37P ROLE OF CALCIUM IN THE M_3 MUSCARINIC RECEPTOR-MEDIATED POTENTIATION OF A_{2B} ADENOSINE RECEPTOR-INDUCED CYCLIC AMP ACCUMULATION IN HEK 293 CELLS

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HEK (Human Embryonic Kidney) 293 cells endogenously express A_{2B} adenosine receptors positively coupled to cyclic AMP (cAMP) accumulation (Cooper *et al.*, 1997). We have previously shown that co-stimulation of these cells with histamine (HA, 100 μM) produces a greater than additive cAMP generation in an HEK 293 cell clone stably over-expressing recombinant human H_1 histamine receptor DNA (568 ± 69 fmol/mg protein) (Walker *et al.*,1998). HA alone (100 μM) did not elicit a significant cAMP generation (Walker *et al.*,1998). In this study, we have investigated this potentiation of A_{2B} adenosine receptor-mediated cAMP generation by $G_{q/11}$ coupled M_3 muscarinic receptors, which are endogenously expressed in these cells (Jackson *et al.*, 1999).

HEK 293 cells stably transfected with recombinant human H_1 histamine receptor DNA (Presland & Hill, 1998) were used for all experimental work and were grown as described previously (Cooper *et al.*, 1997). Competition radioligand binding studies were performed on total cell particulate preparations using [^{3}H]-QNB as radioligand. Non-specific binding was defined using 1 μM atropine. Cyclic AMP and total inositol phosphates (IP) accumulations, and changes in intracellular Ca^{2+} were measured as described previously (Neil *et al.*, 1997, Walker *et al.*, 1998, Iredale *et al.*, 1995).

M_3 muscarinic receptors were expressed at a level of 82 ± 12 fmol/mg protein (n = 4, data are mean ± s.e.m. in this and in all subsequent cases). Carbachol (CCh, 300 μM) did not elicit any cAMP generation (-0.5 ± 1.7% of 10 μM NECA cAMP response, n = 8). Co-stimulation with NECA (10 μM) and CCh (300 μM) produced a significantly greater cAMP response ($P<0.001$, n = 6, paired t-test) than stimulation with NECA alone(218 ± 13 % of 10 μM NECA response). Incubation in Ca^{2+} free medium (containing 0.1 mM EGTA) significantly ($P<0.05$, one-way ANOVA, Neuman Keuls *post-hoc* test) reduced (by 37 ± 11%, n = 3) the cAMP generation following co-stimulation with NECA and CCh. Chelation of intracellular Ca^{2+} with BAPTA/AM (50 μM) under the Ca^{2+} free conditions described above significantly ($P<0.001$, one-way ANOVA, Neuman Keuls *post-hoc* test) reduced (by 80 ± 3%, n = 3) CCh induced potentiation of cAMP accumulation following co-stimulation with NECA and CCh. Maximal IP accumulations elicited by carbachol (300 μM, 27.1 ± 1.5 fold over basal) and histamine (100 μM, 26.3 ± 0.4 fold over basal) were not significantly different (n = 4, paired t-test).

In summary, potentiation of A_{2B} receptor-mediated cAMP generation occurs with activation of either endogenously expressed or stably overexpressed $G_{q/11}$ coupled receptors in HEK 293 cells, dependant predominantly on intracellular calcium elevations. Given the similarities in maximal IP accumulations and levels of cAMP potentiation by H_1 and M_3 receptors, and the differences in expression levels of these receptors, coupling efficiency is also important in this cross-talk phenomenon.

AMJ holds an MRC studentship.

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38P *IN VIVO* INACTIVATION OF IMIDAZOLINE$_2$ BINDING SITES BY A NOVEL IRREVERSIBLE LIGAND

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Our group has recently reported the synthesis of a selective irreversible ligand for the imidazoline$_2$ binding site (I_2-BS) BU99006, Coates *et al.*, (2000). Previously a radio-iodinated photoaffinity ligand ([^{125}I]AZIPI) has been prepared and shown to label at least three subtypes of I_2 sites that differ in their ligand recognition properties, apparent molecular weight and tissue distribution (Lanier *et al.*, 1993). However, since a photo-affinity label requires photoactivation it is not possible to use such a ligand *in vivo*. Here we present evidence of irreversible inactivation of I_2-BS *in vivo*.

Rats (Male, Wistar, 240g) were anaesthetised using sodium pentobarbital (60mg kg^{-1} i.p.) and treated with either vehicle (saline) or BU99006, 15mg kg^{-1}, i.v. 30 minutes post injection the rats were perfused with ice-cold phosphate buffered saline approximately 1ml g^{-1} to wash off any unreacted BU99006. The brains were removed and membranes prepared based on the methods of Lione *et al.*, (1998). Sodium orthophosphate (50mM) was used instead of TRIS HCl (50mM), to prevent the isothiocyanate of BU99006 reacting with the amino group of TRIS. The effect of the BU99006 treatment on the I_2-BS was investigated using [^{3}H]-2BFI binding (Lione *et al.*, 1998). The treated rat brain membranes (~400μg) were incubated in triplicate with increasing concentrations of [^{3}H]-2BFI (0.001 - 5nM) in buffer (50mM sodium orthophosphate, 1mM $MgCl_2$, pH 7.4), in the absence (total binding) or presence (non-specific binding) of 10μM BU224 (Lione *et al.*, 1998) in a final volume of 500μl. The data were analysed using the nonlinear regression analysis supplied with GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA.

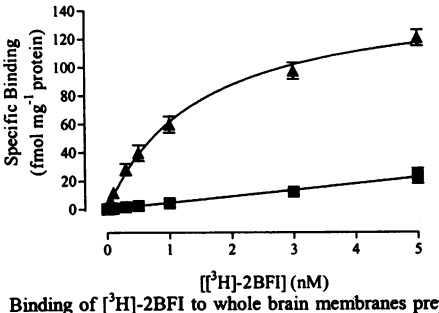

Figure 1. Binding of [^{3}H]-2BFI to whole brain membranes prepared from treated rats. Vehicle saline controls (▲) and BU99006 15mg kg^{-1} (■). Each curve represents the means of three animals (assay performed in triplicate), vertical bars represent the s.e.mean.

Figure 1 shows the saturation binding of [^{3}H]-2BFI to the rat brain membranes prepared from the treated animals. The K_D and B_{MAX} for the vehicle control, 1.6 ± 0.3 nM and 153 ± 8 fmol mg^{-1} protein respectively, agree with previously published data (Lione *et al.*, 1998). However, it was not possible to obtain a true value for the BU99006 treated animals for either K_D or B_{MAX}, also the curve obtained for these animals was the same as the non-specific binding (data not shown) indicating that all the I_2-BS were no longer available for binding. These data show that BU99006 inactivates I_2-BS *in vivo* and will be a very useful tool in the further understanding of their function in brain.

