-6}M) to block α_2 and β adrenoceptors and uptake$_1$ respectively. Tissues were kept at 37°C and bubbled with 5% CO_2 in O_2 throughout the experiment. Tissues were then washed every 15 min during the 3h equilibration period, or until tension was stable. KCl (48mM) was used to prime the tissues, followed by repeated additions of phenylephrine (10^{-6}M), until the contractions attained were within 10% of each other. Contractile concentration response curves were constructed to phenylephrine both before and after 30 min incubation of the tissues with the α_1 adrenoceptor antagonists. Results are expressed as a percentage of the maximal contraction to phenylephrine in each control concentration-response curve.

Phenylephrine caused concentration dependant contraction of the internal mammary artery (pEC_{50} 5.9 ± 0.6 (mean ± s.e.m. respectively) n=7). A time-matched control was used in comparison with antagonist treated preparations (pEC_{50} 4.9 ± 1.0M, n=7). Contraction was mediated via an α_{1H} adrenoceptor subtype as prazosin had a pA_2 of 11.2 (Schild slope 0.7 ± 0.3, n=4). The α_{1A}-adrenoceptor subtype selective antagonist 5-methylurapidil (10^{-8}-10^{-6} M) had an apparent pA_2 of 8.5 (Schild slope 0.3 ± 0.2, n=3; apparent pK_b 8.4 ± 0.4 at 10^{-8}M). The α_{1D}-subtype selective antagonist BMY 7378 (10^{-6} M, n=3) shifted the phenylephrine concentration response curve to the right (apparent pK_B 6.4 ± 0.1). This affinity is about 100-fold lower than that expected for the antagonist at α_{1D}-adrenoceptors.

These data suggest that the α_1 adrenoceptor population mediating contractions of the internal mammary artery is of the α_{1H} subgroup. In addition this data suggests the α_1-adrenoceptors on the internal mammary artery may be of the α_{1A}- but not the α_{1D}-adrenoceptor subtype.

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97P ATP RELEASED BY SYMPATHETIC STIMULATION OF THE HUMAN UTERINE ARTERY: CONTRACTION BY P2 RECEPTORS AND INHIBITION OF NORADRENALINE RELEASE THROUGH A_1 RECEPTORS

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ATP is a cotransmitter with noradrenaline (NA) in the sympathetic nervous system and contributes to neurogenic contractions. The relative importance of ATP in these contractions depends on the tissue and the species. Moreover, ATP may also be released from other tissues, including smooth muscle cells. The aim of this work was to study in the human uterine artery: 1.The relative importance of the ATP released on contraction; 2.The influence of ATP or its breakdown products on the release of NA; 3.The subtype of receptor involved.

From patients undergoing surgery, uterine arteries were carefully obtained, dissected and cut into strips or rings. Then they were suspended in organ baths filled with aerated Krebs-Henseleit solution, maintained at 37°C, and prepared for isometric contractions [by drugs or through field electrical stimulation (1-12 Hz)] or for measurement of endogenous NA release. Since ATP has been known to desensitize its own receptors, when indicated, noncumulative concentration-response curves were performed. To measure the release of endogenous NA, the strips were preincubated with 1 mM pargyline, placed in perifusion chambers and washed out. Cocaine (7.5 μM) and desoxycorticosterone (40 μM) were added and fluid continuously collected; 2 periods of electrical stimulation were applied (S1, S2) and NA measured by HPLC-ECD; 20 min before S2 drugs were added. Results are expressed as a % of the maximal contraction to electrical stimuli or to 60 mM KCl or as S2/S1 ratios; the number of experiments was 5-14.

In the presence of 10 μM prazosin and 10 μM yohimbine the electrical stimulation still induced 33.2±6.2% of contraction which was strongly decreased by 100 μM suramin (13.7±1.7% of contraction) or desensitization by 30 μM α,β-methyleneATP (α,β-meATP) (18.8±2.9%). Purine nucleotides and analogs contracted uterine rings with the following rank order of agonist potency (Emax±s.e.m.): α,β-meATP (62.0±12.2%) >> 2-methylthioATP (2-meSATP) (26.9±12.1%) > ADP (11.4±6.4%) = ATP (7.9±2.3%) > AMP (5.3±2.6%). Inhibition of NO synthase by 100 μM L-NMMA, as well as the pretreatment of the vascular segments with 100 μM suramin, 100 μM PPADS or 30 μM reactive blue 2 did not significantly change the contraction to α,β-meATP. Desensitization by 30 μM α,β-meATP abolished the response to 2-meSATP but not to ATP which was then inhibited by 100 μM suramin.

Concerning the electrically-induced NA release, ATP (200 or 300 μM), 300 μM ADP, 300 μM AMP and adenosine (200 μM) decreased (S2/S1 ratios: 0.56±0.03 or 0.49±0.04, 0.43±0.06, 0.31±0.07, 0.46±0.04, respectively; control: 0.76±0.05) while 100 μM suramin increased NA release (S2/S1: 1.18±0.10); 100 μM α,β-meATP or 100 μM 2-meSATP had no effect on this release. In the presence of 10 μM prazosin the S2/S1 ratio was lower (0.51±0.04), higher concentrations of ATP were needed (300-900 μM) to decrease NA release and suramin was without effect. DPCPX (1 μM), selective A_1 antagonist, abolished the ATP-induced effect.

These results suggest that: 1. The ATP released by the electrical stimulation of the human uterine artery stimulates a mixed population of postsynaptic purinoceptors 2. The ATP released by sympathetic stimulation is hydrolysed to adenosine which then acts upon inhibitory A_1 presynaptic receptors.
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98P CHARACTERISATION OF Kv 1.5 EXPRESSED IN HEK 293 CELLS: A COMPARISON WITH NATIVE RAT PULMONARY ARTERY DELAYED RECTIFIER POTASSIUM CURRENTS

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Studies using reverse transcriptase polymerase chain reaction (RT-PCR) have identified mRNA for Kv1.5 in rat pulmonary artery tissue (Turner *at al.*,1996) suggesting that Kv1.5 α-subunits may represent the structural basis of the native delayed rectifier K-channel (K_V) in this tissue. The whole-cell configuration of the voltage-clamp technique was used to assess the K-current carried by Kv1.5 stably expressed in single HEK 293 cells. Several K-channel modulators were used in the present study to characterise $I_{Kv1.5}$ pharmacologically for comparison with native rat pulmonary artery delayed rectifier K-currents ($I_{K(V)}$, Walker *et al.*, 1996, 1998).

When the cells were held at a potential of -90 mV and stepped to a series of 500 ms voltage pulses from -60 mV to +70 mV, an outward current was observed. The current exhibited activation and inactivation kinetics, which are consistent with previous electrophysiological studies of Kv1.5 channels (Snyders *et al.*, 1993). At depolarising potentials both $I_{Kv1.5}$ and $I_{K(V)}$ activated rapidly. Inactivation of $I_{Kv1.5}$ and $I_{K(V)}$ was best fitted to one exponential with a time constant of 16.2 ± 2.5 s and 5.7 ± 6.4 s, respectively. The more rapid inactivation of the native channel may be due to the presence of β-subunits, an observation consistent with previous reports that show the presence of β-subunits increases the rate of inactivation of several delayed rectifier channels (Rettig *et al.*, 1994).

Kv1.5 was strongly 4-aminopyridine (4-AP)-sensitive yet relatively insensitive to tetreaethylammonium (TEA). 4-AP (1 mM) produced a 58.9 ± 6.4 % (n = 9) reduction in the amplitude of $I_{Kv1.5}$ at a test potential of +50 mV. Both native K_V and Kv1.5 were also similarly sensitive to a wider range of pharmacological agents. The amplitude of $I_{Kv1.5}$, at the end of the +50 mV test pulse was reduced by clotrimazole (30 μM: 92 ± 1.9 %, n = 5); proadifen (50 μM and 100 μM: 72.1 ± 5.3 % and 90 ± 2.8 %, n = 3 - 5; terfenadine (1 μM and 5 μM: 28.6 ± 9.4 % and 47.9 ± 8.1 %, n = 6 - 7); astemizole (10 μM: 41.9 ± 3 %, n = 3); quinidine (20 μM and 100 μM: 52.7 ± 3.4 % and 70.4 ± 4.2 %, n = 5); or S16257-2 (100 μM and 200 μM; 48.5 ± 6.4 % and 63.7 ± 9.4 %, n = 4), respectively. NS 1619 (33 μM) which activates BK_{Ca} and inhibits K_V in smooth muscle (Walker *et al.*, 1996) also produces a 57 ± 5 % (n = 8) reduction in the amplitude of $I_{Kv1.5}$ at +50 mV.

Iberiotoxin (IbTX), and levcromakalim and glibenclamide were used to demonstrate the absence of BK_{Ca} and K_{ATP}, respectively from these cells. Interestingly, levcromakalim (10 μM) which induces $I_{K(ATP)}$ whilst simultaneously inhibiting $I_{K(V)}$ in smooth muscle (Edwards *et al.*, 1993), had no effect on $I_{Kv1.5}$ (n = 4). These findings indicate that levcromakalim does not have a direct effect on K_V-like currents and is consistent with the idea of a levcromakalim-induced 'conversion' of K_V to K_{ATP} via the association of K_V with SUR first proposed by Edwards and co-workers (1993).

The present investigation provides a detailed profile of the pharmacology of Kv1.5 channels expressed in HEK 293 cells. Many similarities in the pharmacology of Kv1.5 and native K_V channels were detected which may suggest that rat pulmonary artery K_V channels comprise Kv1.5 α-subunits.

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99P ENDOTHELIN-1 AND CONTRACTION OF RAT RENAL ARTERIES

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We have further investigated the mechanisms underlying endothelin (ET)-1-induced contractile responses of rat renal arteries, having previously reported that ET-1 has marked electrophysiological effects inhibiting both delayed rectifying and calcium-activated potassium channels in isolated rat renal myocytes (Betts *et al.*, 1998). In the present series of experiments male Wistar rats (250 – 350 g) were killed (pentobarbitone 30 mg/kg i.p.), the kidneys removed and branches of the main renal artery (diameter 100 – 400 μm) proximal to the kidney dissected free. Vessels were either denuded of their endothelium and suspended in a Mulvany myograph (Mulvany & Halpern, 1977) for contraction studies, or treated with collagenase to yield myocytes for patch-clamp experiments. In physiological salt solution ET-1 induced constriction with an EC_{50} of 23.1 nM (n = 7): an effect blocked by BQ-123 (3 μM) confirming ET_A receptor involvement. In order to examine the role of extracellular Ca^{2+} in mediating contraction, we compared the effects of ET-1 with those of elevating extracellular K^+ to 70 mM (high K). ET-1 (30 nM; throughout) constricted arteries by 5.1 ± 0.7 mN/mm^2 (n = 7) while high K constricted arteries by 2.3 ± 0.8 mN/mm^2 (n = 7; 38.1 ± 10.0 % of ET-1-induced response). The time-to-peak for these responses was 405.4 ± 50.8 s (n = 7) and 96.2 ± 37.0 s (n = 5), respectively.

Arteries constricted by either ET-1 or high K were relaxed by nicardipine (1 μM; n = 3). Pretreatment with nicardipine also reduced the ET-1 constriction (n = 3). At a holding potential of –50 mV, voltage-clamp experiments on renal arterial myocytes (cell capacitance: 24.9 ± 3.3 pF; n = 6) revealed that ET-1 produced a transient inward current (I_T) with a time-to-peak of 6.5 ± 1.5 s and a peak current of 2.7 ± 0.8 pA/pF (n = 6) and inhibition of iberiotoxin-sensitive spontaneous transient outward currents (STOCs; n = 7). Inhibition of STOCs occurred 86.7 ± 16.7 s after application of ET-1 while activation of I_T followed a lag of 72.4 ± 30.3 s. Under current-clamp, ET-1 exhibited two effects (i) depolarization of cells from –32.6 ± 1.5 to –14 ± 1.6 mV, (n = 12) and (ii) stabilization of the membrane potential in those cells showing STOCs. These results may suggest that in the rat renal artery ET_A receptor stimulation produces contraction dependent on Ca^{2+}-influx though L-type Ca^{2+} channels which open following activation of an, as yet, uncharacterized depolarizing current coupled with inhibition of Ca^{2+}-activated K^+ channels.

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100P CHRONIC HYPOXIA (CH) AUGMENTS CONVERSION OF BIG ET-1 AND VASOCONSTRICTOR RESPONSE TO ET-1 IN RAT PERFUSED LUNGS

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ET-1 is synthesised in rat lungs from its precursor big ET-1 by endothelin converting enzyme (ECE) (Hisaki et al., 1994) and it has been implicated in CH induced pulmonary hypertension (Elton et al., 1992). In the present study we have examined the effects of CH on conversion of big ET-1 and vascular reactivity to ET-1 and big ET-1 in lungs from CH rats.

CH male Wistar rats (250-270g) were kept in a chamber at pO_2 of 10% for 3 weeks. CH or control rats were anaesthetised with Sagatal (60 mg kg^{-1}, i.p.) heparinised (500 units; i.v), 5 min later lungs were isolated and perfused via the pulmonary artery (Krebs' solution gassed with 20% O_2, 5% CO_2, 75% N_2). Lungs were ventilated via the trachea with room air (28 strokes min^{-1}; 1ml stroke volume). Pulmonary perfusion pressure (PPP) and lung weight (LW) were monitored simultaneously. Bolus injections of ET-1 or big ET-1 were given into the pulmonary artery after 20 min stabilisation.

In another series of experiments bolus injections of big ET-1 were given in control or CH lungs and the perfusate was collected for 10 min. ET-1 was extracted from the perfusate and assayed using human ET-1 enzyme-linked immunosorbent assay as described previously (Smith et al., 1997). Data were analysed statistically with the Students' t-test and are expressed as mean ± s.e.mean.

CH significantly increased the ratios of right ventricular to total ventricular weight when compared with control rats (0.35 ± 0.01 vs. 0.20 ± 0.01, p< 0.01, n=5). Hematocrit values were significantly higher in CH (65 ± 0.8 %, n= 5, p< 0.01) compared to control (43 ± 0.9 %, n=5) rats.

In isolated lungs basal PPP was significantly higher in CH (13.6 ± 1 mmHg, n=6, p< 0.01) than control animals (5 ± 0.7 mmHg, n=5). ET-1 (50-400 pmoles) produced dose-dependent increases in PPP. ET-1 (400 pmoles)-induced increases in PPP were significantly increased in CH (19 ± 2 mmHg, n= 6, p< 0.01) compared to control lungs (8 ± 1 mmHg, n=5). Big ET-1 (50-1600 pmoles) induced dose-dependent increases in PPP in control and CH lungs.

Big ET-1 (1600 pmoles)-induced increases in PPP were significantly higher in CH (21 ± 2.2 mmHg, n=4, p< 0.01) than control lungs (6.6 ± 1.3 mmHg, n=3).

ET-1 (400 pmoles)-induced increases in LW in control vs. CH lungs (5 ± 2.1 g, n=5; 8.2 ± 3g, n=6) were not different. Similarly, big ET-1 (1600 pmoles) produced similar increases in LW in control (0.87 ± 0.3 g, n=4) and CH lungs (1.2 ± 0.16g, n=4).

Basal perfusate levels of ET-1 in control vs. CH lungs were not different (0.028 ± 0.01 pg ml^{-1} vs. 0.056 ± 0.022 pg ml^{-1}, n=4). However, after big ET-1 (1600 pmoles) the perfusate levels of ET-1 were significantly higher in CH (12.5 ± 1.8 pg ml,$^{-1}$ n =4, p< 0.01) than control lungs (1.2 ± 0.34 pg ml^{-1}, n=4).