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39P INHIBITION OF IMIDAZOLINE I_2 SITE BINDING BY POTASSIUM CHANNEL MODULATORS

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The I_2 class of imidazoline receptors is heterogenous with at least one binding protein being associated with monoamine oxidase while the nature of other I_2 binding proteins is unknown (Eglen *et al.*, 1998). As binding to I_2 receptors is K^+ sensitive and at least one other imidazoline receptor, the I_3 receptor, is known to be linked to a K_{ATP} channel (Eglen *et al.*, 1998), we investigated whether potassium channel modulators affect the binding of [^{3}H]2-BFI (2-benzofuran-2-yl-4,5-dihydro-1H-imidazoline) to I_2 sites in rat brain membranes.

Competition binding studies were based on the methods of Hudson and Lione (1998). Brain membranes were prepared from Wistar rats (male, 250-300g) and aliquots incubated with 1nM [^{3}H]2-BFI (approximately K_D concentration) in a Tris-HCl buffer (50mM, pH 7.4). The assays were terminated after 30 minutes by rapid filtration. The competing ligands used were glibenclamide (a K_{ATP} channel blocker), diazoxide (a K_{ATP} channel activator) and 4-aminopyridine (4-AP) (a voltage-activated potassium channel blocker). Non-specific binding was determined using 10μM 2-(4,5-dihydro-1H-imidazol-2-yl)-quinoline (BU224). Data were analysed by GraphPAD Prism.

The K_i values obtained from the competition assays demonstrated that both glibenclamide (K_i = 77±14μM) and diazoxide (K_i = 41±5μM) have similar affinities for displacing [^{3}H]-2BFI. 4-AP displayed a biphasic curve (K_i = 22.5±5μM; 350±123μM). KCl proved to have low affinity (K_i = 23±3.5mM). The results demonstrate that K_{ATP} channel modulators and voltage-activated potassium channel modulators are able to inhibit [^{3}H]-2BFI binding in rat brain

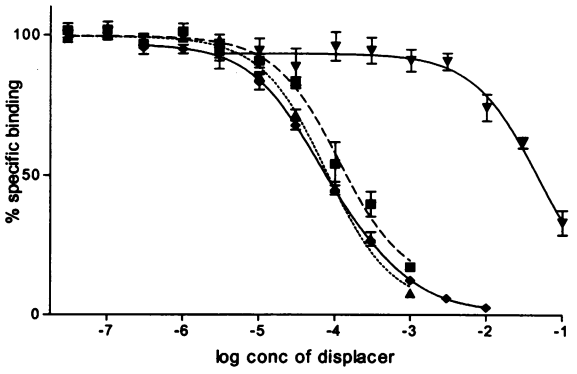

Figure 1. Displacement of [^{3}H]-2BFI from rat brain membranes by (▲) diazoxide (n=6), (◆) 4-AP (n=7), (■) glibenclamide (n=6) and (▼) KCl (n=4).

membranes. BaCl was the only modulator that did not display affinity for the I_2-binding site. That these non-imidazoline compounds retain any affinity is somewhat unexpected, although this may have implications in resolving the actions of I_2 binding sites *in vivo*. Further studies are required to determine whether a functional link exists between I_2 binding sites and potassium channels.

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D.A. Slattery is a BBSRC Case Award student with Organon Laboratories Ltd.

40P [^{3}H]-SB-269970 RADIOLABELS 5-HT$_7$ RECEPTORS IN HUMAN BRAIN MEMBRANE HOMOGENATES

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The selective 5-HT$_7$ receptor antagonist radioligand [^{3}H]-SB-269970 ((R)-3-(2-(2-(4-Methyl-piperidin-1-yl) ethyl) -pyrrolidine-1-sulfonyl)-phenol), has been reported to label the human recombinant 5-HT$_{7(a)}$ receptor and 5-HT$_7$ receptors in guinea pig, mouse, rat, and pig brain (Thomas *et al.*, 2000 ; Atkinson *et al.*, 1999). In the present study we have investigated the binding of [^{3}H]-SB-269970 to human brain membrane homogenates.

Radioligand binding to washed membranes prepared from *post mortem* human thalamus, hippocampus and cerebral cortex (no history of neurological disease) was carried out according to the method of Thomas *et al.* (2000) using 0.1-10nM [^{3}H]-SB-269970 for saturation studies and 1nM [^{3}H]-SB-269970 for drug competition studies. 10μM 5-HT was used to define non-specific binding.

[^{3}H]-SB-269970 bound saturably and apparently monophasically to human thalamic (K_d 1.6 ± 0.2nM, B_{max} 33 ± 0.2 fmoles mg protein^{-1}) and hippocampal (K_d 1.8 ± 0.5nM, B_{max} 12 ± 3.3 fmoles mg protein^{-1}) membranes. In contrast, the level of [^{3}H]-SB-269970 binding to cerebral cortex membranes was very low and unquantifiable. Specific binding at 1nM [^{3}H]-SB-269970 represented 36 and 61 % of total binding in hippocampal and thalamic membranes, respectively and K_d values were similar to that reported for binding to the human recombinant 5-HT$_{7(a)}$ receptor. The relative level of [^{3}H]-SB-269970 binding to human thalamus, hippocampus and cerebral cortex membranes correlated well with the relative level of 5-HT$_7$ mRNA determined in these human brain regions by TaqMan RT-PCR (Hagan *et al.*, 2000). The rank order of affinities (pK_i) for a range of 5-HT$_7$ receptor agonists and antagonists correlated well with that for [^{3}H]-SB-269970 binding to the human recombinant 5-HT$_{7(a)}$ receptor (correlation coefficient 0.96) (Table 1). However, the agonist affinities were somewhat higher in the human thalamus compared to the recombinant system. Hill slopes for drug inhibition of [^{3}H]-SB-269970 binding to thalamus were not significantly different from 1, consistent with binding to a single population of receptors.