In summary, ET-1-induced increases in PPP were significantly potentiated in lungs from CH rats. This is in contrast to our previous studies in isolated large pulmonary arteries and veins, where ET-1-induced contractions were reduced in CH vessels (Lal et al., 1998). This suggests that CH differentially alters the responses to ET-1 in resistance vessels in perfused lungs compared to large pulmonary blood vessels. Big ET-1-induced increases in PPP were significantly potentiated in CH lungs. In addition after big ET-1 injection the perfusate ET-1 levels were higher in CH compared to control lungs. This suggests that ECE activity is enhanced in lungs from CH rats. The fact that ET-1 and big ET-1-induced increases in LW in CH vs. control lungs were not different, suggests that enhanced ET-1-induced pulmonary vasoconstriction in CH is due to pulmonary arterial rather than venous constriction. The underlying mechanisms for this enhanced responsiveness to ET-1 in CH lungs are under investigation.

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101P THE STABLE NITROXIDE RADICAL TEMPOL REDUCES HYDROGEN PEROXIDE-MEDIATED CELLULAR INJURY IN PRIMARY CULTURES OF RAT RENAL PROXIMAL TUBULAR CELLS

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Reactive oxygen species (ROS)-mediated cellular injury and death has been implicated in the pathogenesis of renal ischaemia-reperfusion injury (Weight *et al.*, 1996). Within the kidney, the proximal tubule (PT) appears to be particularly susceptible to reperfusion-injury (Weight *et al.*, 1996). Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable water-soluble free radical of low molecular weight, which permeates biological membranes and scavenges ROS (Laight *et al.*, 1997). We have recently discovered that tempol reduces renal dysfunction in rat models of shock induced by wall fragments of Gram-negative (endotoxin) and Gram-positive bacteria (Leach *et al.*, 1998, Olbrich *et al.*, 1999).

The aims of this study were to investigate the effect of tempol on cellular injury in cultures of rat PT cells exposed to oxidant stress in the form of hydrogen peroxide (H_2O_2) and to compare the effects of tempol with those of other agents known to reduce the generation and/or the actions of ROS.

Kidneys were obtained from male Wistar rats (240-300 g) anaesthetised using sodium thiopentone (120 mg·kg^{-1}, i.p). PT cells were isolated from kidney cortex using collagenase digestion, differential sieving and Percoll density centrifugation and cultured on 24 well plates (1x10^5 cells/well) in Minimum Essential Medium containing 10% (v/v) fetal calf serum. Once confluent, monolayers were incubated with H_2O_2 (1 mM) for 4 hours in the presence or absence of increasing concentrations of tempol (0.03-10 mM), deferoxamine mesylate (0.03-10 mM) or catalase (0.03-10 U·ml^{-1}). Cellular injury was assessed spectrophotometrically by measurement of the mitochondrial-dependent conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan. Data are expressed as mean ± s.e.mean from triplicate measurements from 4 separate isolations and were analysed using one-way ANOVA followed by a Dunnett's post significance test. A p value of less than 0.05 was considered to indicate significance.

Treatment		tempol	deferoxamine	catalase
[H_2O_2] (mM)	[Drug] (mM or U·ml^{-1})	% control	% control	% control
0 (Control)	0	100	100	100
1 (H_2O_2 only)	0	22 ± 1	23 ± 1	22 ± 1
1	0.03	30 ± 2	32 ± 4	41 ± 5
1	0.1	30 ± 2	36 ± 4	42 ± 6
1	0.3	31 ± 3	36 ± 4	43 ± 6
1	1	35 ± 4*	55 ± 9*	44 ± 4*
1	3	39 ± 5*	41 ± 5*	68 ± 7*
1	10	33 ± 3	38 ± 6	71 ± 6*

Table 1: Effect of increasing concentrations of tempol, deferoxamine and catalase on H_2O_2-mediated inhibition of mitochondrial respiration (mean ± s.e.mean, *p<0.05 vs. 1 mM H_2O_2 only, n=4).

Incubation of rat PT cells with 1 mM H_2O_2 for 4 hours inhibited mitochondrial respiration (Table 1). Tempol (1, 3 mM), deferoxamine (1, 3 mM) and catalase (1, 3 and 10 mM) reduced the H_2O_2-mediated decrease in mitochondrial respiration (Table 1). Higher concentrations of tempol and deferoxamine were toxic to rat PT cells.

These results demonstrate that H_2O_2 produces significant cellular injury in primary cultures of rat PT cells and that tempol can reduce H_2O_2 (and therefore ROS)-mediated PT cell injury.

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102P THE POLY (ADP-RIBOSE) SYNTHETASE INHIBITOR 1,5-DIHYDROXYISOQUINOLINE REDUCES RENAL ISCHAEMIA-REPERFUSION INJURY IN THE RAT *IN VIVO*

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Generation of reactive oxygen species (ROS) has been implicated in the pathogenesis of renal ischaemia-reperfusion injury (Paller *et al.*, 1984). ROS produce DNA strand breaks which leads to the activation of the DNA-repair enzyme poly(ADP-ribose) synthetase (PARS) (Schraufstatter *et al.*, 1986). During episodes of oxidant stress, excessive PARS activation can result in depletion of its substrate NAD and subsequently of ATP, leading to cellular dysfunction and eventual cell death (Schraufstatter *et al.*, 1986). We have recently reported that the PARS inhibitor 3-aminobenzamide reduces renal reperfusion-injury in anaesthetised rats (Chatterjee *et al.*, 1999). The aim of this study was to investigate the effect of 1,5-dihydroxyisoquinoline (ISO), a more potent PARS inhibitor, in a model of renal ischaemia-reperfusion injury in the anaesthetised rat.

Twenty-four male Wistar rats (240-300 g) were anaesthetised with sodium thiopentone (120 mg·kg^{-1} i.p.) and tracheotomised. The carotid artery was cannulated to monitor mean arterial blood pressure and the jugular vein was cannulated for administration of drugs. Following a midline laparotomy, isolation of the renal pedicles and recovery for 30 min, the rats were divided into four groups. Group 1 (sham rats, n=6) were maintained under anaesthesia for the duration of the experiment (45 min + 6 h). Group 2 (control rats, n=6) were subjected to bilateral renal pedicle clamping (45 min) followed by reperfusion (6 h). Group 3 (ISO-treated rats, n=6) were manipulated as described for Group 2, but were given a bolus injection of ISO [1 mg·kg^{-1} in 10 % (v/v) DMSO in saline, i.v.] 1 min prior to beginning reperfusion followed by an infusion of ISO [0.5 mg·kg^{-1}·h^{-1} 10 % (v/v) DMSO in saline, i.v.]. Group 4 (vehicle treated rats, n=6) were administered 10 % (v/v) DMSO only in saline, i.v. as described for Group 3. Renal function and reperfusion injury was assessed by measurement of the plasma levels of creatinine, aspartate aminotransferase (AST) and γ-glutamyl transferase (γ-GT).

	Creatinine (μmol/l)	AST (iu/l)	γ-GT (iu/l)
Group 1 (sham, n=6)	36 ± 2	166 ± 19	0.7 ± 0.4
Group 2 (control, n=6)	213 ± 14*	1370 ± 123*	4.9 ± 0.8*
Group 3 (ISO, n=6)	115 ± 9* +	510 ± 35* +	1.0 ± 0.4+
Group 4 (vehicle, n=6)	186 ± 14*	1532 ± 262*	2.3 ± 0.7*

Table 1: Effect of ISO on biochemical markers of ischaemia-reperfusion injury (*p < 0.05 vs. sham, +p < 0.05 vs. control, n=6). Data are expressed as mean ± s.e.mean and were analysed using one-way ANOVA followed by the Bonferroni's post significance test. A p value of less than 0.05 was considered to be significant.

Bilateral renal clamping (45 min) followed by reperfusion (6 h) of rat kidneys produced significant increases in plasma levels of creatinine, AST and γ-GT (Table 1). Infusion of ISO prior to and during reperfusion significantly reduced plasma concentrations of creatinine, AST and γ-GT (Table 1). Thus, the PARS inhibitor ISO reduced the degree of renal dysfunction (creatinine) and reperfusion injury (AST, γ-GT) caused by ischaemia-reperfusion of the rat kidney *in vivo*. This suggests that activation of PARS may contribute to renal reperfusion-injury.

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103P REPERFUSION THERAPY AND PLASMA CATECHOLAMINE LEVELS AFTER ACUTE MYOCARDIAL INFARCTION

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Potentially harmful activation of the adrenergic nervous system occurs in the early hours of acute myocardial infarction (AMI). The rise in plasma catecholamines is temporally related to the decrease in arterial pressure and the magnitude is directly related to the size of the infarct that results from coronary occlusion; these findings suggest that sympathoadrenal activation compensates for decreased cardiac output and arterial pressure (Cryer, 1980). However, stimulation of sympathetic nerves lowers the threshold for ventricular fibrillation and increases coronary vascular resistance in patients (pts) with ischemic heart disease (Foy et al., 1995; Teerlink, 1996).

The use of thrombolytic therapy in pts with AMI has resulted in better short-term and medium-term survival and better recovery of left ventricular function; an alternative, equally effective aproach to the treatment of AMI is immediate coronary angioplasty (PTCA).

In order to test the hypothesis that reperfusion (RP) therapy (thrombolysis and primary PTCA) can improve the neuroendocrine profile after AMI we measured plasma concentrations of dopamine (DA), adrenaline (AD) and noradrenaline (NA), by liquid chromatography and electochemical detection (HPLC-ECD), on admission and at 6h, 24h and 48h, in 27 pts admitted in the first 24 hours (h) after onset of symptoms. Nineteen pts were treated with RP (12 with thrombolysis and 7 with primary PTCA); 8 pts were conventionally managed.

Pts previously treated with diuretics, beta blockers or ACE inhibitors were excluded as well as pts on Killip class > 1. The baseline demographic characteristics were not different. Plasma levels were compared considering two groups: RP *versus* non RP group (control).

TABLE 1 - Plasma levels (Mean ±S.E.M.) of NA, AD and DA (pg/ml)

	NA		AD		DA	
h	control	reperfusion	control	reperfusion	control	reperfusion
6	516±80	474±62	139±58	92±33	73±25	177±119
12	524±62	385±55	117±38	59±24	166±90	50±28
24	475±91	391±37	117±54	10±6*	45±14	61±27
48	574±147	310±38*	79±40	5±4*	92±35	27±13*

* $p < 0.05$ related to control (ANOVA and Student's T-test for unpaired data)

Compared with conventional therapy, RP induced a steep decline in plasma catecholamines, with statistically significant different levels of NA and DA at 48h, and AD at 24h and 48h (Table 1).

The findings of the present series of experiments suggest that sympathetic nervous system response to acute myocardial infarction is significantly modified by reperfusion therapy. Further studies are needed to confirm these data which can provide adjuvant support to the open artery concept (Fears, 1990).

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104P EVIDENCE OF INCREASED ENDOTHELIUM-MEDIATED VASODILATATION FOLLOWING CHRONIC AORTIC CONSTRICTION

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Left ventricular (LV) out-flow obstruction increases afterload and masks the impact of LV ejection on the systemic circulation. We hypothesise that this may alter the properties of vessels distal to the stenosis and this is supported by evidence of forearm vasodilatation in response to leg exercise in patients with aortic stenosis, in contrast to the vasoconstriction observed in normal subjects (Mark *et al* 1973). To test this we examined vasomotor responses in isolated thoracic aortae following chronic constriction of the ascending aorta.

Male Dunkin-Hartley guinea-pigs (600-800g) underwent constriction of the ascending aorta (n=10) or sham (n=10) operation using a modification of the method of Ling and deBold (1976). 150 days after surgery animals were sacrificed, the thoracic aorta removed, cut into 3 mm rings and mounted in 10ml tissue baths. After equilibration at a 3g resting tension, responses to 60mM K^+ solution and cumulative doses of: phenylephrine (10^{-7} - 10^{-4} M), angiotensin II (10^{-7} -3x10^{-6} M), isoprenaline (10^{-9} - 10^{-4} M), acetylcholine (10^{-9} - 10^{-4} M), sodium nitroprusside (10^{-9} - 10^{-4} M), forskolin (10^{-9} - 10^{-4} M) and cromakalim (10^{-9} - 10^{-4} M) were determined. Constriction was expressed as a percentage of maximum response to a 60mM K^+ solution and dilatation as percentage relaxation from submaximal preconstriction with phenylephrine (3x10^{-5} M). Dose response curves were analysed by fitting sigmoidal curves using non-linear regression analysis; EC_{50} and maximum values were obtained for each experiment and analysed using unpaired t-tests with Welch's correction for unequal variance.

Aortic rings from both the aortic constriction and sham control groups showed similar constrictor responses to phenylephrine and angiotensin II, and dilator responses to sodium nitroprusside, forskolin and cromokalim were also unchanged. However maximal vasodilator responses to acetylcholine and isoprenaline were significantly increased (144% and 48% respectively, n=10, $p<0.001$) following aortic constriction, while EC_{50} values were unchanged (acetylcholine 3.5±0.3 vs. 2.7±0.2 x10^{-7} M, isoprenaline 9.2±1 vs. 7.4±0.8 x10^{-7} M). The increased vasodilator response to both acetylcholine and isoprenaline was partially attenuated by the nitric oxide synthase inhibitor N omega-nitro-L-arginine methyl ester (L-NAME, 3x10^{-5} M, $p<0.001$).

Chronic (149±6 days) constriction of the ascending aorta resulted in increased vasodilator responses to acetylcholine and isoprenaline in the distal aorta. As the responses to sodium nitroprusside, forskolin and cromokalin were unchanged this is unlikely to reflect a general increase in vasodilator efficacy or concern an increase in cyclic AMP or cyclic GMP mediated vasodilatation. The increase in response is consistent with increased endothelium mediated vasodilatation as isoprenaline mediated aortic relaxation has been shown to be partly via NO release from the endothelium (Graves & Poston, 1993). This is further supported by the attenuation of both the acetylcholine and isoprenaline response by the nitric oxide synthase inhibitor L-NAME. Thus we have evidence of increased endothelium mediated vasodilatation in the aorta distal to a chronic constriction. We hypothesise that this may contribute to hypotension and syncope in patients with left ventricular outflow obstruction.

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105P ENDOTHELIUM-DEPENDENT AND -INDEPENDENT CORONARY VASODILATOR RESPONSES ARE REDUCED IN LEFT VENTRICULAR HYPTERTROPHY

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Reduced coronary reserve is a hallmark of left ventricular hypertrophy (LVH) and limits the heart's ability to meet increased demand, making it more vulnerable to ischaemia. Available evidence suggests that while coronary rarefaction, vascular remodelling and increased myocardial forces are important, they do not fully explain the reduced reserve. To test the hypothesis that impaired vasodilator responsiveness may contribute to the reduced coronary reserve in hypertrophy we studied vascular responses in isolated perfused control and hypertrophied hearts.

LVH was induced in male Dunkin-Hartley guinea-pigs (600-800g) by banding the ascending aorta using a modification of the method of Ling and deBold (1976). Animals were sacrificed 55±3 days after banding or sham control operation and Langendorff perfused hearts taken. Vascular reactivity was assessed by constructing dose-response curves to acetylcholine , bradykinin , sodium nitroprusside and adenosine. Reactive hyperaemic vasodilatation (RH) was also assessed after periods of global ischaemia (5-120s). Curves were fitted and maximum and ED_{50} values were compared with unpaired t-tests.