Table 1. Inhibition of [^{3}H]-SB-269970 binding to human cloned 5-HT$_{7(a)}$ receptors and human thalamic membranes.

	pK_i (mean ± s.e. mean; n ≥ 3)	
Compound	Human 5-HT$_{7(a)}$	Human Thalamus
Agonists		
5-CT	8.86 ± 0.15	9.59 ± 0.28
5-HT	8.10 ± 0.19	9.10 ± 0.10
8-OH-DPAT	6.94 ± 0.08	7.26 ± 0.14
Antagonists		
SB-269970-A	8.61 ± 0.10	8.92 ± 0.05
Mesulergine	7.63 ± 0.06	7.58 ± 0.10
Ritanserin	7.12 ± 0.12	6.80 ± 0.12
Clozapine	6.83 ± 0.12	6.78 ± 0.02

In summary, [^{3}H]-SB-269970 has been shown to radiolabel 5-HT$_7$ receptors in human thalamic and hippocampal membranes. The pharmacological profile of [^{3}H]-SB-269970 binding to brain homogenates appears to be consistent across species. In addition, the density of 5-HT$_7$ receptors in these human brain regions is relatively low and comparable to that reported previously in mouse, rat and pig brain tissue.

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41P INVESTIGATION OF 5-HT$_{1B}$ RECEPTOR FUNCTION IN RAT STRIATAL MEMBRANES USING [^{35}S]GTPγS BINDING

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Agonist stimulated [^{35}S]GTPγS binding is a well developed method used to measure G protein activation, the first step in translating an extracellular signal into an intracellular response. This method has been used in cell lines transfected with human 5-HT$_{1B}$ receptors (Selkirk *et al.*, 1998), but there are few studies relating this technique for native tissue 5-HT$_{1B}$ receptors. We have therefore used rat striatal membranes to investigate agonist activation of native 5-HT$_{1B}$ receptors using [^{35}S]GTPγS binding.

For receptor binding studies, membranes were assayed as described by Scott *et al.* (2000), with the following modifications. Assay buffer contained 100nM 8-OH-DPAT, 100nM pizotifen, 100nM SB-269970 (Lovell *et al.*, 2000), and 100nM SB-258585 (Hirst *et al.*, 2000) to preclude binding to 5-HT$_{1A}$, 5-HT$_2$, 5-HT$_7$ and 5-HT$_6$ receptors respectively. For GTPγS studies, striatal tissue from adult male Sprague Dawley (approx 250g) rats was dissected and prepared as described by Watson *et al.* (1998) with the following modifications: striatal membranes were pre-incubated in the presence of 300μM GDP followed by a second incubation in the presence of 0.2nM [^{35}S]GTPγS. Results were expressed as a percentage of basal [^{35}S]GTPγS binding, and agonist activity expressed as a fraction of the maximal 5-HT response to give a measure of intrinsic activity. For inhibition studies, antagonist concentration response curves were carried out in the presence of 3μM 5-HT and an apparent pA$_2$ calculated using the Gaddum equation (Lazareno *et al.*, 1993). Receptor binding assays gave similar pK$_i$ values for all compounds when tested with both the agonist ([^{3}H]5-CT) and the antagonist ([^{3}H]GR125743) radioligands (see Table 1). From the [^{35}S]GTPγS binding studies, 5-HT was defined as a full agonist (Table 1), 5-CT, sumatriptan and GR125743 showed partial agonism, wheras GR127935 and methiothepin had no intrinsic activity up to 10μM. SB-224289 and SB-236057, selective 5-HT$_{1B}$ receptor antagonists (Selkirk *et al.*, 1998 and Middlemiss *et al.*, 1999), decreased [^{35}S]GTPγS binding below basal levels (Table 1). From antagonist studies, SB-224289 and SB-236057 yielded apparent pA$_2$ values of 9.3 ± 0.2, and 8.7 ± 0.0 respectively.

Table 1. Summary of Radioligand Binding and [^{35}S]GTPγS Binding Data in Rat Striatal Membranes

	pK$_i$ [^{3}H]5-CT	pK$_i$ [^{3}H]GR125743	pEC$_{50}$	Intrinsic Activity	E$_{max}$ (% Basal Binding)
5-HT	8.1 ± 0.0	7.9 ± 0.1	6.5 ± 0.1	1.0 ± 0.0	139 ± 2
5-CT	8.6 ± 0.0	8.5 ± 0.1	7.1 ± 0.1	0.7 ± 0.1	125 ± 3
Sumatriptan	6.8 ± 0.0	6.6 ± 0.1	8.1 ± 0.2	0.7 ± 0.2	122 ± 3
GR127935	8.6 ± 0.1	8.5 ± 0.0	-	0.0 ± 0.0	-
GR125743	8.2 ± 0.0	8.0 ± 0.0	7.9 ± 0.1	0.7 ± 0.0	126 ± 1
Methiothepin	6.6 ± 0.1	6.7 ± 0.1	-	0.0 ± 0.0	-
SB-224289	8.0 ± 0.0	8.1 ± 0.0	5.2 ± 0.1	*	64 ± 2
SB-236057	8.0 ± 0.0	8.2 ± 0.0	6.5 ± 0.2	*	73 ± 4

* reduced below basal [^{35}S]GTPγS binding levels
All values are mean ± SEM from 3 independent experiments

These data suggest that 5-HT stimulated [^{35}S]GTPγS binding in rat striatal membranes is mediated predominantly via 5-HT$_{1B}$ receptors, and that this technique may be used to investigate the function of 5-HT$_{1B}$ selective compounds in native tissue. Further studies will be needed to define whether the inhibition of [^{35}S]GTPγS binding by SB-224289 and SB-236057 is due to blockade of an endogenous 5-HT response, or negative intrinsic activity.

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42P NORADRENERGIC MODULATION OF 5-HT RELEASE IN THE RAT DORSAL RAPHE NUCLEUS VIA α$_1$ AND α$_2$ ADRENOCEPTORS: *IN VITRO* VOLTAMMETRIC EVIDENCE

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The rat dorsal raphe nucleus (DRN) receives catecholaminergic innervation from noradrenergic areas such as the locus coeruleus as well as from adrenergic cell groups such as the dorsomedial medulla (Baraban & Aghajanian, 1981). Furthermore, activation of α$_1$ receptors present on the DRN 5-HT cell bodies is known to increase cell firing (Vandermaelen & Aghajanian, 1983). In the present study, we used fast cyclic voltammetry (FCV) to investigate a possible noradrenergic modulation of local 5-HT release in rat DRN.

DRN slices (350 μm thick) from male Wistar rats (150-200 g) were superfused with artificial cerebrospinal fluid (aCSF). The DRN was locally stimulated (0.1 ms pulses, 10 mA) and 5-HT efflux was monitored at carbon fibre microelectrodes using FCV (Stamford, 1990). All drugs were administered via the aCSF. Agonists were added for 40 min. Antagonists were allowed to equilibrate for a minimum of 90 min before the first stimulation. With long stimuli, the drug or vehicle was added for 2 h following 3 control release events.

When short stimulus trains (10 pulses, 200 Hz) were applied, the α$_1$-adrenoceptor agonist phenylephrine (5 μM) decreased 5-HT release to 49 ± 7 % of pre-drug controls at minimum (mean ± s.e.m., n = 4). This effect was blocked by the α$_1$-selective antagonist prazosin (1 μM) ($P < 0.001$ vs. phenylephrine; One Way ANOVA with post-hoc Tukey-Kramer test) and by the selective α$_{2A}$-antagonist BRL 44408 (2-[2H-(1-methyl-1,3-dihydroisoindole) methyl]-4,5-dihydroimidazole, 1 μM) (n = 4, $P < 0.01$), but was unaffected by the 5-HT$_{1A}$-receptor antagonist WAY 100635 (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide, 0.1 μM). The α$_2$-selective agonist dexmedetomidine (0.01 μM) also decreased stimulated DRN 5-HT efflux on short trains, to 44 ± 3 % of pre-drug values at minimum (n = 4). This response was antagonised by BRL 44408 (1 μM) ($P < 0.001$ vs. dexmedetomidine) but not by the selective α$_{2B/C}$-adrenoceptor antagonist ARC 239 (2-[2[4-(o-methoxyphenyl)piperazin-1-yl] ethyl]-4,4dimethyl-1,3-(2H,4H)-isoquinolinedione, 0.5 μM), WAY 100635 (0.1 μM) or prazosin (1 μM), suggesting that the effect of dexmedetomidine is attributable to α$_{2A}$-receptor activation. On long stimuli (30 pulses, 10 Hz), BRL 44408 (1 μM) increased evoked 5-HT efflux to 187 ± 17 % of pre-drug values at maximum (n = 4) ($P < 0.001$ vs. vehicle, t-test).

These data show that activation of both α$_1$ and α$_{2A}$-adrenoceptors can mediate a decrease in extracellular 5-HT levels in the DRN. Since the effect of dexmedetomidine was not antagonised by α$_1$ adrenoceptor blockade, this implies that its effect was mediated directly, possibly through α$_2$ receptors located on 5-HT cell bodies. This is in contrast to the data of Adell and Artigas (1999) which suggested that the effects of α$_2$ agonists in the median raphe nucleus were mediated via α$_2$ autoreceptors on afferent noradrenergic terminals.

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43P DOPAMINE ACTING AT D_4 RECEPTORS MODULATES NITRIC OXIDE RELEASE IN THE RABBIT RETINA

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Dopamine is an amacrine cell neurotransmitter in the mammalian retina. It is released when the retina is stimulated with light and is thought to be involved in light adaptation. Nitric oxide (NO) is also thought to contribute to the light adaptation process and interactions between the two transmitters have been demonstrated. Thus, in the rabbit retina NO inhibits the depolarization-induced release of endogenous dopamine (Djamgoz et al, 1995). In the present study, we examine the effects of dopamine receptor blockade on the release of NO from the retina.

Adult New Zealand white rabbits were anaesthetized with urethane (1.5g/kg I.P.) and an eye-cup was prepared. The dark-adapted eye-cup was filled with Krebs bicarbonate medium (0.5ml) which was replaced every 10 minutes. The samples collected were analysed for NO using a nitrate reductase assay and a NO meter (Neal *et al,* 1998).

Exposure of the retina to the specific D_4-receptor antagonist U-101,958 (10μM) caused a striking increase in the spontaneous resting release of NO to 175.8±10.8% of control values ($P<0.001$, n=17). In contrast, the specific antagonists at D_1 and D_2 receptors SCH23390 (10μM) and L-741,626 (10μM) respectively had no effect on the resting release of NO (94.7±4.7% of control, n=3 and 85.3±6.2% of control, n=3 respectively). In the retina, cis-2,3, piperidine dicarboxylic acid (PDA) (2mM) blocks all glutamate receptors except those on the depolarizing bipolar cells and L-2-amino-4-phosphonobutyric acid (APB) (20μM) specifically blocks transmission between the photoreceptors and depolarizing bipolar cells. Exposure of the retina to a combination of PDA and APB or to the non-selective glutamate antagonist kynurenic acid (500μM) blocked the U-101,958-induced release of nitric oxide (102.5±2.9% (n=4) and 101.5±1.2% (n=4) of control release respectively). PDA alone was found to have no effect on the U101,958-evoked release of NO causing an increase of 234±14% of control values ($p<0.05$, n=3). This suggests that the D_4-antagonist induced release of NO may originate from the depolarizing bipolar cells.

Our results suggest that in dark-adapted conditions, the resting release of nitric oxide is under tonic inhibitory control caused by dopamine acting on dopaminergic-D_4 receptors, probably located on the cone photoreceptors (for review see Nguyen-Legros *et al,* 1999).

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44P ANTAGONISM OF METABOTROPIC GLUTAMATE RECEPTOR-MEDIATED RESPONSES AND NOCICEPTIVE RESPONSES BY THE mGlu5-SELECTIVE RECEPTOR ANTAGONIST MPEP IN THE RAT THALAMUS

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Previous work from this laboratory has shown that postsynaptic excitatory responses of rat ventrobasal thalamus neurones to mGlu agonists can be blocked by antagonists which act non-selectively at Group I metabotropic glutamate receptors (*i.e.* mGlu1 and mGlu5), and by selective mGlu1 antagonists. These antagonists also cause a parallel reduction of nociceptive responses of thalamic neurones (Salt & Turner 1998). The recent development of the mGlu5-selective antagonist 6-methyl-2-(phenylethynyl)-pyridine (MPEP) (Gasparini *et.al.*, 1999) now allows the direct probing of possible mGlu5 involvement in thalamic nociceptive responses (Salt *et.al.* 1999).

Extracellular recordings were made from single neurones in the ventrobasal thalamus and immediately overlying dorsal thalamic nuclei of adult male Wistar rats anaesthetised with urethane (1.2 g/kg, I.P.) using multi-barrel electrodes (Salt & Turner, 1998). Responses of neurones to iontophoretic applications of the mGlu5-selective agonist (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG) were reduced during continuous iontophoretic application of MPEP (20-120 nA, from a 1mM pH5 solution in saline) whereas responses to either N-methyl-D-aspartate (NMDA) or 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (ACPD) were less affected in 17 neurones (Table 1A). Six of these neurones were also investigated using noxious thermal sensory stimuli (52°C, 20-30sec, applied to either the tail or contralateral hindpaw). Responses to such stimuli were reduced to 53±9.5 % of control responses by MPEP when the antagonist was applied with iontophoretic currents which reduced CHPG responses of the same neurones (Table 1B).