After 55±3 days aortic banding there was significant LVH as evidenced by an increase in heart weight to body weight ratio (42%, $p<0.001$) and LV myocyte size (19%, $p<0.01$). LV arteriolar wall thickness was also significantly increased (51%, $p<0.01$). These changes were associated with a characteristic decrease in coronary reserve, assessed by maximal RH vasodilatation (182 ±12% vs. 105 ±8%, $p<0.001$). All the vasodilators produced a dose dependent increase in flow but showed a significant ($p<0.01$) attenuation of maximum flow (≈70%) in LVH (Table 1). The duration of RH response and the ischaemic flow debt repayment were significantly ($p<0.001$) reduced in hypertrophied hearts. However, while the peak flow response to RH was attenuated (50%, $p<0.001$) the flows achieved in hypertrophied hearts were well above those obtained with the exogenous vasodilators (Table 1).

Drug	Sham control		LVH	
	ED_{50}	Maximum	ED_{50}	Maximum
Acetylcholine (n=25)	157 ±36 pmol	4.5 ±0.3	107 ±17 pmol	1.4 ±0.1
Bradykinin (n=14)	236 ±76 pmol	3.7 ±0.1	264 ±19 pmol	0.9 ±.04
Sodium nitroprusside (n=14)	3.9 ±2.9 nmol	3.6 ±0.1	1.2 ±12.6 nmol	0.5 ±.01
Adenosine (n=25)	3.4 ±0.8 nmol	5.0 ±0.2	3.0 ±0.9 nmol	1.6 ±0.1
Reactive Hyperaemia (n=30)	-	7.8 ±0.2	-	3.9 ±0.2

Table 1: showing ED_{50} values and maximum flow (ml/min/g) for the computer fitted dose response curves.

The reduced coronary reserve in LVH would be expected to limit maximal coronary flow and thus limit responses to a variety of stimuli. However the maximal hyperaemic response was greater than that of exogenous vasodilators, therefore the depressed responses to these agents is not simply due to exhaustion of vasodilator reserve. Furthermore, while reduced coronary reserve in LVH could explain the attenuated peak hyperaemic flow, the shortening of its duration at the expense of flow debt repayment suggests a disturbance in factors that mediate the response. Coronary vasodilator responses to endogenous and exogenous stimuli are reduced in LVH and this is true of both endothelium dependent and independent stimuli. This is likely to contribute to the impaired coronary reserve in LVH and has implications for clinical tests of myocardial perfusion based on vasodilator stress.

Ling, E.T. & deBold, A.J. (1976) Lab. Animals **10**, 285-289.

106P ROLE OF ENDOTHELIUM ON ABNORMAL VASCULAR RESPONSE TO ADENOSINE IN PORTAL HYPERTENSION

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Several studies have shown that there is a decreased response to the vasoconstrictors and an increased response to the endothelium-dependent vasodilators, both in cirrhotic and portal hypertensive animals. These responses are reversed in the first group when the endothelium of the vessel is removed, and in the latter, when the production of nitric oxide (NO) is inhibited (Clària *et al.*, 1994; Atucha *et al.*, 1996; Gadano *et al.*, 1997). In a previous study we have showed that the vascular response to adenosine (ADO) is decreased in rabbits with portal hypertension (PH), and that in normal rabbits (nPH) and rabbits with PH, this response is diminished by the overproduction of NO (Villa de Brito *et al.*, 1998). The aim of the present study is to assess the role of endothelium in the hyporesponsiveness to ADO in rabbits with PH.

Vascular responses to ADO increasing concentrations (1 ρM to 1 mM) were measured in isolated cranial mesenteric, renal and femoral arterial rings, with (n=7) and without endothelium (n=6) mounted for isometric recording in oxygenated Krebs-Henseleit solution baths at 38° C, after pre-contraction with norepinephrine (10 μM). The arterial rings were obtained from male New Zealand rabbits (3.0 Kg ± 0.250) in normal conditions (n=13) and with PH (n=13) (induced by partial portal vein ligation) These procedures were carried out under anaesthesia with medetomidine 0.5 mg/kg and ketamine 25 mg/kg. Successful removal of the endothelium was confirmed by the absence of response to acetylcholine (1μM to 10mM). In another group of vessels from normal rabbits (n=8) the responses to increasing doses of ADO 0.01 μM to 10 mM were evaluated before and 20 minutes after incubation with N^{ω}-Nitro-L-Arginine-methyl-ester (L-NAME) (100 μM). Dose-response curves parameters (E_{max} and the ED_{50}) were calculated by a non-linear regression analysis (Enzfitter software). Statistical analysis of the results were performed with *f*-test and paired and unpaired Student's *t* test when appropriate. Results are given as mean ± S.E.M. and considered significant at $p< 0.05$.

The maximum effect to ADO on the arterial vascular rings without endothelium from nPH decreased significantly when compared with the intact arterial rings. The E_{max} from nPH vessels with (n=7) and without endothelium (n=6) were respectively: cranial mesenteric artery: 100.7 ± 2.6 %; renal artery: 112.0 ± 1.1 %; femoral artery: 86.0 ± 1.8 %, and cranial mesenteric artery: 32.9 ± 1.0 %; renal artery: 21.9 ± 1.1 %; femoral artery: 51.1 ± 4.5 %. A significant decreased of the maximum effect of ADO was also observed on the vessels without endothelium from PH animals. The Emax from PH vessels with and without endothelium were respectively: cranial mesenteric artery: 59.0 ± 3.5 %; renal artery: 67.9 ± 3.2 %; femoral artery: 81.5 ± 4.9 %; and cranial mesenteric artery: 13.6 ± 1.1 %; renal artery: 13.5 ± 3.4 %; femoral artery: 24.2 ± 3.8 %. The responses of the denuded arteries of nPH and PH rabbits were significantly different, although the responses of the different vascular territories without endothelium in nPH rabbits decreased significantly when compared with PH intact vessels. Pre-incubation with L-NAME did not modify the response to ADO in the nPH rabbits (E_{max} cranial mesenteric artery: 108.5 ± 1.2 % versus 109.0 ± 1.0 %; E_{max} renal artery: 115.0 ± 2.0 % versus 121.3 ± 1.1 %; E_{max} femoral artery: 99.0 ± 0.8 versus 103.0 ± 1.0 %) ($p>0.05$).

This study demonstrates that the endothelium plays an important role in the response to ADO in the arterial vessels, but this response does not depend on NO production. The results also show that the hyporesponsiveness to ADO in PH vessels is not solely due to endothelium factors. In fact, the significant difference between the denuded vessels of normal and PH animals strongly suggests that the vascular smooth muscle response to ADO is modified in PH.

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107P A ROLE FOR K^+ IN RELAXATION RESPONSES TO ACETYLCHOLINE IN RABBIT ISOLATED ARCUATE ARTERY

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In small arteries, raising extracellular K^+ concentration ($[K^+]_o$) from 5-15 mM causes smooth muscle hyperpolarization and dilation and, if released from endothelial cells, can act as an endothelium-derived hyperpolarizing factor (EDHF) (Edwards *et al.*, 1998). Rabbit arcuate arteries exhibit vasodilatory responses to modest increases in $[K^+]_o$ (Prior *et al.*, 1998). The mechanism of dilation to K^+ appears to be via the stimulation of Na^+/K^+ ATPase, as indicated by blockade with ouabain. The aim of this study was to determine whether the endothelium-dependent relaxation to EDHF released with acetylcholine (ACh) could be explained by K^+ acting through smooth muscle Na^+/K^+ ATPase.

Arcuate artery segments (D_{100}=373 ± 39 μm, n=12) from male NZ white rabbits were mounted in a Mulvany-Halpern myograph containing modified Krebs-Ringer bicarbonate solution (37°C, aerated with a 95% O_2:5% CO_2 gas mixture). Indomethacin (2.8 μM) and N^ω-nitro-L-arginine methyl ester (100 μM) were added to block prostacyclin and nitric oxide synthesis. After normalization and equilibration, all arteries used maximally relaxed to ACh (1 μM, in pre-contracted vessels). In these studies, Ba^{2+} (30 μM) or ouabain (10 μM) were added to the organ bath at least 20 min before addition of PE, and had no effect on basal tension. In some arteries, a hair was used to destroy the endothelium, assessed as a complete loss of relaxation to ACh (1-3 μM). Data are means ± SEM (expressed as % of maximum dilation).

Both ACh (3 nM–3 μM) and K^+ (plus 5-10 mM) caused concentration-dependent dilation of phenylephrine contracted arteries. Ba^{2+} had no effect on the maximum dilation to ACh or K^+. Ouabain reduced the maximum dilation to ACh by 15.9 ± 9.9%, and abolished K^+-mediated dilation. The combination of Ba^{2+} plus ouabain further reduced the maximum dilation to ACh (Fig. 1). Relaxation to K^+ was not altered by removal of the endothelium.

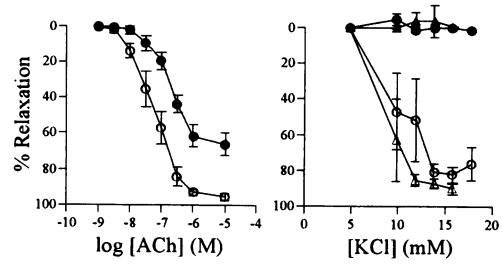

Fig. 1. ACh and K^+ concentration response curves in the presence (filled symbols) and absence (open symbols) of Ba^{2+} plus ouabain (n=4-5). K^+ concentration response curves were also obtained in endothelium-denuded arteries (n=4, triangles).

The profile of inhibition of ACh relaxation by Ba^{2+} and ouabain is consistent with a role for K^+ as EDHF (Edwards *et al.*, 1998). Further, the use of the blocker combination has unmasked a role for the inward rectifier K^+ channel, which is present in this artery (Prior *et al.*, 1998). The blockade-insensitive relaxation may reflect the spread of hyperpolarization from the endothelium to the smooth muscle (Dora *et al.*, 1999).

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108P THE MODULATION OF VASCULAR TONE IN RAT PIAL ARTERIOLES *IN VITRO* BY NITRIC OXIDE AND BK_{CA}-ACTIVATION

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Numerous studies have demonstrated the importance of nitric oxide (NO) in the control of blood vessel diameter. This occurs via its ability to activate guanylyl cyclase in smooth muscle, increase cyclic GMP accumulation and so activate cyclic GMP-dependent protein kinase (G kinase). The targets for phosphorylation by G kinase that are known to be important for its vasodilator actions include plasmalemmal Ca^{2+} channels and Ca^{2+} ATPases. In spite of the fact that recent work has shown that the large-conductance, Ca^{2+}-activated K^+ channel (BK_{Ca}) is also a target for G kinase (Archer et al, 1994; Holland et al, 1996), the physiological role of BK_{Ca}-activation in NO-induced vasodilatation is controversial. This is because BK_{Ca} blockade has little effect on NO potency in many blood vessels. However, since these data were obtained from large diameter blood vessels and, the importance of hyperpolarisation is thought to be greater in small vessels (Félétou & Vanhoutte, 1996), we decided to investigate the role, if any, of BK_{Ca} activation in NO-induced relaxation of resistance arterioles (30-80μm) from rat pia mater.

Complete sheets of pia mater were dissected from the brain of adult WS male rats (200-300g) and pinned out in a chamber mounted on an inverted microscope. The chamber was continuously perfused with hepes buffered physiological salt solution (30°C, 1 $mlmin^{-1}$). Test solutions were applied at the same rate and temperature by switching to different perfusion reservoirs. Arteriolar diameter was continuously monitored and recorded using a computerised video tracking system.

75mM K^+ contracted the arterioles by 34.9 ± 0.7%, n=50 (K_{75}). This value was taken as 100% contraction. The NO synthase inhibitor N^ω-Nitro-L-Arginine Methyl Ester (L-NAME; 200μM) contracted the vessels to 77.5 ± 3.2%, of K_{75} (n=16) and induced phasic activity (vasomotion) in most preparations. The further addition of 10μM indomethacin contracted the vessel by a further 9 ± 0.5%, n=7, and reduced vasomotion. This gave a total contraction, in the presence of both inhibitors, of 84.9 ± 2.9% of K_{75}, n=13.

The NO donor compound SIN-1 (10μM), in the presence of superoxide dismutase (50U/ml), caused an 85.5 ± 3.0%, n=4 relaxation of the L-NAME contraction. SIN-1 (10μM) failed to produce any relaxation when applied to resting arterioles (n=16). The possible contribution of BK_{Ca}-activation to the effects of NO was investigated using tetraethylammonium (TEA). TEA (1mM) applied to resting arterioles produced a constriction which was inversely proportional to arteriolar diameter (maximum 50% in 30μm arterioles; minimum 0% in 80μm vessels; $r = -0.89$, $p<0.05$, n=16). TEA failed to produce any contraction after treatment with L-NAME.

This study provides evidence that NO plays a major role in modulating arteriolar diameter in the rat cerebral circulation. There appears to be tonic release of NO and a prostanoid, probably prostacyclin, which dilates arterioles and inhibits vasomotion. Furthermore, these data suggest that, in the cerebral circulation, the importance of BK_{Ca}-activation by NO as a component of NO-induced vasorelaxation increases as vessel diameter decreases.

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109P QUALITATIVE DIFFERENCES IN ANGIOTENSIN II-MEDIATED MODULATION OF TOLERANCE TO DETA NONOATE AND SODIUM NITROPRUSSIDE IN RAT AORTIC SMOOTH MUSCLE CELLS

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There is substantial evidence for involvement of angiotensin II (AII) in the development of tolerance to nitrates; for example such tolerance can be prevented by treatment with angiotensin-converting enzyme inhibitors or AII type I receptor antagonists; it has also been proposed that AII may contribute to tolerance via stimulation of NADH/NADPH oxidases, thus increasing cellular superoxide (O_2^-), a potent scavenger of NO (Münzel & Harrison, 1997). This study investigated modulation of tolerance to NO donors by AII in an *in vitro* cell culture model.

Rat aortic smooth muscle cells (passage numbers 2-8) cultured according to Redmond *et al.* (1996) were used. Tolerance was assessed as a decrease in NO donor-stimulated cGMP production (measured by radioimmunoassay) following a 12 h exposure to the same drug. Two NO donors were compared, DETA NONOate (DETA) and sodium nitroprusside (SNP). In some experiments AII was present during the 12 h incubation period alone, or in combination with superoxide dismutase (SOD) or the NADH/NADPH oxidase inhibitor diphenylene iodonium (DPI). Data are presented as fold increases over basal cGMP production (mean ± s.e.m. of 6-8 (DETA) or 4-6 (SNP) observations). The significance ($p<0.05$) of differences between treatments was assessed by ANOVAand a Bonferroni post test.

A reduction in cGMP responsiveness was observed in cells pre-exposed for 12 h to 100 µM DETA or 20 µM SNP and then rechallanged with donor (10 µM in each case). The DETA-mediated reduction in responsiveness was further enhanced in the presence of 0.1 µM AII; this enhancement was unaffected by the presence of 1000 U/ml SOD. In contrast, significant protection against the SNP-mediated reduction was evident in cells pre-exposed in the presence of AII alone, but was less in the presence of AII + SOD. Both the enhancing or protective effects of AII in combination with DETA or SNP respectively, were abolished in the presence of 100 µM DPI (results summarised in Table 1).