Table 1. Reduction of responses by iontophoretic MPEP. Values are % of control responses ± s.e.m. from *n* neurones.
* $P<0.05$; ** $P<0.01$

		CHPG	ACPD	NMDA	Noxious
A	*n*=17	32±3.3**	85±7.8*	101±8.7	-
B	*n*=6	29±5.3*	83±9.0	101±8.9	53±9.5*

When MPEP was given intravenously (1mg/kg bolus), responses to noxious stimuli of ten neurones were reduced to a mean of 24±4 % of control within 10 minutes, whereas saline injections had no significant effect (100±11%). In addition, MPEP caused a decrease in the peak frequency of the EEG power spectrum, and an increase in the amplitude of the peak frequency component of the EEG (231±43 % of control).

These results suggest that nociceptive responses of thalamic neurones are mediated in part by mGlu5 in addition to the known role of mGlu1 and NMDA receptors. Furthermore, the effectiveness of intravenous MPEP suggests that such antagonists may have analgesic properties.

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45P FACILITATION OF FAST (5-50 Hz) AND SLOW (0.1-1 Hz) OSCILLATIONS IN THALAMOCORTICAL NEURONES BY ACTIVATION OF GROUP 1 METABOTROPIC GLUTAMATE RECEPTORS

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The activity of thalamocortical (TC) neurones is critically dependent on the state of arousal. During sleep, these neurones exhibit recurring groups of delta (1-4Hz) and spindle (7-14Hz) oscillations at frequencies close to 0.1Hz. However, during wakefulness, the behaviour of TC neurones is characterised by epochs of oscillatory activity in the alpha (8-15Hz), beta (16-24Hz) and gamma (25-80Hz) bands. Although it is well known that delta and spindle oscillations can occur in the isolated thalamus, faster oscillations and 0.1Hz rhythmicity are thought to originate in cortical areas.

Here we show, however, that following activation of metabotropic glutamate receptors (mGluRs) with the non-specific Group I/II mGluR agonist, trans-ACPD (50-200mM), TC neurones are able to display robust oscillations at both high (5-50 Hz) and low (0.1 Hz) frequencies. This facilitation could be mimicked by application of the Group I specific mGluR agonist DHPG (50-100mM), but not by either the Group II specific agonist DCG-IV (100mM) or the Group III specific agonist L-AP4 (100mM).

The appearance of either oscillatory type was strongly voltage-dependent, with low-frequency activity occuring at hyperpolarized membrane potentials (<-65mV) and high-freqeuncy activity at depolarized membrane potentials (>-55mV). Abolition of low-frequency oscillations by Ni^{2+} (250-500mM), but not Cd^{2+} (250-500mM) or Co^{2+} (500mM), confirmed that they were dependent on the 'window' component of the T-type Ca^{2+} current, I_{Tw}, as observed previously in a small proportion (15%) of TC neurones in normal conditions.

In contrast, whilst high-frequency oscillations were also sensitive to Ni^{2+} (250-500mM) they additionally showed a slight sensitivity to both Cd^{2+} (250-500mM) and Co^{2+} (500mM) suggesting a minor involvement of high-threshold Ca^{2+} channels but a primary involvement of T-type Ca^{2+} channels.

Thus, activation of Group I mGluRs on TC neurones supports the generation of both fast and slow rhythms through a facilitation of similar ionic mechanisms.

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46P BACLOFEN INHIBITS ELECTRICALLY EVOKED GABA RELEASE FROM RAT SUBSTANTIA NIGRA SLICES WITHOUT EVIDENCE FOR AUTORECEPTORS

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The presence of $GABA_B$ autoreceptors on synaptosomes and slices prepared from many regions of the rat brain is well established. However, their existence in the substantia nigra (SN) has been questioned. Whilst Giralt et al (1990) state that they are present on GABA nerve terminals in the SN and that GABA controls its own release in this region, Waldmeier et al (1989) indicate that "GABA release from the SN is not controlled by GABA autoreceptors". We have therefore examined this further in an attempt to resolve the apparent discrepancy. SN slices (250μm x 250μm) were prepared from male Wistar rats and incubated in [^{3}H]-GABA(45nM for 20min at 35^{0}C). Aliquots of 6 slices were then rinsed in artificial cerebrospinal fluid containing tiagabine (10μM), aminooxyacetic acid (50μM) and β-alanine (100μM), and superfused (0.4 ml.min^{-1}, 35^{0}C, Brandel 2000) for 35 min before collection of consecutive 4 min samples. The slices were stimulated (40mA, 4Hz for 3.5min) 48 min (S1) and again 88 min (S2) after the start of superfusion. The $GABA_B$ receptor agonist, (-) baclofen and/or the antagonists, CGP54626A and CGP55845A (Froestl and Mickel 1997) were added to the superfusion solution 30 min before S2. Results were then expressed as S2:S1 ratios where the values of S1 and S2 represented amounts released above basal. The mean control S2:S1 ratios obtained from slices from 20 rats was 0.88± 0.09 (±s.e.mean). This value was significantly reduced to 0.40 ± 0.08(n=9) in the presence of (-) baclofen (10μM) and to a lesser extent by 1μM (-) baclofen (S2/S1= 0.58). The addition of the $GABA_B$ antagonists CGP55845A (10μM) or CGP54626 (10μM) significantly reduced the response to baclofen (10μM). In the presence of CGP55845A, baclofen only reduced S2/S1 to 0.85 ± 0.13 (n=6) and in the presence of CGP54626A (plus baclofen) the ratio was 0.66± 0.13 (n=3). These data would suggest that baclofen acts through $GABA_B$ receptors to suppress the evoked release of GABA. This effect was not detected by Waldmeier et al (1989). If autoreceptors are involved in this action then it might be expected that the antagonists applied in the absence of baclofen would increase the stimulated release of [^{3}H]-GABA. However, the S2:S1 ratios obtained in the presence of the antagonists alone (0.73± 0.17 n=5, 0.79± 0.14 n=4, CGP55845 and CGP54626 respectively) were not significantly different from the control S2:S1 value. Thus no direct evidence for the presence of autoreceptors was obtained.

Alternatively $GABA_B$ receptor-mediated inhibition of the evoked release may be produced via an intermediary mechanism within the slice. We can conclude that $GABA_B$ receptor activation can reduce the evoked release of [^{3}H]-GABA from the SN contrary to the results of Waldmeier et al (1989). Nevertheless we would support their conclusion regarding the apparent lack of autoreceptors in this brain region.

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47P SPINAL CAPSAZEPINE REDUCES Aδ- AND C-FIBRE-EVOKED RESPONSES OF DORSAL HORN NEURONES IN NON-INFLAMED AND CARRAGEENAN-INFLAMED ANAESTHETISED RATS

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The capsaicin activated vanilloid receptor subtype 1 (VR1) is present on peripheral and central endings of primary afferent nociceptors (Guo *et al.*, 1999). The physiological role of peripheral VR1 has been widely studied (see Cesare *et al.*, 1999). However, the role of spinal VR1 remains unknown. Here, the effects of spinal administration of a VR1 antagonist, capsazepine, on innocuous and noxious evoked responses of dorsal horn neurones have been studied in non-inflamed and carrageenin-inflamed rats.

Extracellular recordings of convergent dorsal horn neurones were made in aneasthetised (1% halothane in 66% N_2O / 33% O_2) male Sprague Dawley rats (Chapman *et al.*, 1994). Neuronal responses to transcutaneous electrical stimulation (3x C-fibre threshold, trains of 16 stimuli at 0.5Hz) of the receptive field were recorded and post-stimulus histograms were constructed. Responses were separated and quantified on the basis of post stimulus latencies: Aβ-fibre 0-20ms; Aδ-fibre 20-90ms; C-fibre 90-300ms and post-discharge 300-800ms. The last three recordings were taken as controls and 50µl of capsazepine (0.5- 30µM) was applied to the cord (n=6). Drug effects were followed every 10 minutes for 60 minutes per dose. In a separate group of rats (n=4), λ–carrageenan (100µl, 2% in saline) was injected into the plantar surface of a hind paw 3 hours before the capsazepine (0.5-30µM) dose-response data was obtained. Data are presented as mean maximal effects; statistical analysis was performed using repeated measures ANOVA and Dunnett's *post hoc* test.

The neurones studied were at a depth of 890 ± 58µm (mean ± s.e.m; n=10) and the C-fibre threshold and latency of responses was 1.6± 0.1mA and 207± 11ms, respectively. Control mean Aβ-, Aδ-, C-fibre and post-discharge responses of the neurones were 93 ± 11, 65 ± 9, 336 ± 47 and 229 ± 60 action potentials, respectively. Spinal administration of capsazepine (30µM) did not alter Aβ-fibre evoked responses of spinal neurones in non-inflamed (99±7% of control) or carrageenin inflamed rats (81±6% of control). However, capsazepine (0.5, 5, 10 and 30µM) reduced Aδ-fibre evoked responses of dorsal horn neurones in non-inflamed (65±8%; 52±9%; 46±8%; 31±8% of control, $p<0.0001$) and carrageenin-inflamed (74±9%; 60±13%; 52±12%; 43±7% of control, $P<0.01$) rats. Capsazepine reduced C-fibre evoked responses of dorsal horn neurones in both non-inflamed (77±6%; 73±12%; 72±13%; 58±10% of control, $P<0.001$) and carrageenin-inflamed (80±4%; 63±10; 62±9; 63±7% of control, $P<0.001$) rats. Finally, spinal capsazepine also reduced post-discharge responses of dorsal horn neurones in non-inflamed (67±8%; 58±11%; 61±11%; 41±14% of control, $p<0.0001$) and carrageenin-inflamed (56±16%; 48±18%; 40±16%; 26±14%, $p<0.001$) rats.

Antagonism of spinal VR1 significantly reduced both Aδ-fibre and C-fibre evoked responses of dorsal horn neurones. This functional role of VR1 corresponds with the location of VR1-immunoreactivity in small and medium sized dorsal root ganglia neurones (Guo *et al.*, 1999). Marked differences between the effect of capsazepine on evoked responses in non-inflamed and carrageenin inflamed rats were not observed, suggesting that this receptor system is not altered, at the level of the spinal cord, following peripheral inflammation.

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48P EFFECTS OF HYDROCORTISONE TREATMENT IN DOPAMINERGIC RECEPTOR BINDING IN RAT BRAIN STRIATUM

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Long term exposure to an increased level of glucocorticoids leads to psychosis, behavioral abnormalities, and substance abuse (Deroche et al., 1992, and Schimmer and Parker, 1996). Altered dopaminergic activity is implicated in psychosis, behavioral abnormalities, and substance abuse (Piazza and LeMoal, 1996). The purpose of this investigation is to study the effects of dihydrocortisone treatment on dopaminergic (D_2) receptor binding in rat brain striatum. Male Sprague-Dawley rats (200-225g) were divided in 2 groups having 10 rats in each group. Group 1 served as a control. Rats from Group 2 were given dihydrocortisone (100mcg/ml) in drinking water for 10 days. All the rats were allowed to have food and water ad libitum. Five rats from each group were sacrificed by decapitation at 9:00AM (for light phase), and five rats were sacrificed at 9:00PM (for dark phase). Dopamine (D_2) receptor binding was assayed in membrane preparations isolated from rat brain striatum using [^{3}H]-spiperone as a ligand and butaclamol to define non-specific binding (Tayebati et al., 1992). Data is presented in Table 1. Dihydrocortisone treatment (100mcg/ml in drinking water for 10 days) significantly increased ($P<0.05$) D_2 receptor binding in both light and dark phases in rat brain striatum. D_2 receptors may be involved in corticosteroid-induced behaviors. Supported by SDEPSCoR.

Table 1.

Group	Treatment	D_2 receptor binding (fmole spiperone bound/mcg protein/hr)	
		Light Phase	Dark Phase
1	Control	5.95±0.16	6.1±1.34
2	Dihydro-cortisone	12.52±1.87*	16.57±2.69*

*Significantly higher than control ($P<0.05$, students' t-test)

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49P BIA 3-202 DOES NOT POTENTIATE LOCOMOTOR HYPERACTIVITY DURING INCREASED DOPAMINERGIC STIMULATION