The enhancing effect of AII in the development of tolerance to DETA and the abolition of this effect by DPI, supports a role for delayed and prolonged AII-mediated O_2^- production during tolerance and therefore increased scavenging of NO; the inability of SOD to counter this may reflect its lack of access to the intracellular site of O_2^- generation. The protective effect of AII versus SNP may have been due to (i) redox cycling of SNP by cellular oxido-reductases, with rapid concomitant formation of O_2^- (Ramakrishna, & Cederbaum, 1996) + NO and therefore (ii) continuous formation of $ONOO^-$ which is modified and inactivated by AII-derived superoxide.
Supported by the Health Research Board of Ireland.

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Treatment	DETA NOnoate	SNP
Basal	1	1
Control	47.9 ± 2.5	13.2 ± 0.9
Tolerant	27.1 ± 4.7**	7.0 ± 1.0**
Tolerant + AII (0.1 µM)	19.7 ± 3.3**	10.3 ± 1.5*
Tolerant + AII + DPI	35.9 ± 6.9**	7.6 ± 1.7*
Tolerant + DPI (100 µM)	33.2 ± 2.5	6.7 ± 0.8

Table 1. The effect of AII on the development of tolerance to DETA and SNP alone and in combination with DPI. *$p<0.05$, **$p<0.001$ w.r.t. preceding measurement in each case.

110P EFFECTS OF ANGIOTENSIN II IN THE MESENTERIC ARTERY OF DPSPX (1,3-DIPROPYL-8-SULFOPHENYLXANTHINE)-HYPERTENSIVE RATS

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The prolonged infusion of DPSPX, a non-selective antagonist of adenosine receptors, causes a long-lasting hypertensive state (Albino-Teixeira *et al.*, 1991). This effect is accompanied by an increase in plasma renin activity (Albino-Teixeira & Osswald, 1994) which suggests an involvement of the renin-angiotensin system in this experimental model of hypertension. The aim of this study was to evaluate the pre- and postjunctional effects of angiotensin II in DPSPX-hypertensive rats.

Male Wistar-Kyoto rats of 250-300 g were used in this study. DPSPX (90 $\mu g.kg^{-1}.h^{-1}$) was given i.p. through an Alzet 2ML1 minipump. A group of rats was treated for 3 days and then killed. Another group was treated for 7 days and killed on day 15. Controls received an infusion of saline i.p.. For the prejunctional study, isolated segments of the rat mesenteric artery, preincubated with ^{3}H-noradrenaline (0.2 µM), were mounted in perifusion chambers, washed-out for 100 min and then submitted to two periods of electrical stimulation (2 Hz, 2 ms, 600 pulses) at 110 and 160 min. Angiotensin II was added to the perifusion fluid between these two stimulations. For the postjunctional study, two steel wires were introduced in the lumen of the arteries and used to stretch them to a resting tension of about 200 mg. One of the wires was fixed at the bottom of the organ bath while the other was connected to an isometric transducer. After a stabilisation period of 90 min, non-cumulative concentration-response curves were obtained for increasing doses of angiotensin II. Statistical analysis was done by unpaired Student's t-test.

In rats treated with DPSPX for 3 days, systolic blood pressure increased from 107±3 mmHg in controls to 135±5 mmHg ($n=12$; $p<0.0001$). Angiotensin II caused a concentration-dependent increase in tritium overflow evoked by electrical stimulation and was more potent on arterial segments from DPSPX-treated rats than on those from controls ($pEC_{50\%}=9.23\pm0.24$ and 8.49 ± 0.09, $n=4$, respectively; $p<0.05$). There were no differences in postjunctional angiotensin II responses between controls ($pD_2=8.40\pm0.05$, $n=35$) and DPSPX-hypertensive rats ($pD_2=8.33\pm0.08$, $n=21$). In rats treated for 7 days and killed on day 15, systolic blood pressure increased from 105±4 mmHg in controls to 142±4 mmHg ($n=12$; $p<0.0001$). In this group of rats, there were no differences between controls and DPSPX-treated rats either for the pre- ($pEC_{50\%}=8.55\pm0.20$, $n=4$) or for the postjunctional ($pD_2=8.32\pm0.06$, $n=21$) effect of angiotensin II.

These results show that the continuous infusion of DPSPX increases the prejunctional angiotensin II-mediated facilitation of noradrenaline release in the early phase of the hypertensive state but not in the chronic phase. Furthermore, there are no changes in postjunctional angiotensin II responses.

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Supported by JNICT (PECS/P/SAU/95 and Praxis/2/2.1/sau/1293/95)

111P COMPARISON OF THE EFFECTS OF SOTALOL ON THE CARDIAC ACTION POTENTIAL RECORDED IN OVINE AND CANINE ISOLATED PURKINJE FIBRES

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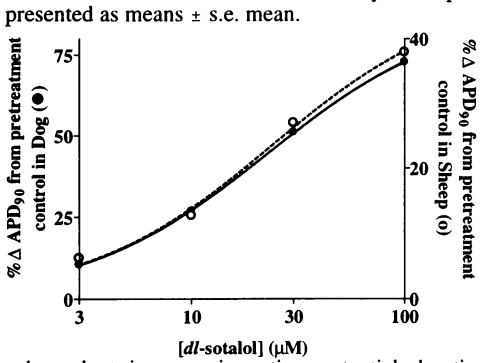
Sotalol is a clinically available beta-adrenergic blocking agent, which also has class III antiarrhythmic properties (Singh & Vaughan Williams, 1970). In the present experiments we have studied the effects of *dl*-sotalol in sheep and dog Purkinje fibres.

Intracellular recordings (at ~36°C) were made from electrically paced (1 Hz) left ventricular Purkinje fibres isolated from hearts of either adult Suffolk sheep or Beagle dogs (obtained at post-mortem). The effects of *dl*-sotalol (3, 10, 30 and 100 μM; 30 min at each concentration) were assessed on the following parameters: action potential duration at 90% repolarization (APD_{90}), maximum rate of depolarization (MRD), upstroke amplitude (UA) and diastolic membrane potential (DMP). *dl*-Sotalol was formulated as a 10 mM stock solution in distilled water, serially diluted to 1 mM in distilled water. These stock solutions were diluted directly in the perfusant. Data are presented as means ± s.e. mean.



A dose dependent increase in action potential duration was observed with increasing concentrations of *dl*-sotalol with the greatest prolongation of action potential duration seen at a dose of 100 μM *dl*-sotalol. The increase in action potential duration was greater in canine Purkinje fibres, with a maximum prolongation of 73 ± 16% compared with 38 ± 31% in ovine Purkinje fibres. The concentration of *dl*-sotalol required to produce a 15% prolongation of the action potential was 3 μM in canine Purkinje fibres and 11 μM in ovine Purkinje fibres. In the ovine and canine groups (n = 5 for both) the pre-treatment values and values after exposure to 30 μM *dl*-sotalol hydrochloride for APD_{90}, MRD, UA and DMP are shown in the table (mean difference ± SEM).

These experiments indicate that *dl*-sotalol, at all concentrations, prolonged action potential duration in canine and ovine Purkinje fibres. It is therefore probable that an inhibitory action on the rapid component of the delayed rectifier K^+-channel underlies the effect of *dl*-sotalol on action potential duration observed in this study (Carmeliet, 1985; Lynch *et al.*, 1995).

	Ovine		Canine	
	Pretreatment	**30 μM Sotalol**	**Pretreament**	**30 μM Sotalol**
APD_{90}	294 ± 33 ms	+27 ± 17.2%*	+273 ± 35 ms	+51 ± 10.4%**
MRD	515 ± 157 V/s	-2 ± 5.7%	378 ± 57 V/s	-0.5 ± 18.4%
UA	114 ± 6 nV	0 ± 0.4 mV	112 ± 3 mV	2.6 ± 7.2 mV
DMP	-77 ± 6 mV	-2 ± 0.5 mV	-85 ± 2 mV	-0.6 ± 2.5 mV

*P < 0.05; ** P < 0.005 (paired 2-tailed t-test; n = 5 per group)

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112P RESPONSE OF RETINAL ARTERIOLES *IN VIVO* TO SENSORY NEUROPEPTIDES IN THE RABBIT

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This work is aimed to study "in vivo" the vascular response of retinal vessels of New Zealand White (NZW) normal and alloxan-induced diabetic rabbits to Substance P (SP), Neurokinin-A (NKA), Neurokinin-B (NKB), Senktide, Capsaicin (CAPS) and Calcitonin Gene-Related Peptide (CGRP), before and after selective antagonists administration, because the effects and receptors for these neuropeptides were not yet characterized in this experimental model.

Direct measurement of retinal arteriole diameters was performed using digital angiography after intravitreous perivascular microinjection of drugs into the eye ball. Quantification of the relaxing effect is expressed in percentage of basal vascular diameter. Diabetic rabbits were obtained after 3 months of hyperglycemia induced by alloxan. The number of experiments for each dose was 6 or more. The results expressed are means ± s.e.m. and the comparison between groups was made by using the Student's t test.

Microinjection of Substance P (NK1 receptor agonist) induced a dose-dependent arteriolar dilating effect. The Emax was obtained with 1 nmol [maximal vascular diameter enlargement (mean±s.e.m.): 21.3±0.3%]. After perivascular preinjection of 0.1 nmol L-668,169 (Cyclo(Gln-D-Trp(NMe)Phe(R)Gly-[ANC-2] Leu-Met)2), a NK1 receptor antagonist, the dilating response decreased, shifting the SP dose-response curve to the right. The same results were obtained with NKA (NK2 receptor agonist) which induced the most potent effect of all neuropeptides used (Emax: 53.3±2.5%). After the NK2 antagonist L-659,877 (Cyclo(Gln-Trp-Phe-Gly-Leu-Met) (0.1 nmol) the vascular diameter enlargement was 41.3±0.7%. As for CGRP is concerned, doses up to 1 nmol induced a marked vasodilation (Emax: 41.1±0.4%) which decreased with the antagonist $CGRP_{8-37}$ (33.6±0.7%). NK3 agonists (Senktide and NKB) showed a less proeminent vasodilating effect (Emax: 5.1±1.2% and 8.0±0.9%, respectively). Capsaicin showed a marked dose-dependent vasodilating effect (Emax: 43.2±2.9%) antagonized by tachykinin receptor antagonists (L-668,169 and L-659,877) and $CGRP_{8-37}$. In the diabetic rabbit, dilating responses to all tested neuropeptides were abolished.

These results suggest for the first time, the presence of capsaicin-sensitive sensory nerves which contain CGRP, SP and NKA and the existence of neuropeptide receptors (NK1, NK2 and CGRP receptors) on the retina vascular wall of the rabbit. Furthermore, they suggest the existence of postsynaptic dysfunction on diabetic retinal vessels.

113P THE INVOLVEMENT OF KININ B1 RECEPTORS IN THE HYPOTENSIVE RESPONSE TO ENDOTOXIN IN RATS

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Endotoxin causes a delayed hypotension in part mediated by NO, however there is a significant NO-independent component. Hypotensive kinin B_1 receptors are induced 6-24 h after endotoxin but the role of these receptors in endotoxin-induced hypotension is not known. We investigated whether hypotensive doses of endotoxin caused B_1 receptor induction *in vivo* and whether endogenous activation of these receptors is involved in endotoxin-induced hypotension.

Male Wistar rats (310-370g) either untreated or previously treated with lipopolysaccharide (LPS, *E. Coli* 055:B5, 2.5-10 mg kg^{-1}, i.p.) were anaesthetized with thiopentone sodium (120 mg kg^{-1}, i.p.), the trachea cannulated and rectal temperature maintained at 37°C. The right common carotid artery was cannulated for mean arterial blood pressure (MAP) recording and for administration of noradrenaline (3-10 μg min^{-1}). The left jugular vein was cannulated for the administration of drugs. After base line hemodynamic parameters were recorded, animals were given either i.v. saline, des-arg[9] bradykinin (DABK), des-arg[9]leu[8] bradykinin (DALBK) or des-arg[10]HOE140.

LPS (6h) caused significant ($P < 0.05$) hypotension (MAP=125.9 ± 2.5 mmHg, (control, n=8); 82.7±5.4 mmHg (LPS 2.5 mg kg^{-1}, n=10); 66.1 ± 6.3 mmHg (LPS 10 mg kg^{-1}, n=7). Bolus doses of DABK (1-100 nmol kg^{-1}) had no effect in control rats (n=4) but in LPS (2.5 mg kg^{-1}, 6 h)-treated rats with noradrenaline-elevated blood pressure (MAP=103.9±3.3 mmHg, n=10) DABK produced a dose-dependent hypotensive response (EC_{50}=29.7 (8.3-105) nmol kg^{-1}, Emax=45.1(24-66) mmHg, n=10). DALBK (3, 6 nmol $kg^{-1}min^{-1}$, 5 min) significantly ($P < 0.05$) reduced the hypotensive effect of DABK (Figure 1). DALBK (3 nmol $kg^{-1}min^{-1}$, 5 min) produced no effect *per se* in control animals but increased MAP by 5.3±1.2 % (n=6, P<0.05) in LPS (10 mg kg^{-1}, 6 h)-treated rats. Similarly, des-arg[10]HOE 140 (30 nmol $kg^{-1}min^{-1}$, 5 min) increased MAP by 8.8±0.34% (n=5, P<0.05) in LPS-treated rats.

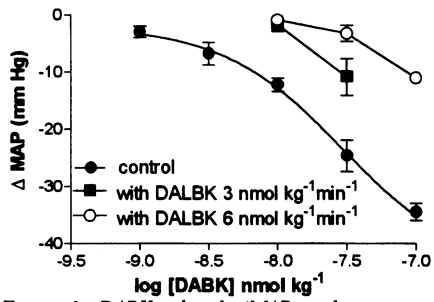

Figure 1. DABK-induced ΔMAP and antagonism by DALBK in LPS (2.5 mg kg^{-1}, 6 h)-pretreated rats (n=4-7).

These results suggest that hypotensive doses of endotoxin causes B_1 receptor induction *in vivo* the endogenous activation of which may play a role in endotoxin-induced hypotension. The inhibition of ACE/kininase II does not appear to markedly effect the role of B_1 receptors in this hypotension.

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114P INFLUENCE OF α_2-AUTORECEPTOR BLOCKADE ON THE A_{2A}-MEDIATED FACILITATION OF NORADRENALINE RELEASE IN RAT ISOLATED TAIL ARTERY

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Adenosine inhibition of noradrenaline release is mainly mediated by prejunctional A_1-adenosine receptors, an effect influenced by activation of "neighbouring" prejunctional receptors, namely α_2-autoreceptors (Limberger et al., 1988). Several lines of evidence suggest that adenosine modulation of noradrenaline release is not limited to the "classical" inhibitory effect, since adenosine can also facilitate noradrenaline release through activation of A_2-adenosine receptors. In the present study we have investigated the influence of α_2-autoreceptors on the facilitation of noradrenaline release mediated by A_2-adenosine receptors in the rat tail artery.

Tail arteries of Wistar rats (290 – 400 g) were incubated in 1.5 ml medium containing 0.1 μM ^{3}H-noradrenaline and perfused at a constant rate of 1ml min^{-1}. The medium contained (mM) NaCl 118.6, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.23, $NaHCO_3$ 25.0, glucose 10.0, ascorbic acid 0.3, dissodium EDTA 0.031, saturated with 95% O_2 and 5% CO_2 and kept at 37 °C. Desipramine (400 nM) was added throughout superfusion [in some experiments 1 μM of yohimbine or 5 mM of tetraethylammonium (TEA) was also added]. A total of 5 identical periods of stimulation were applied (5Hz, 1ms, 50mA, 100 pulses) every 30 min, starting at t=30 min (S_0, - S_4; t=0 was the onset of perfusion). The stimulation evoked overflow of tritium was taken to reflect action-potential-evoked release of noradrenaline. Effects of drugs on tritium overflow was expressed as % of respective control. Results are expressed as mean ± s.e.mean and n represents the number of tissue preparations. The results were analysed using the Student's unpaired t test; P<0.05 was taken to be statistically significant.