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The central effects of catechol-*O*-methyltransferase (COMT) inhibition are very small when given alone, but when given with L-DOPA the risk of inhibition of brain COMT may be associated with the appearance of symptoms related to increased dopaminergic stimulation. BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone) is a new long acting COMT inhibitor with limited access to the brain. The present study was aimed at determining the potentiation of amphetamine-induced hyperactivity of brain dopaminergic systems by BIA 3-202 and two other COMT inhibitors differing in their ability to access the brain (entacapone and tolcapone). For this purpose 128 rats (Harlan, U.K.) were divided into 16 groups, and were given by gastric tube the vehicle (4 ml kg^{-1} 0.5% carboxymethylcellulose) or 30 mg kg^{-1} of one of the three COMT inhibitors (BIA 3-202, entacapone and tolcapone) 6 h prior to behavioural evaluation); maximal effective doses were used (this meeting). In all groups of rats, behavioural testing started 15 min after the s.c. injection of vehicle (2 ml kg^{-1} saline) or increasing doses of amphetamine (0.5, 2.0 or 4.0 mg kg^{-1}). Spontaneous locomotor activity was measured using a San Diego Instruments rodent activity monitor (model Flex Field, San Diego Instruments, San Diego, CA) with 48 infrared motion sensors, as previously described (Parada & Soares-da-Silva, 2000). Stereotypical behaviour (intense sniffing, repetitive head and limb movements, licking and biting) was quantified by an independent observer after being recorded on tape by means of video tracking system (VP200, HVS Image, Ltd) placed 70 cm above the test field. Results are arithmetic means with s.e.mean. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. As predicted, low doses of amphetamine (0.5 and 2.0 mg kg^{-1}, s.c.) were found to produce dose-dependent increases in horizontal activity and rearing, with no evidence of stereotyped behaviour (Table 1). By contrast, a high dose of amphetamine (4.0 mg kg^{-1}, s.c.) was found to produce no further increase in locomotor activity, but resulted in the appearance of stereotypies that lasted for 250 s during the 600 s observation period. Tolcapone administered 6 h before amphetamine challenge was found to significantly increase locomotor activity in rats treated with 0.5 and 2.0 mg kg^{-1} amphetamine. In rats given 4.0 mg kg^{-1} amphetamine, tolcapone produced a marked decrease in locomotor activity and increased two-fold the duration of the stereotyped behaviour. Rats treated with BIA 3-202 or entacapone 6 hours before the amphetamine challenge presented the same pattern of locomotor activity and stereotyped behaviour as their corresponding controls. At 6 hours after 30 mg kg^{-1} BIA 3-202, entacapone and tolcapone, brain COMT activity was reduced by 32±3 %, 20±7 % and 86±8 %, respectively. In conclusion, limited access of BIA 3-202 to the brain may be associated with lack of ability to potentiate excessive dopaminergic stimulation by amphetamine.

Table 1. Total counts and duration of stereotypies 6 h after vehicle, BIA 3-202, tolcapone and entacapone (all at 30 mg kg^{-1}) administration in rats challenged with amphetamine (Amph). Results are means ± s.e.mean (n=8).

Amph	Vehicle	BIA 3-202	Entacapone	Tolcapone
(mg kg^{-1})		Total counts		
0	505±42	187±41	368±48	320±70
0.5	1266±94	1476±164	1067±107	1648±109 *
2.0	2433±95	2479±160	2766±186	3157±277 *
4.0	2216±250	2466±337	2216±248	1409±120 *
		Duration of stereotypies (s)		
4.0	266±88	286±87	206±66	558±17 *

Significantly different from corresponding values in vehicle trated rats (P<0.05).

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50P BIA 3-202 DOES NOT POTENTIATE AMPHETAMINE-INDUCED DOPAMINERGIC HYPERACTIVITY

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BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone) is a new long acting peripheral COMT inhibitor with limited access to the brain. The present study was aimed at determining the potentiation by COMT inhibitors differing in their ability to access the brain (BIA 3-202, entacapone and tolcapone) on rat striatal dopaminergic stimulation induced by amphetamine. Male Wistar rats (Harlan, U.K.; 280-320 g) were implanted (rostrocaudal 0.0 mm; lateromedial -3.4 mm; dorsoventral -6.0 mm, relative to bregma) with intracerebral guide cannulas for microdialysis probes (CMA/12 Guide Cannula, CMA Microdialysis, Stockholm, Sweden) under sodium pentobarbitone (60 mg kg^{-1}, i.p.) anaesthesia. On the day of the experiment (48 h after surgery), a probe was inserted into the intracerebral guide and rats transferred to a plastic bowl cage with a moving arm (CMA/120). The microdialysis probes (CMA/12) were perfused continuously with artificial cerebrospinal fluid, at a flow rate of 2 µl min^{-1}. The collection of the dialysate was started at the end of the 2 h stabilisation period (t=0 min) and lasted for 120 min. At t=30 min amphetamine (4 mg kg^{-1}, s.c.) or vehicle (saline, 2 ml kg^{-1}, s.c.) were administered. Dialysate samples were collected every 20 min. COMT inhibitors (30 mg kg^{-1}, p.o.) and vehicle (0.5% carboxymethylcellulose, 4 ml kg^{-1}) were administered 1 h or 6 h prior to the start of dialysate collection (t=0 min); maximal effective doses were used (this meeting). Dopamine (DA) and metabolites (DOPAC and HVA) were assayed by h.p.l.c. with eletrochemical detection (Vieira-Coelho & Soares-da-Silva, 1999). Results are arithmetic means with s.e.mean. Statistical differences were determined by ANOVA followed by the Newman-Keuls test. The administration of amphetamine increased the extracellular levels of DA, which peaked at t=100 min (995±173 and 594±213 fmol 20 min^{-1}, 1 h and 6 h after vehicle administration, respectively), without significant changes in extracellular levels of DOPAC and HVA. Extracellular levels of DA, DOPAC and HVA, before and after the stimulatory effect of amphetamine, in rats treated for 1 h and 6 h with BIA 3-202 and entacapone were similar to those in corresponding controls. A similar picture occurred in rats treated for 1 h with tolcapone. In rats given tolcapone 6 h before the amphetamine challenge, there was a significant (P<0.05) increase in the extracellular levels of DA following the administration of amphetamine (figure 1). Significant (P<0.05) increases in DOPAC and decreases in HVA levels accompanied this effect of tolcapone. At 6 hours after 30 mg kg^{-1} BIA 3-202, entacapone and tolcapone, brain COMT activity was reduced by 32±3%, 20±7% and 86±8%, respectively. In conclusion, limited access of BIA 3-202 to the brain may be associated with lack of ability to potentiate excessive dopaminergic stimulation by amphetamine.

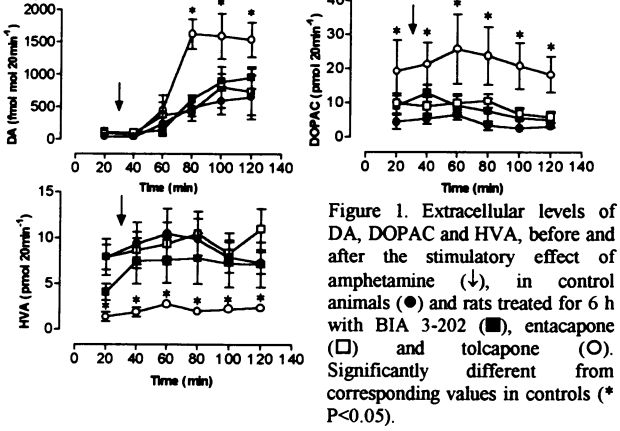

Figure 1. Extracellular levels of DA, DOPAC and HVA, before and after the stimulatory effect of amphetamine (↓), in control animals (●) and rats treated for 6 h with BIA 3-202 (■), entacapone (□) and tolcapone (○). Significantly different from corresponding values in controls (* P<0.05).