The overflow of tritium averaged 0.52 ± 0.04 % (n=7) of tissue content was increased to 1.84 ± 0.12 % (n=10) in the presence of 1μM yohimbine (an α_2-adrenoceptor antagonist) and to 1.83 ± 0.29 % (n=6) in the presence of 5 mM TEA (a K^+ channel blocker). The effects of the A_{2A}-adenosine receptor agonist CGS 21680 in the absence or in the presence of yohimbine or TEA are shown in the table.

		n	% of control
CGS 21680	1 nM	7	119.5 ± 5.3*
	100 nM	7	144.3 ± 8.9*
Yohimbine 1μM+CGS 21680	1nM	10	101.9 ± 3.1*
	100 nM	10	97.4 ± 6.1*
TEA 5 mM + CGS 21680	1 nM	6	138.3 ± 11.8*
	100 nM	6	139.8 ± 6.2*

Significant differences from the respective control (*P<0.05); from CGS 21680 alone (* P<0.05).

In conclusion, the facilitation of noradrenaline release mediated by A_{2A}-adenosine receptors was evidenced when α_2-autoreceptors were activated, irrespectively of the magnitude of noradrenaline release.

Supported by FCT/PRAXIS XXI and Associação Nacional das Farmácias.

Limberger N. et al. (1988) Naunyn-Schmied. Arch. Pharmacol., 338, 53-61

115P FACILITATION BY ADENOSINE OF NORADRENALINE RELEASE IN RAT ISOLATED VAS DEFERENS IS PREVENTED BY BLOCKADE OF α_2-AUTORECEPTORS

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Adenosine seems to exert an opposite modulation on noradrenaline release: an inhibition mediated by A_1-adenosine receptors and a putative facilitation mediated by A_2-adenosine receptors. However, evidences for this facilitation were obtained using exogenous agonists and antagonists and not its natural ligand, adenosine. The aim of the present study was to investigate the effects of adenosine on noradrenaline release in the rat vas deferens, a tissue where the presence of facilitatory adenosine receptors has been described (Gonçalves & Queiroz, 1993); the influence of α_2-autoreceptor blockade on the effects of adenosine was also investigated.

Vasa deferentia of male Wistar rats (310 – 400 g) were incubated in 1.5 ml medium containing 0.1 μM ^{3}H-noradrenaline and perfused at a constant rate of 1ml min^{-1}. The medium contained desipramine (400 nM; in some experiments was also added 1 μM yohimbine, an α_2-adrenoceptor antagonist), was saturated with 95% O_2 and 5% CO_2 and kept at 37 °C. A total of 5 identical periods of stimulation were applied (8Hz, 1ms, 50mA, 100 pulses) every 30 min (S_0-S_4). The stimulation evoked overflow of ^{3}H was taken to reflect action-potential-evoked release of noradrenaline. Effects of adenosine on ^{3}H overflow (in the absence or in the presence of DPCPX, a selective A_1-adenosine receptor antagonist) are expressed as % of respective control. Results are expressed as mean ± s.e.mean and *n* represents the number of tissue preparations. The results were analysed using the Student's unpaired *t* test; $P<0.05$ was taken to be statistically significant.

The overflow of ^{3}H averaged 0.19 ± 0.02 % (n=6) of tissue content and was increased to 0.93 ± 0.06 % (n=10) in the presence of 1 μM yohimbine ($P<0.05$). DPCPX (20 nM) increased ^{3}H overflow (130 ± 9 %, n=10; $P<0.05$) only in the presence of yohimbine. The effects of adenosine on noradrenaline release are shown in figure 1.

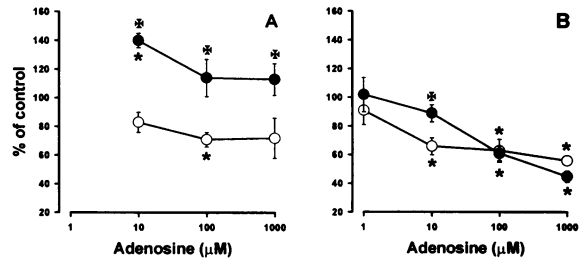

Figure 1. Effects of adenosine on electrically evoked ^{3}H overflow in the absence (○) and in the presence of 20 nM DPCPX (●) either when α_2-autoreceptors were operating (**A**) or blocked (**B**): Shown are mean ± s.e.mean of 6 (**A**) and 10 (**B**) experiments. Significant differences from control: * $P<0.05$; from adenosine alone ⊛$P<0.05$.

In conclusion, the present results support the existence of an adenosine-receptor mediated facilitation of noradrenaline release in rat vas deferens, evident only when α_2-autoreceptors are activated.

Supported by FCT/PRAXIS XXI and Associação Nacional das Farmácias.

Gonçalves J. & Queiroz G. (1993) *Naunyn-Schmied. Arch. Pharmacol.* 348, 367-371.

116P ROLE OF N^G-NITRO-L-ARGININE IN THE RAT VAS DEFERENS INDUCED CONTRACTILITY BY NORADRENALINE AND PHENYLEPHRINE

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The vasodilator nitric oxide (NO) is a corpus cavernosum potent dilator (Kim *et al.*, 1991) contributing to the penile smooth muscle relaxation and subsequent penile erection (Saenz de Tejada *et al.*, 1991). Corpus cavernosum smooth muscle relaxation can be inhibited *in vitro* by NO synthase inhibitors like N^G-nitro-L-arginine (L-NOARG) and N^G-methyl-L-arginine (Pickard *et al.*, 1991). Also it is well established that the contractile movements of vas deferens are involved in the sperm progression up to the urethra during ejaculation. In order to further clarify the modulatory effect of NO at the male reproductive tract smooth muscle (at least for corpus cavernosum) we investigated the influence of NO synthase pathway in the rat vas deferens contractility under adrenergic stimulation by noradrenaline (NE) and phenylephrine (PhE).

Effect of NO synthase inhibition by L-NOARG in the NE and PhE dose responses curves was therefore evaluated in order to further clarify the role of NO in the male reproductive function.

Male Wistar rats (250 ± 30 g body weight) were lightly anaesthetised with ether and then killed by exsanguination. Abdomen was opened and the vas deferens were removed, freed from the serous mesenteric coat and placed in organ bath with Krebs-Henseleit solution at 37 °C. The dose response curves were obtained with through the addition of increasing single concentrations of NE or PhE (0.8 – 26.0 μM) according to the protocol, in the absence (control) and subsequently in the presence of L-NOARG (1 nM and 10 nM). After inclusion of L-NOARG the preparation was kept for, at least, 30 min till stabilisation. Contractile responses were recorded by a force transducer LETICA coupled to a LETICA preamplifier and polygraph. NE and PhE dose responses curves parameters (E_{max} and $ED_{50\%}$) were calculated by a non-linear regression analysis (Enzfitter software) and compared by paired Student's *t* test.

Results: Effect of L-NOARG on the NE-dose responses curves parameters (Mean ± s.e.mean)

	Control	L-NOARG (1nM)	L-NOARG (10nM)
E_{max}(%)	100 (1070 ± 80 mg)	78.8 ± 3.5*	56.2 ± 7.7*
$ED_{50\%}$ (μM)	1.8 ± 0.6	3.4 ± 1.6	1.8 ± 0.4

(n=5; *P *vs* control <0.05)

Effect of L-NOARG on the PhE-dose responses curves parameters (Mean ± s.e.mean)

	Control	L-NOARG (1nM)	L-NOARG (10nM)
E_{max}(%)	100 (1686 ± 130 mg)	83.9 ± 2.5*	78.9 ± 7.5*
$ED_{50\%}$ (μM)	0.5 ± 0.3	0.4 ± 0.2	0.6 ± 0.4

(n=5; *P *vs* control <0.05)

L-NOARG (1 and 10 nM) reduced the NE and PhE maximal responses at concentrations much lower than those reported to NO synthase inhibition in other systems, like the vascular smooth muscle (10-100μM) (Rees *et al.*, 1990).
Contrariwise to the relaxant effect recognised for NO synthase pathway in several smooth muscles, particularly the vascular (Bucher *et al.*, 1992), our results showed that NO synthase inhibitors (L-NOARG) induced a decrease in the vas deferens contractility.

Bucher, B., *et al.* (1992) *Br. J. Pharmacol.* **107**, 976-982.
Kim, N., *et al.* (1991) *J. Clin. Invest.* **88**,112-118.
Pickard, R.S., Powell, P.H. and Zar, M. (1991) *Br. J. Pharmacol.* **104**, 755-759.
Rees, D.D., *et al.* (1990) *Br. J. Pharmacol.* **101**, 746-752.
Saenz de Tejada, I., et al. (1991) *Am. J. Physiol.* **260**, H1590-1595.

117P NORADRENALINE RELEASES Ca^{2+} FROM RYANODINE-SENSITIVE INTRACELLULAR STORES IN RAT ISOLATED EPIDIDYMAL VAS DEFERENS WHICH CAN BE FILLED BY A HIGH K^+ KREBS SOLUTION

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The rat epididymal vas deferens contracts to noradrenaline (NA) via α_{1A}-adrenoceptors (Burt et al., 1995). The contraction is dependent on activation of PKC and is abolished in Ca^{2+}-free Krebs solution (Burt et al., 1996). However this does not rule out the possibility that Ca^{2+} release from intracellular stores may play a part in the contraction to NA as the stores may be depleted quickly with time in Ca^{2+}-free Krebs. High K^+ Ca^{2+}-containing Krebs can potentiate responses in Ca^{2+}-free Krebs as can lower temperature in some tissues. The aim of this study was therefore to see if a contraction to NA could be obtained after short periods in Ca^{2+}-free Krebs at 25°C and if this might be potentiated by high K^+ Krebs.

Epididymal portions of rat vas deferens (Sprague Dawley 350-500g) were set up in Krebs solution under 0.5g resting tension at 25°C and gassed with 95%O_2/5%CO_2. In some experiments a modified high K^+ (50mM) Krebs was also used. Ca^{2+}-free Krebs contained 1mM EGTA. A single dose of NA (10^{-4}M) was added initially to all tissues in normal Krebs, producing a maximum contraction. NA (10^{-4}M) was used in all subsequent additions and ryanodine or nifedipine was incubated for 30 min. Responses were calculated as a percentage of the initial NA response and shown as the mean±s.e.m. for n=4.

NA (10^{-4}M) in normal Krebs produced a single phasic contraction (2.04±0.1g). After 2 min in Ca^{2+}-free Krebs a biphasic contraction to NA was obtained which was much smaller. It consisted of an initial phasic response (18±2%) and a second smaller phasic response (7±1%). After 5 min in Ca^{2+}-free Krebs there was no response left. Ryanodine (10^{-4}M) abolished the initial phasic response to NA but not the second phasic response (5±1%). When tissues were incubated in high K^+ Krebs and then changed to high K^+ Ca^{2+}-free Krebs for 2 min a single large phasic contraction to NA (131±7%) was obtained. Similar but smaller contractions were still produced after 10 min (113±9%) and 30 min (66±9%). Ryanodine (10^{-4}M) and nifedipine ($3x10^{-7}$M) both reduced the response to NA after 2 min in high K^+ Ca^{2+}-free Krebs (after high K^+ Ca^{2+}-containing Krebs) to 45±3 and 67±4% respectively.

It appears that Ca^{2+} is released from intracellular stores during contraction to NA in this tissue but these store(s) are depleted quickly in the absence of extracellular Ca^{2+}. The initial phasic response after 2 min in Ca^{2+}-free Krebs involved Ca^{2+} from a ryanodine-sensitive store while the second phasic response was ryanodine-insensitive. High K^+ Ca^{2+}-containing Krebs greatly potentiated the Ca^{2+}-free contractions to NA so that the two responses merged into one, and was still present after 30 min. The results suggest that high K^+ Ca^{2+}-containing Krebs fills Ca^{2+} store(s) via a nifedipine-sensitive pathway and which are normally relatively empty. The potentiated response in Ca^{2+}-free Krebs was partly reduced by ryanodine which may reflect it's effect only on the initial phasic response to NA first seen in Ca^{2+}-free Krebs. However the role of the Ca^{2+} stores in this tissue for the NA contraction in normal Krebs is not clear as this is not inhibited by ryanodine (Burt *et al.*, 1996).

Burt, R.P. *et al.*, (1995). *Br. J. Pharmacol.*, **115**, 467-475.

Burt, R.P. *et al.*, (1996). *Br. J. Pharmacol.*, **117**, 224-230.

118P TIME-DEPENDENT UP-REGULATION OF NEURONAL 5-HYDROXYTRYPTAMINE BINDING SITES IN THE DETRUSOR OF A RABBIT MODEL OF PARTIAL BLADDER OUTLET OBSTRUCTION

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Serotonin (5-hydroxytryptamine; 5-HT), a vasoactive bioamine, with potent contractile activity is thought to act indirectly in the urinary bladder by the stimulation of its presynaptic receptors. This results in the release of acetylcholine (ACh) which then acts on muscarinic receptors to produce bladder contractility. Bladder outlet obstruction (BOO) can lead to detrusor instability associated with denervation supersensitivity with altered response to ACh. Using a rabbit model of partial BOO we investigated whether there are any associated changes in the neuronal 5-HT binding sites.

Under general anaesthesia (using 1-2% halothane in O_2) partial BOO was induced in six adult male New Zealand White rabbits as previously described (Buttyan *et al.*, 1992). Six sham operated age-matched rabbits acted as controls. After one, three and six weeks, following cervical dislocation, the urinary bladders were excised. Detrusor sections were incubated with [^{3}H]-5-HT. Autoradiographs were generated and analysed densitometrically. The presence of nerves was detected by using immunohistochemistry (NF200).

Autoradiography demonstrated [^{3}H]-5-HT binding to nerves within the detrusor smooth muscle. Image analysis revealed a significant upregulation of this neuronal binding in the six-week partial BOO ($p<0.0001$; Mann-Whitney, Table 1).

Table 1. Number of nerves/mm^2 binding [^{3}H]-5-HT in control, 1, 3 and 6-week partial BOO rabbit detrusor smooth muscle. Results are expressed as median and (range). *$p<0.0001$.

CONTROL	1-week BOO	3-week BOO	6-week BOO
3	3.5	4	*16
(1-9)	(1-8)	(1-8)	(11-58)

In the rabbit model of partial BOO there was a significant time-dependent upregulation of neuronal 5-HT binding sites in the detrusor. This change may influence 5-HT-mediated ACh release, resulting in increased bladder contractility. This in turn may play a role in detrusor instability associated with denervation post-junctional supersensitivity. These results provide a possible rationale for further investigation into the use of 5HT antagonists in the treatment of obstructive detrusor instability.

Buttyan, R. *et al.*, (1992) Neurourol. Urodyn., **11**, 225-238.

119P ROLE OF PROTEIN KINASE G ON EXCITATORY NANC CONTRACTIONS IN GUINEA-PIG ISOLATED BRONCHUS

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A number of studies have demonstrated that an elevation of cyclic-GMP evokes relaxation in airway smooth muscle (Torphy *et al*, 1994). Nitric oxide (NO) has been associated with this pathway and known to activate guanylyl cyclase and subsequently activate protein kinase G. We have previously shown that a number of protein kinase A inhibitors (H-89 and KT5720) failed to attenuate the excitatory nonadrenergic noncholinergic (eNANC) response evoked by electrical field stimulation (EFS). However, these protein kinase A inhibitors significantly reversed the inhibitory effect of Ro-20-1724 (phosphodiesterase 4 inhibitor) on the eNANC contractile response (Harrison *et al.*, 1998). The aim of the present investigation was to observe the role of protein kinase G against eNANC contractile responses in guinea-pig isolated airway smooth muscle.

Guinea-pigs were killed by cervical dislocation and the lungs removed. Isolated main bronchus was cut into 2 mm rings and suspended on two 'L' shaped stainless steel holders and placed in 8 ml organ bath under a resting tension of 500 mg. The tissues were placed in an 10 ml organ bath and bathed in Krebs-Henseleit solution, aerated with 95% O_2 at 37°C in the presence of indomethacin (5 μM). eNANC responses were elicited by EFS [3 Hz, 20 sec, 0.5 ms at max. voltage: in the presence of atropine (1 μM) and thiorphan (10 μM)] in the absence (DMSO 0.01 %) or presence of the protein kinase G inhibitor, KT5823 (10 μM), and/or the nitric oxide synthase inhibitor, L-NAME (30 μM). Cumulative concentration curves to substance P (0.0001 - 1 μM) and sodium nitroprusside (0.01 - 1000 μM) were performed in the absence or presence of KT5823 and L-NAME. Results are expressed as mean ± s.e.mean. Concentration-effect curves to substance P and sodium nitroprusside was analysed and differences between the means were assessed using Student's non-paired t-test. Values were considered significant if $P < 0.05$.

The protein kinase G inhibitor, KT5823, significantly attenuated the eNANC contractile response (% inhibition of control) evoked by EFS (control, 16.8 ± 8.2 % vs. KT5823, 62.5 ± 10.9 %; $P < 0.05$, $n = 4$). In the presence of the nitric oxide synthase inhibitor, L-NAME, the response to KT5823 was abolished (control, 6 ± 6 vs. KT5823/L-NAME, 2.7 ± 1.9; $P > 0.05$, $n = 4$). In contrast, KT5823 failed to significantly alter the contractile potency (pD_2) to substance P (control, 6.4 ± 0.4 vs. 7.3 ± 0.4; $P > 0.05$, $n = 4$). Furthermore, the relaxant response evoked by sodium nitroprusside was not altered by KT5823 (control, 5.7 ± 0.1 vs. KT5823, 5.9 ± 0.2, $P > 0.05$, $n = 5 - 6$) or L-NAME (control, 5.7 ± 0.1 vs. L-NAME, 6.3 ± 0.3, $P > 0.05$, $n = 5 - 6$).

The results indicate that KT5823 inhibits eNANC contractile responses via an L-NAME-sensitive pathway, although the mechanism of this effect remains to be established.

HARRISON, S. *et al,* 1998 *Eur. Respir. J.,* **12(28),** 10s (0175)
TORPHY, T. J. *et al.*, 1994 *Trends Pharmacol. Sci.,* **15 (10),** 370-374.

120P CHARACTERISATION OF THE PARASYMPATHETIC DEPENDENT RELEASE OF AN HEPATIC INSULIN SENSITIZING SUBSTANCE (HISS) IN THE RAT

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The whole body disposal of glucose in response to insulin is determined, in part by, a hepatic parasympathetic reflex release of a humoral factor referred to as hepatic insulin sensitizing substance (HISS) (Xie and Lautt, 1996). Intra-portal administration of atropine produces a dose dependent increase in insulin resistance (Xie and Lautt, 1995) which is of similar magnitude to that induced by surgical denervation of the liver (Xie et al, 1993). Therefore, insulin action consists of an hepatic parasympathetic nerve (HPN) independent and of a variable HISS-mediated HPN-dependent component (Xie and Lautt, 1996).

The aim of this study was to characterize the profile of HISS release. Insulin sensitivity was evaluated by a new Rapid Insulin Sensitivity Test (RIST) (Lautt et al., 1998) with the RIST index defined as the amount of glucose required to be infused over a period of time following intra-venous (I.V.) insulin administration (5-200 mU/Kg, over 5 minutes) using an euglycemic clamp. The HISS effect was quantitated as the differences in the RISTs obtained with insulin alone (HPN-independent plus HPN-dependent components) or with insulin in the presence of atropine (3 mg/kg, I.V.) (HPN-independent component). Insulin infusion (5–200 mU) promoted a dose dependent increase in HISS release (26.1 ± 3.5 to 351.0 ± 5.1 mg/kg glucose)($p<0.001$, $n=18$). When two sequential RISTs were performed in the same animal, at insulin doses of 10 and 100 mU the percentage of inhibition of the RIST after atropine was not statistically different between the two doses (64.27 ± 13.15 % at 10 mU of insulin and 69.24 ± 4.71 % at 100 mU of insulin, n=4).

These results suggest that the HPN-dependent component of the insulin action *i.e.* the proportion of HISS released is constant in the same animal and is independent of the dose of insulin used. In addition, the HPN-independent component of insulin action had a shorter duration of action (10.3 ± 0.6 to 33.0 ± 3.2 min) than the HISS component (16.0 ± 1.0 to 43.3 ± 4.5 min) for insulin doses of 5 to 200mU, ($n=18$, $p<0.001$), which is an indication that HISS may also act through insulin independent mechanisms. An impairment of the HISS mediated HPN-dependent component of the insulin action may contribute to the insulin resistance observed in non-insulin dependent diabetes mellitus.

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Xie, H. and Lautt, W.W., (1996) Am. J. Physiol., 270: E858-863.
Lautt, W.W.; Wang, X.; Sadri, P.; Legare D.; Macedo, M.P. (1998);Can. J. Physiol. Pharmacol. 76 : 1080-1086..

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121P INVESTIGATION OF THE EFFECT OF 5-HYDROXYTRYPTAMINE AS AN EMETIC AND ANTI-EMETIC AGENT IN *SUNCUS MURINUS* (MUSK SHREW)

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Suncus murinus is an insectivore which is sensitive to the emetic effects of a number of drugs and motion sickness (Matsuki *et al.*, 1992; Javid *et al.*, 1998a, b). The aim of the present study was to investigate the emetic effect of 5-hydroxytryptamine (5-HT) and its influence on motion induced emesis in *Suncus murinus*.

Adult male Japanese House Musk shrew, *Suncus murinus* (65-80 g) were used. In the initial experiments animals first received saline (i.p.) 10 min prior to a horizontal motion stimulus of 1 Hz and 40 mm amplitude. After 10 days, they received 5-HT (1, 2 or 4mg/kg, i.p.) (and were observed for emesis) 10 min prior to a motion stimulus. In the second experiments, animals were firstly injected i.p. with saline 10 min prior to a motion stimulus (MS, 1Hz and 40mm). After 10 days, the animals received 5-HT (4mg/kg, i.p.) (and were observed for emesis) 10 min prior to a motion stimulus. On the third occasion the animals were injected with either granisetron (gran, 0.5mg/kg, i.p.), GR12548D (GR, 1mg/kg, i.p.), methysergide (meth, 1mg/kg, i.p.) or saline 35 min prior to the administration of 5-HT (4 mg/kg, i.p.) (and were observed for emesis) 10 min prior to a motion stimulus (1Hz, 40mm). In all the experiments, the number of emetic episodes (vomits/retches) were recorded over a 10 min period. Data were expressed as the mean±s.e.mean of n=12 and analysed using one-way ANOVA which was followed by Bonferronni-Dunnett's t-test.

The administration of 5-HT (1, 2 and 4 mg/kg, i.p.), 10 min prior to a motion stimulus, produced a dose-dependent emetic response in its own right and attenuated the emetic response to a motion stimulus (Table 1). Granisetron (a 5-HT_3 receptor antagonist) reduced significantly ($p<0.05$) the emesis induced by 5-HT (4 mg/kg) and also ameliorated the reduction in motion sickness caused by 5-HT. Pre-treatment with methysergide (a 5-$HT_{1/2}$ receptor antagonist) and GR125487D (a 5-HT4 receptor antagonist, Gale *et al.*, 1994) also significantly ($p<0.05$) attenuated the emesis induced by 5-HT but not the reduction in motion sickness caused by 5-HT (Table 2).

Table1. The number of emetic episodes induced by (A) motion stimulus, (B) vehicle or 5-HT and (C) the effect of the 5-HT treatment on the response to a subsequent challenge with MS.

5-HT (mg/kg)	A-MS	B-(vehicle/5-HT)	C-MS
-	11.6±1.8	0.0	9.8±0.6
1	12.3±4.0	0.5±0.5	14.5±3.0
2	13.5±2.0	1.3±0.2	*7.0±2.0
4	10.6±3.0	2.4±1.0	*1.8±1.0

Table 2. The effect of 5-HT receptor antagonists (A, gran, GR or meth) to inhibit the emesis induced by 5-HT (4mg/kg) and to influence the actions of 5-HT to attenuate emesis induced by MS.

Group	Control	5-HT	MS (after 5-HT)	5-HT+A	MS (after 5-HT+A)
Vehicle	11.6±1.8	-	9.8±0.6	-	8.5±2.3
Gran	9.3±1.0	1.5±0.4	*1.3±0.7	#0.37±0.2	5.0±2.0
GR	13.6±2.2	2.7±0.8	*3.6±1.2	# 0.7±0.4	5.3±1.6
Meth	9.1±1.7	1.8±0.5	*1.1±0.5	# 0.0	2.9±1.9

*$p<0.05$ compared to motion stimulus applied after saline (control).
#$p<0.05$ compared to animals treated with 5-HT.

These data suggest that 5-$HT_{1/2}$, 5-HT_3 and 5-HT_4 receptors are involved in mediating the emetic response to 5-HT. In addition, 5-HT_3 but not 5-$HT_{1/2}$ and 5-HT_4 receptors may play a role in mediating an inhibitory action of 5-HT to reduce emesis induced by a motion stimulus in *Suncus murinus*.

Gale *et al.* (1994). Br. J. Pharmacol., 123, 120P.
Javid, F. A. & Naylor, R. J. (1998a). Br. J. Pharmacol., 123, 255P.
Javid, F. A. & Naylor, R. J. (1998b). Br. J. Pharmacol., 123, 340P.
Matsuki *et al.* (1992) Mechanisms and control of emesis. Colloque Inserm. vol. 223, 323-329.

122P INVESTIGATION OF TACHYPHYLAXIS TO DOI-INDUCED CONTRACTION IN THE PROXIMAL REGION OF THE *SUNCUS MURINUS* INTESTINE

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In previous studies we have shown that the 5-HT_2 receptor agonist DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, Hoyer *et al.*, 1994) produced a bell-shaped concentration-dependent contraction curve in the *Suncus murinus* intestine (Javid & Naylor, 1998). The present study investigated whether the decreasing response to higher concentrations of DOI was due to a 5-HT receptor desensitisation and if so, whether desensitisation to DOI would affect the contraction response to 5-HT.

Segments (1 cm length) taken from the intestine (1 cm distal to the pyloric sphincter) of adult Japanese House Musk shrew, *Suncus murinus* (28-64 g) of either sex were mounted in 10 ml organ baths containing Krebs' solution (37°C, 95% O2, 5% CO2). The tissues were allowed to equilibrate for 60 min and washed every 20 min. The resting tension was maintained at 0.5 g and contractions were recorded isometrically. Tissues were challenged repeatedly with a single concentration of 3 μM DOI with a 1 min contact time and 22 min intervals. The procedure was repeated in the presence of SB206553 (1μM). In separate experiments tissues were challenged with 5-HT (0.3 and 10μM) before and after 3 additions of DOI (3 μM). The procedure was also repeated in the presence of SB206553 (1μM). Tension changes were expressed as a percentage of KCl-induced contraction (0.12 M) of n=6 and analysed using Student's t-test.

The first challenge with DOI at 3 μM produced a robust contraction response. However, subsequent challenges with 3 μM of DOI produced a significantly ($p<0.05$) smaller contraction response. SB206553, a selective 5-HT_{2C} antagonist (Forbes *et al.*, 1996), did not influence the contractile response to the first challenge to DOI, but prevented the attenuation of the contraction to subsequent challenge to DOI (Figure 1). Repeated challenges to DOI (3 μM) also reduced the contractile response induced by 0.3 μM 5-HT, but not 10 μM 5-HT. SB206553 also prevented the attenuation of response to 0.3 μM 5-HT (Figure 1).

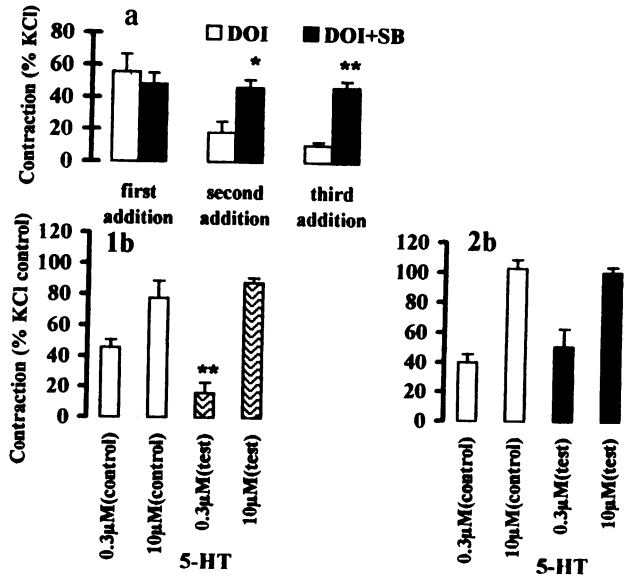

Figure 1. The contractile effect of: (a) a repeated challenge to DOI (3μM) in the absence and presence of SB206553 (SB, 1μM), and (b) 5-HT (0.3 and 10μM) added before (control) and following (test) repeated challenge to DOI (3μM) in the (1b) absence and (2b) presence of SB206553 (1μM) in the proximal region of *Suncus murinus* intestine, n=6; *$p<0.05$ and **$p<0.01$ compared to the control values.

These data suggest that the decrease in the contraction response to DOI may be due to the desensitisation of 5-HT receptors involving the 5-HT_{2C} receptor subtype.

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Javid, F.A. & Naylor, R.J. (1998). Br.J.Pharmacol. In press
Hoyer, D. et al. (1994). Am.Soc.Pharm.Exp.Ther., 125, 158-193.

123P STRUCTURE-ACTIVITY STUDIES OF PHORBOL ESTER COMPOUNDS ON PKCα-ACTIVATION USING AN *IN VIVO* PHENOTYPIC YEAST ASSAY: INFLUENCE OF SUBSTITUENTS AT C-12 AND C-13

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The functional analysis of protein kinase C (PKC) activation in experimental mammalian systems is complicated by the co-existence of multiple isoforms (Nishizuka, 1988). Therefore the actual role of each individual isoform remains largely unknwon. An *in vivo* alternative method, based on growth inhibition of *Saccharomyces cerevisiae* transformed with individual mammalian PKC isoforms, has been recently proposed (Shieh *et al.*, 1995). The aim of the present study was to analyse the differential activation of PKCα isoform by phorbol esters with different substituents at C-12 and C-13 using the yeast phenotypic assay.

Yeast expression plasmid containing the bovine PKCα gene or YEp51 were introduced into *S. cerevisiae* (strain CG379). Cells were routinely grown at 30°C with continuous aeration in leucine-free synthetic medium containing 2% glucose in liquid culture or 1.5% agar plates. Transformed yeast were diluted to 0.05 OD_{600} in 2% galactose and 3% glycerol. One mililitre cultures were incubated for 48 h to OD_{600} 0.5-0.7 in the presence of phorbol esters or solvent (dimethylsulfoxide; DMSO; 0.1% final concentration). The OD_{600} of each culture was measured and effects of drugs expressed as % growth inhibition (effect of solvent was considered as 0% growth inhibition). Values presented are mean ± s.e.mean of *n* duplicate measurements. The following compounds were assayed: phorbol 12-myristate 13-acetate (PMA), phorbol 12-myristate (PM), phorbol 12,13-diacetate (PDA), phorbol 13-acetate (PA) and phorbol 12,13-didecanoate (PDD).

In yeast expressing PKCα, PMA and PDD exerted a concentration dependent inhibition of growth. The maximal effects were obtained for 10^{-6} M PMA and 10^{-6} M PDD and reached 49.4 ± 1.2 % (n=33) and 53.8 ± 2,3% (n=8), respectively (Figure 1). On interaction experiments it was observed that the inactive phorbol esters did not alter the effects of sub-maximal concentrations of PMA.

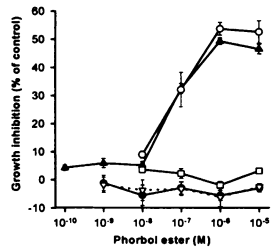

Figure 1. Effect of phorbol esters on growth inhibition of yeast expressing PKCα: PMA (▲), PDD (O), PM (□), PDA(●) and PA (V). Values are mean ± s.e.mean of 8-33 duplicate measurements.

In yeast transformed with YEp51 (control) only PDD caused growth inhibition (maximum effect was 31.2 ± 1.6%, n=17 for 10^{-5} M PDD).

The results obtained indicate that structural changes at C-12 and C-13 are critical for PKCα activation. When compared to PMA, compounds with smaller substituents showed a loss of activity while those with larger substituents at C-13 (as in PDD) showed a loss of selectivity towards the PKCα isoform. Furthermore, the inactive phorbol esters used do not seem to compete for the PKCα activation site. These results reinforce the importance of the yeast phenotypic assay to study the pharmacology of PKC activators.

Supported by FCT/PRAXIS/PSAU/C/SAU/71/96

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Shieh *et al*, (1995) *Mol. Carcinog.* 12, 166-176

124P CHELERYTHRINE REVERTS THE PHENOTYPIC EFFECTS OF PKCα ACTIVATION ON AN *IN VIVO* YEAST PHENOTYPIC ASSAY

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An *in vivo* alternative method based on growth inhibition of *Saccharomyces cerevisiae* expressing individual mammalian protein kinase C (PKC) isoforms has been proposed as a rapid and general screen for the PKC-activating potential of drugs (Shieh *et al.*, 1995). The aim of the present work was to adapt this method to screen for PKC inhibitors, and study interactions between activators and inhibitors of PKCα. Chelerythrine (CHL), a PKC inhibitor, and 4β-phorbol 12-myristate 13-acetate (PMA), a PKC activator, were used as test drugs.

Yeast expression plasmids containing the bovine PKCα gene were introduced into *S. cerevisiae* (strain CG379). The cells were routinely grown at 30°C with continuous aeration in leucine-free synthetic medium containing 2% glucose in liquid culture or 1.5 % agar plates. Transformed yeast were diluted to 0.05 OD_{620} in 3% glycerol and 2% galactose (induces the expression of PKCα) and 200 µl transferred into a 96-well microtitre plate. The cells were incubated for up to 100 hours in the presence of chelerythrine and/or PMA; control cells were incubated with solvent (dimethylsulfoxide; DMSO; 0.1% final concentration). Quadruplicate samples were used for each treatment. The OD_{620} of each well was measured and effects of drugs expressed as % of control (growth on control conditions was considered as 100%).

In the absence of PKCα expression (medium without galactose) neither CHL nor PMA modified yeast growth.

Figure 1 shows the effects of chelerythrine and/or PMA on growth of transformed yeast.

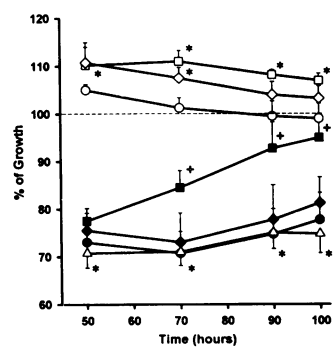

Figure 1: Influence of CHL (circles for 10^{-7}M; diamonds for 10^{-6} M; squares for 10^{-5} M) on yeast growth in the absence (open symbols) and in the presence of 10^{-7}M PMA (filled symbols; Δ shows the effects of PMA alone). Shown are mean ± s. e. mean (n = 16 to 20 values). Significant differences from control: *P<0.05; from PMA alone: +P<0.05; paired Student's t-test.

In conclusion, chelerythrine stimulated growth (suggesting basal PKCα activity) and antagonised the growth inhibition caused by PMA in transformed yeast. These results indicate that the phenotypic yeast assay can be used for the screening of potential PKCα inhibitors either by a direct effect on the basal PKCα activity or by experiments with PKC activators.

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125P P-GLYCOPROTEIN PHOSPHORYLATION/DEPHOSPHORYLATION AND CELLULAR ACCUMULATION OF L-DOPA

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Phorbol esters increase phosphorylation of P-glycoprotein (P-gp) and decrease the accumulation of P-gp substrates, whereas inhibition of PKC decreases phosphorylation of P-gp and impair transport of P-gp substrates (Fine et al., 1996; Germann et al., 1995). Protein-serine/threonine phosphatases-1 and -2A (PP1 and PP2A) are active in dephosphorylation of P-gp and increase the accumulation of P-gp substrates (Chambers et al., 1992; Lelong et al., 1994). L-DOPA, the immediate precursor of intrarenal natriuretic hormone dopamine, is a substrate for P-gp in LLC-PK_1 cells, and its intracellular availability may dependent on P-gp state of activation (Soares-da-Silva et al., 1998). The present work was aimed to study the effect of PKC activation and PP1/PP2A inhibition on P-gp mediated transport of L-DOPA in LLC-GA5 Col300 cells, a renal cell line expressing the human P-gp in the apical membrane (Saeki et al., 1993). LLC-GA5 Col300 cells (Riken Cell Bank; passages 16-25) were grown in Medium 199 supplemented with 300 ng ml^{-1} colchicine, 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B, 100 µg ml^{-1} streptomycin, 3% foetal bovine serum and 25 mM HEPES. For 24 hours prior to each experiment, the cell medium was free of foetal bovine serum. Results are given as arithmetic means ± s.e. mean, n=5-8. Statistical analysis were performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P-value less than 0.05 was assumed to denote a significant difference. In uptake studies, cells were preincubated (30 min) with Hanks' medium with added pargyline (100 µM), tolcapone (1 µM) and benserazide (50 µM). L-DOPA accumulation was a time- and concentration-dependent process with the following kinetic characteristics: k_{in}, 57.3±1.2 pmol/mg protein/min; k_{out}, 3.3±0.1 pmol/mg protein/min; A_{max}, 10.6±0.8; K_m, 342±48 µM; V_{max}, 9.7±0.7 nmol/mg protein. Verapamil (25 µM), a P-gp inhibitor, markedly increased (~40% increase) the accumulation of a non-saturating concentration of L-DOPA (2.5 µM) at both initial rate of uptake (IRU, 6 min incubation) and at steady-state (SS, 30 min incubation). PKC activation with phorbol 12,13-dibutirate (PDBu, 1, 3 and 10 nM) produced a concentration-dependent decrease in L-DOPA accumulation at SS, but not at IRU. The inactive phorbol ester 4α-phorbol 12,13-didecanoate (100 nM) produced no change in L-DOPA accumulation. The effect of PDBU was completely reverted by staurosporine (100 nM). The phosphatase inhibitor okadaic acid (100 nM) significantly reduced ($P<0.05$) by 26±2% the accumulation of L-DOPA at IRU, but not at SS. It is suggested that P-gp plays a role in regulation of intracellular availability of L-DOPA in renal epithelial cells, and phosphorylation/dephosphorylation of P-gp may be involved in the regulation of the transporter.

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126P NEUROPEPTIDE Y RELEASE AND RECEPTORS IN HUMAN CHROMAFFIN CELLS

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Neuropeptide Y (NPY) is a 36-aminoacid peptide neurotransmitter present in the central and peripheral nervous system. NPY acts through different G-proteins coupled receptors termed Y_1, Y_2, Y_3, Y_4 and Y_5 (Michel *et al.*, 1998). NPY is co-stored and co-released from the adrenal medula with the catecholamines (Majane *et al.*, 1985). Studies in bovine chromaffin cells showed that these cells express Y_1 and the Y_3 receptors (Norenberg *et al.*, 1994) and NGF-differentiated rat PC-12 pheocromocytoma cells expressed Y_1, Y_2 and Y_3 subtypes of NPY receptors (McCullough & Westfall, 1996). Bovine chromaffin cells release immunoreactive NPY (Kataoka et al., 1985). The aims of this work were: 1. To characterize the NPY receptor subtypes by measuring calcium increase, on primary cell cultures of human chromaffin cells; 2. To study the release of endogenous NPY from perifused chromaffin cells.

Cell Culture: the adrenal glands were obtained from kidney transplant donors and the isolation and culturing of human chromaffin cells was performed with modifications as described previously (Joseph *et al.*, 1994). Determination of $[Ca^{2+}]_i$: $[Ca^{2+}]_i$ was determined with the fluorescent probe FLUO-3/AM (2.5 µM) using confocal microscopy as described by Grouzmann *et al.* (1997). Release experiments: the cells plated on glass coverslips, at 37°C, were perifused at 0.75 ml/min, with Krebs or Krebs with drugs, and samples were collected every minute. The content of amidated NPY was measured by an amplified enzyme immunoassay (Grouzmann *et al.*, 1992). The results are expressed as mean ± SEM.

The basal $[Ca^{2+}]_i$ levels were 157±12.3 nM (n=312). NPY caused an increase in $[Ca^{2+}]_i$, 10 nM and 100 nM NPY stimulated 22 and 40 % of the cells, respectively. NPY13-36 and TASP-V (selective Y_2 agonists, Malis *et al.*, 1999), PYY, 34ProNPY (selective agonist Y_1/ Y_5) increased $[Ca^{2+}]_i$ in human chromaffin cells. The exposure to PP 100 nM (Y_4 agonist) did not raise the $[Ca^{2+}]_i$. These results suggest that human chromaffin cells expressed functional Y_2 and Y_1 or/and Y_5 receptors but not Y_4 receptors.

Table 1

	$[Ca^{2+}]_i$ (nM) in the total cell population	$\Delta[Ca^{2+}]_i$ (nM) in responding cells	% Responding cells
h-NPY 100 nM	213.2±43.9 (n=77)	288.4±92.6 (n=29)	40.1±6.6
h-NPY 10 nM	201.7±38.1 (n=34)	187.8±78.7 (n=7)	21.9±0.3*
h-PYY 100 nM	187.9±33.8 (n=33)	214.1±85.7 (n=9)	24.9±3.9*
h-34ProNPY 100 nM	237.8±47.2 (n=39)	190.5±86.3 (n=6)	22.5±1.5*
h-NPY13-36 100 nM	150.1±23.8 (n=44)	183.1±87.7 (n=6)	17.1±1.1*
r -PP 100 nM	147.0±23.2 (n=40)	–	0
TASP-V 100 nM	283.5±103.4 (n=27)	225.9±155.2 (n=11)	30.5±15.5

* $p<0.05$ compared to NPY 100 nM

In perifused chromaffin cells, nicotine (100 µM) increased the release of amidated NPY by 10 times (basal=3.0±0.1 fmoles/fraction, 0.31±0.04 % of the total content of NPY; peak of stimulation = 38 fmol/fraction).
In conclusion, this work shows that NPY could act in an autocrine manner to stimulate its own receptors in primary cell cultures of human chromaffin cells.

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127P EXTRACELLULAR Ca^{2+} MODULATES AGONIST-STIMULATED PHOSPHOINOSITIDE SIGNALLING IN CHINESE HAMSTER OVARY CELLS INDUCIBLE EXPRESSING HUMAN RECOMBINANT mGluR1α

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Extracellular Ca^{2+} ($[Ca^{2+}]_e$) can act as an agonist of the rat type 1α metabotropic glutamate receptor (mGluR1α) (Kubokawa *et al*, 1996, Kubo *et al*, 1998), or a modulator of agonist-stimulated signalling via mGluR1α (Saunders *et al*, 1998). Here we have examined the effects of $[Ca^{2+}]_e$ on signalling via human mGluR1α expressed in an inducible Chinese hamster ovary cell system (CHO-lac-hmGluR1α) in comparison to the M_3-muscarinic receptor expressed in CHO cells (CHO-m3).

Cells were cultured as described previously (Hermans *et al*, 1998). Inositol phosphate (IP_1) accumulation was measured in cells incubated in the presence of 2.5 μCi ml^{-1} [^{3}H]-*myo* inositol for 48 h (CHO-lac-hmGluR1α cells were induced with 100 μM IPTG for the last 20 h) prior to agonist stimulation in the presence of 10 mM LiCl (CHO-lac-hmGluR1α cells were treated with 3 U ml^{-1} glutamic-pyruvic transaminase and 5 mM pyruvate for 30 min prior to agonist stimulation). The IP_1 fraction was resolved using ion exchange chromatography, as previously described (Challiss *et al*, 1993). Data are expressed as means ± s.e. mean for $n \geq 4$ experiments. Statistical analysis was performed using ANOVA followed by Duncan's multiple range test.

Increasing $[Ca^{2+}]_e$ from nominally Ca^{2+} free - 4 mM had little effect on IP_1 accumulation in either cell line (CHO-lac-hmGluR1α, $p>0.05$, nominally Ca^{2+}-free, 414 ± 65, 4 mM Ca^{2+}, 852 ± 203 d.p.m. $well^{-1}$). As $[Ca^{2+}]_e$ was increased across the nominally Ca^{2+}-free - 0.9 mM range the agonist-stimulated IP_1 responses to a maximal concentration of quisqualate (Quis, 30μM) increased in CHO-lac-hmGluR1α cells from; nominally Ca^{2+}-free, 936 ± 316 to; 0.5 mM, 5984 ± 580, ($p<0.05$) to; 0.9 mM, 8499 ± 804 d.p.m. $well^{-1}$ ($p<0.05$). Increasing $[Ca^{2+}]_e$ above 0.9 mM caused no further increases in IP_1 accumulation. Similar trends were seen upon stimulation of the cells with a submaximal concentration of Quis (1μM), and both maximal (1 mM) and submaximal (30 μM) concentrations of a partial agonist of mGluR1α; **1S, 3R-ACPD** (e.g, 1 mM **1S,3R-ACPD**; nominally Ca^{2+}-free, 560 ± 115 to; 0.5 mM, 4682 ± 235, ($p<0.05$) to; 0.9 mM, 7585 ± 357 d.p.m. $well^{-1}$ ($p<0.05$). Increasing $[Ca^{2+}]_e$ over the range from nominally Ca^{2+}-free to 4 mM had no effect on IP_1 accumulation in response to a maximal concentration MCh (100 μM). IP_1 accumulation in response to a maximal concentration of the partial agonist arecoline, and submaximal concentrations of both arecoline (3 and 10 μM) and MCh (0.1 and 1 μM), was reduced under nominally Ca^{2+} free conditions ($p<0.05$), however increasing Ca^{2+} from 0.5 mM to 4 mM had no effect on IP_1 accumulation in comparison to CHO-lac-hmGluR1α cells. Analysis of membrane phospholipids suggests that the differences in agonist-stimulated IP_1 accumulation in CHO-lac-hmGluR1α cells seen in the presence of different $[Ca^{2+}]_e$ are not due to membrane phospholipid depletion.

Although this study contradicts those which suggest a role for $Ca^{2+}{}_e$ as an agonist of mGluR1α, this study indicates that, in common with our previous studies in BHK cells expressing rat mGluR1α, varying $[Ca^{2+}]_e$ in the millimolar range has differential effects on agonist-stimulation of mGluR1α and the M_3-muscarinic receptor expressed in a common cell background. We conclude that these differences are receptor- rather than cell-specific. This study supports a role for $Ca^{2+}{}_e$ as a modulator of mGluR1α function and could be of both physiological and patho-physiological significance (Erecinska and Silver, 1992).

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128P A DOMAIN IN THE POTASSIUM CHANNEL β_3 SUBUNIT INVOLVED IN LACK OF INACTIVATION IN OOCYTES

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The β subunit co-assembles with the Shaker-related potassium channel, and contributes to channel diversity. In particular, the β_1 (Kvβ1) subunit binds to the Kv1.5α channel subunit to confer the property of rapid inactivation via an amino-terminal "ball" domain on β_1 (Heinemann *et al.*, 1996). On the other hand, the β_3 subunit does not confer inactivation to the Kv1.5α subunit in oocytes, despite possessing a ball domain and apparently binding to Kv1.5α. Here we have investigated the possibility of a further domain on the β_3 subunit which may underlie the non-inactivating property of this subunit when expressed with Kv1.5α in oocytes.

For this, we constructed chimaeras of β_3 (N-terminal part) and β_1 (C-terminal part) and expressed them in *Xenopus* oocytes. Two days after injection of cRNA for Kv1.5α subunit, with or without the β subunit, two-electrode voltage-clamp recordings were made using step-depolarisations from a holding potential of -80mV in 10mV increments to +120mV, 1s duration, 0.1Hz.

As expected, expression of Kv1.5α or Kv1.5α+β_3 produced currents with little inactivation, while expression of Kv1.5α with β_1 produced rapidly inactivating currents (τ=25±4ms, n=6). Using a chimaera with the first 229 amino-acids from β_3 and the last 179 amino acids from β_1, rapidly inactivating currents were observed (τ=45±5ms, n=6). On the other hand, using a chimaera with a longer stretch of 381 amino acids from β_3 and only the last 27 from β_1, rapid inactivation was absent. This suggests that a sequence 229-381 is responsible for loss of inactivation in β_3. This was narrowed down further by replacing 28 amino acids of β_3 (residues 354-381) by the equivalent β_1 sequence (347-374); this conferred rapid inactivation (τ=30±4ms, n=6) to Kv1.5α currents. Therefore the results suggest that residues 354-381 of β_3 underlie the loss of inactivation of β_3.

Given the presence of the functional ball domain on the β_3 subunit, one possible explanation of our results might be that the β_3 subunit contains an inactivation-inhibitory domain like the previously-described N-type inactivation-prevention (NIP) domain (Roeper *et al.*, 1998) in the α subunit of Kv1.6. Other possibilities are being explored.

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129P EFFECTS OF REBOXETINE, FLUVOXAMINE AND AMITRIPTYLINE UPON SPONTANEOUS PUPILLARY FLUCTUATIONS IN HEALTHY HUMAN VOLUNTEERS

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Pupil diameter fluctuates spontaneously in darkness: these fluctuations are accentuated when subjects are drowsy (Lowenstein *et al.*, 1963, Yoss *et al.*, 1970). The recently developed "pupillographic sleepiness test" (PST) records and quantifies "pupillary fatigue waves" (Lüdtke *et al.*, 1998), and has been used to detect daytime sleepiness both in healthy sleep-deprived subjects (Wilhelm *et al.*, 1998a) and in patients suffering from sleep disorders (Wilhelm *et al.*, 1998b). We have used this test to compare the sedative effects of amitriptyline, a tricyclic antidepressant of known sedative property, and of fluvoxamine and reboxetine, two novel antidepressants with little clinical sedative potential (Szabadi & Bradshaw 1995).

13 healthy males (19-30 years) participated in 4 weekly sessions, associated with one oral drug condition (placebo, fluvoxamine 100 mg, reboxetine 4 mg and amitriptyline 100 mg), according to a balanced double-blind design. Pupil diameter was recorded continuously over 11 min in darkness using a dedicated monocular television pupillometer (AMTech Weinheim Germany). Average pupil diameter, power of pupil diameter fluctuations (obtained by Fast Fourier transformation), and the pupillary unrest index (PUI), a measure of cumulative changes in pupil size, were computed (Lüdtke *et al.*, 1998). Subjective "alertness", "anxiety" and "contentedness" were rated using visual analogue scales (Bond & Lader 1974). Measurements were carried out 30 min after arrival in the laboratory and 3 h after drug ingestion. Table 1 shows the effects of the 4 treatments on pupillary measures and self ratings, defined as the differences between pre- and post-treatment values.

Amitriptyline increased pupillary fatigue waves evidenced by increases in both the power of fluctuations and the PUI, consistent with the sedative property of the drug. The two other antidepressants failed to enhance these pupillary measures. The effect of amitriptyline upon pupillary fatigue was parallelled by decreases in "alertness" and "contentedness" ratings. The small reduction in alertness rating after reboxetine is in contrast with the reduction of the PUI caused by the drug which in fact may rather suggest a mild alerting effect. The PST seems to be suitable for the detection of drug-induced changes in the level of arousal.

Table 1. Effects of treatments (mean ± s.e. mean)

	Placebo	Fluvoxamine	Reboxetine	Amitriptyline
Δ Pupil diameter (mm)	0.15 ±0.16	0.32 ± 0.13	0.87 ± 0.28	-0.56 ± 0.27
Δ Power of fluctuations (arbitrary units)	-60.10 ±207.80	-450.57 ±288.48	-79.17 ±281.53	760.73 * ±267.08
Δ PUI (mm min^{-1})	-0.02 ±0.71	-0.77 ±0.55	-0.86 ** ±1.14	3.03 ** ±1.26
Δ Alertness Rating (mm)	0.49 ±2.75	-4.69 ±1.93	-9.56 * ±3.25	24.42 ** ±2.38
Δ Anxiety rating (mm)	2.94 ±2.56	0.40 ±2.90	5.78 ±2.29	-4.68 ±3.52
Δ Contentedness rating (mm)	2.08 ±1.74	-2.94 ±1.82	-3.00 ± 2.59	-10.56 ±1.87

Significance of difference from placebo treatment (ANOVA [repeated measures] followed by Dunnett's corrected t-test): * P<0.05 **P<0.01.

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130P THE EFFECTS OF SOME ANTIDEPRESSANTS ON PREPULSE INHIBITION OF THE ACOUSTIC STARTLE REFLEX IN MAN

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The acoustic startle response can be suppressed by presentation of a brief low-intensity auditory stimulus 30-500 ms before the 'startle-eliciting' stimulus ('prepulse inhibition', PPI) (Swerdlow *et al.*, 1992). We have examined the effects of three antidepressant drugs on the electromyographic (EMG) component of the startle eyeblink response and the N1/P2 complex of the auditory evoked potential (Mauguiere *et al.*, 1995), and on PPI of these responses: amitriptyline (tricyclic antidepressant), fluvoxamine (selective serotonin reuptake inhibitor) and reboxetine (selective noradrenaline reuptake inhibitor) (Szabadi & Bradshaw, 1995).

15 males (19-30 years) participated in 4 weekly sessions, in which they received placebo, fluvoxamine 100 mg, reboxetine 4 mg and amitriptyline 100 mg (p.o.) according to a balanced double-blind design. EMG recording via electrodes placed on the orbicularis oculi muscle and electroencephalographic recording via a vertex electrode were carried out 195 min after drug ingestion. Subjects received 40 trials separated by variable intervals (mean 25 s, range 15-35 s); the acoustic stimuli (1 kHz) were: (i) 40 ms, 115 dB ('pulse alone' [PA] trials), and (ii) 40 ms, 85 dB, followed after 120 ms by 40 ms, 115dB ('prepulse/ pulse' [PP] trials) (20 trials of each type, in random sequence). Mean amplitudes [A] of the EMG response and the N1/P2 complex were derived from the PA trials, and in each case, percent PPI was calculated as $100.[A_{PA}-A_{PP}]/A_{PA}$ (Swerdlow *et al.*, 1992). Results were analyzed by repeated-measures ANOVA, followed by comparisons of active treatments with placebo by Dunnett's test.

Under the placebo condition, both the EMG response and the N1/P2 complex showed >50% PPI. Fluvoxamine and reboxetine did not significantly alter the amplitude of either response, nor PPI of either response. Amitriptyline significantly reduced the amplitudes of both responses; it had no effect on PPI of the EMG response, but significantly attenuated PPI of the N1/P2 complex (Table 1).

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

	eyeblink EMG		N1/P2 complex	
Treatment	amplitude, mV	%PPI	amplitude, μV	%PPI
Placebo	0.73±0.10	51.0±10.0	47.3±4.0	65.7±9.8
Fluvoxamine	0.89±0.20	45.8±10.2	42.4±5.0	61.0±9.6
Reboxetine	0.60±0.10	56.5±9.7	41.6±5.0	72.0±7.8
Amitriptyline	0.20±0.04*	50.4±15.0	30.2±4.0*	30.9±16.2*

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

The results confirm the susceptibility of the N1/P2 complex to PPI (Abduljawad *et al.*, 1999). The reduction of the amplitudes of the EMG response and N1/P2 complex by amitriptyline may be related to its sedative action (Szabadi & Bradshaw, 1995). The selective effect of amitriptyline on PPI of the N1/P2 complex supports the suggestion that different mechanisms may be involved in PPI of this response and PPI of the EMG response (Abduljawad *et al.*, 1999).

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131P ANTICONVULSANT PROPERTIES OF THE SELECTIVE 5-HT$_6$ RECEPTOR ANTAGONIST SB-271046 IN THE RAT MAXIMAL ELECTROSHOCK SEIZURE THRESHOLD TEST

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There is growing evidence that serotonergic neurotransmission modulates a variety of experimentally induced seizures, but little is known about the 5-HT receptor subtypes involved (see Upton, *et al*, 1998). The present study was undertaken to determine the effect of the selective 5-HT$_6$ receptor antagonist 5-Chloro-3-methylbenzo[b]thiophene-2-sulfonic acid (4-methoxy-3-piperazin-1-ylphenyl)amide (SB-271046) (Bromidge *et al.*, 1999) in the rat maximal electroshock seizure threshold (MEST) test.

In this model, male Sprague Dawley rats (100-150g) were assessed for production of tonic hindlimb extensor seizures following application of a single corneal electroshock. CC$_{50}$ values (current producing tonic seizure in 50% of animals) were determined for each treatment group (n=12-14) using an 'up and down' method of shock titration (Upton *et al.*, 1998).

SB-271046 (0.1-30mg/kg p.o, 4 hour pretest) was found to elevate the threshold for generalised seizures in the rat MEST test, in a largely dose-related fashion, with a minimum significantly effective dose of ≤0.1mg/kg ($P<0.05$ compared to 1% methylcellulose vehicle control; Litchfield and Wilcoxon, 1949) and a maximum increase of 167±3% at 30mg/kg p.o. ($P<0.001$). At 10mg/kg p.o., this anticonvulsant action was prolonged (≥8 hours), with a peak rise in seizure threshold of 125±26% from a control CC$_{50}$ value of 33.3±2.7mA, at 4 hours post-dose. Furthermore, there was no evidence that tolerance developed to the anticonvulsant properties of SB-271046 following repeated administration at 10mg/kg p.o. b.i.d. for 7 days (increases in seizure threshold of 58±6% and 60±13% for a single 10mg/kg p.o. dose, 4 hours post-dose and repeated treatment, 4 hours after the last dose, respectively; $P>0.05$).

In all studies, the anticonvulsant properties of SB-271046 were evident in the absence of any observed overt behavioural depressant effects.

In summary, SB-271046 has been shown to produce potent and long-lasting anticonvulsant activity in the rat MEST test although the magnitude of this effect was modest in comparison to that of known antiepileptic drugs, such as carbamazepine, evaluated in the same model (Upton *et al.*, 1997). The present finding is of particular interest in view of previous evidence indicating that agents which elevate serotonergic neurotransmission are more generally associated with anticonvulsant properties (see Upton *et al.*, 1998).

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132P EFFECTS OF VEHICLE INJECTIONS ON THE PREFERENCE OF C57 STRAIN MICE FOR ALCOHOL

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The inbred C57 strain of mice has been used for many years for studies on their high voluntary alcohol consumption (Unwin and Taberner, 1982). We recently found that, given a free choice between 8% ethanol or water, these mice vary considerably in their alcohol intake, some having very low preference, which is increased by minor stress, such as saline injections (Wilson et al., 1997). In this study we investigate further the effect of vehicle injections and determine the actions of diazepam on ethanol preference.

The mice (25-30g) were male or female C57/Bl0 mice from an in-house breeding programme, housed singly, with ad lib access to food. Mice were first identified as high or low preference mice on the basis of a screening procedure in which the ratio of 8% v/v ethanol to water consumed was monitored 3 days per week for 3 weeks. Mice with a low ratio (less than 0.4) over the last 3 screening measurements were used for the subsequent studies.

The first study compared the effects of intraperitoneal injections of saline (0.3 ml), compared with handling the mice in the same manner as for the injections, but without the actual injection (n=9 per group). The second experiment, on different mice, compared the effects of intraperitoneal injections of Tween vehicle (0.3 ml of 0.5% Tween 80 in distilled water) (n=8) with the handling procedure (n=9), and with corresponding injections of diazepam in Tween vehicle (n=7). Diazepam was given at 1 mg/kg, a dose with clear effects against anxiety-related behaviour (Cole and Rodgers, 1995). In both studies, tap water or 8% ethanol was continuously available, and the amount of each fluid consumed was measured. After one week of baseline measurements, the treatments were carried out once daily for 3 weeks. The mean ratio of ethanol to water was calculated, for each mouse for each week. Analysis was by paired and unpaired Student's t-test.

In the third week (Table 1) the alcohol preference after intraperitoneal saline injections was significantly higher than that after the handling procedure ($P<0.05$). By the third week of the second experiment (Table 2) the ethanol preference after Tween injections or diazepam showed significant increases from baseline (both $P<0.01$) but there was no effect of handling compared with baseline ($P>0.1$). Within the third week, the mean preference after Tween injections was higher than that after handling, but the difference did not reach significance. The difference between diazepam and handling was significant, but there was no significant effect of diazepam compared with Tween vehicle. There were no changes in total fluid intake in any of the studies.

The results indicate that it is the procedure of saline injection which increases the alcohol consumption of low ethanol preference C57 mice. In contrast to our previous results with a CCKB antagonist (Wilson et al., 1997), diazepam did not reduce the alcohol preference of these mice.

Table 1: Ratio of alcohol to total fluid consumed mean ± s.e.m. P values for comparisons within week 3

Treatment	Baseline	Week 3	
Handling	0.26±0.03	0.24±0.03	
Saline	0.23±0.03	0.4±0.06	$P < 0.02$

Table 2: Ratio of alcohol to total fluid consumed, mean ± s.e.m. P values are for comparisons within week 3

Treatment	Baseline	Week 3	cf handling	cf Tween
Handling	0.31±0.03	0.35±0.04		
Tween	0.28±0.03	0.46±0.04	0.1>P>0.05	
Diazepam	0.31±0.03	0.48±0.03	$P < 0.05$	P>0.1

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