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51P AGMATINE, HARMANE, IBOGAINE AND THE MONOAMINE OXIDASE INHIHBITOR RO41-1049 SUBSTITUTE FOR THE IMIDAZOLINE I_2 SITE LIGAND 2-BFI IN THE RAT DRUG DISCRIMINATION PARADIGM

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The specific imidazoline I_2 site ligand 2-(-2-benzofuranyl)-2-imidazoline (2-BFI) generates a 'cue' in rat drug discrimination (Jordan *et al.*, 1996) and structurally related compounds dose dependently substitute for 2-BFI (MacInnes & Handley, 1999). Endogenous substances that bind to imidazoline sites include agmatine (decarboxylated arginine) (Li *et al.*, 1995). Certain endogenous (e.g. harmane) and non-endogenous (e.g. ibogaine) beta carbolines have a high affinity *in vitro* for I_1 and I_2 sites (Moncrieff, 1989; Hudson *et al.*, 1999). Additionally, some I_2 specific ligands, including 2-BFI inhibit MAO (Carpene *et al.*, 1995). We have examined the ability of such substances to substitute for 2-BFI in drug discrimination.

Eight male Hooded Lister rats were taught to discriminate 2-BFI ($7mgkg^{-1}$ i.p. -20 min) from saline vehicle, in two lever skinner boxes with condensed-milk reward as described previously (Jordan *et al.*, 1996; MacInnes & Handley, 1999). Fig. 1 shows that harmane, ibogaine and the MAO-A inhibitor RO 41-1049, administered i.p. at 3, 6 and 9 $mgkg^{-1}$ dose-dependently increased 2-BFI-appropriate responding; all doses were significantly different from saline controls ($P<0.05$ in each case, *post-hoc* contrasts after Friedman non-parametric ANOVA), except $3mgkg^{-1}$ RO41-1049. At 30 and 50 $mgkg^{-1}$, ($p<0.05$), but not at 10 $mgkg^{-1}$ ($p>0.05$), agmatine also showed significant 2-BFI-appropriate responding. Deprenyl, being an irreversible MAO-B, inhibitor was tested in a single ($4mgkg^{-1}$) dose at the end of the experiments and the increase in 2-BFI-appropriate responding was not significant ($p>0.05$).

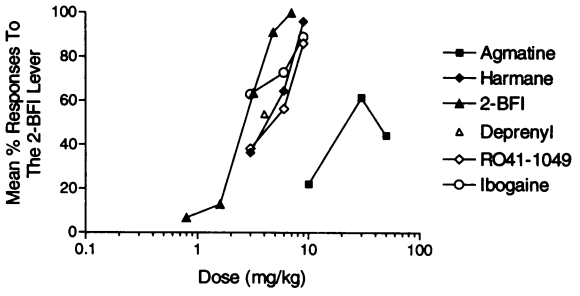

Figure 1. Discriminative properties of imidazoline associated ligands.

These results indicate that agmatine exerts bioactivity in the rat. The ability of the beta carbolines to substitute potently for 2-BFI *in vivo* confirms their high affinity for I_2 sites *in vitro*. RO41-1049 also showed high levels of substitution suggesting the 2-BFI cue may also be associated with the inhibition of MAO-A. *This work was supported by the BBSRC. 2-BFI was kindly donated by Pierre Fabre.*

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52P DIFFERENTIAL EFFECTS OF 5-HT_{1A} RECEPTOR ACTIVATION IN A COMBINED DELAYED-MATCHING/ NON-MATCHING-TO-POSITION TASK

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Delayed matching/non-matching-to-position (DM/DNMTP) tasks have become standard psychopharmacological tools for the investigation of drug effects on short-term memory function in rodents. However, they are usually conducted in isolation or, at least, with two separate groups of animals, one trained to a DMTP routine and the other to a DNMTP routine. 8-Hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) has previously been shown to improve performance in a DMTP task (Cole et al., 1994), but is also associated with an impairment of accuracy in short-term memory tasks (e.g. Deacon, 1991; Carli and Samanin, 1992). In this study we have used a combined DM/NMTP task to examine more closely the effect of 8-OH-DPAT-induced $5HT_{1A}$ receptor activation on both matching and non-matching performance accuracy. By combining the tasks, depending on the relative effects on trial performance accuracy, it would indicate the relative importance of $5HT_{1A}$ receptors for a given style of trial.

Standard operant conditioning chambers equipped with two retractable levers situated either side of a food panel flap were used. Male hooded Lister rats, weighing 150-200 g at the start of the study, were trained to press a sample lever (right or left) inserted into the operant chamber following the presentation of a 5 s stimulus (flashing houselight or darkness). Once pressed, the sample lever retracted and the animals were required to press the food panel flap until a randomly determined delay (1, 4, 8 or 16 s) had elapsed. Upon delay expiry, both levers were extended into the operant chamber and the animal required to press a lever according to the stimulus, e.g. the right lever if they had been exposed to darkness and the left lever if the stimulus had been the flashing houselight. Thus according to which lever had been the sample lever, animals were exposed to matching-to-position or non-matching-to-position styles of trial. Importantly, the animal would not be in a position to associate the sample lever or the stimulus with the style of trial.

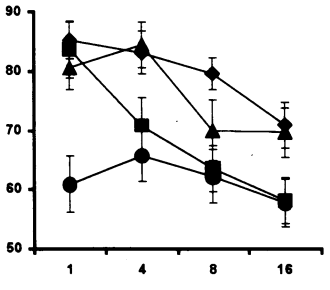

Figure 1. Effect of 0.3 mg/kg 8-OH-DPAT on response accuracy (%, y-axis) and delay (seconds, x-axis). ◆ DNMTP (saline), ▲ DMTP (saline), ■ DNMTP (8-OH-DPAT) and ● DMTP (8-OH-DPAT). Points are mean ± sem (n = 16).

8-OH-DPAT at 0.3 mg/kg but not 0.1 mg/kg dissociated choice accuracy between trial styles, as can be seen at the 1 s delay and by the DMTP/DNMTP response accuracy slopes (figure 1). Two-way ANOVA with both factors repeated (delay x treatment) indicated a significant interaction for DNMTP trials ($F[2,44] = 3.5$, $p<0.05$), i.e. the deficit induced was delay dependent. No such interaction was observed with DMTP trials ($F[2,44] = 1.4$), indicating that the deficit was delay-independent. Importantly, although some sensorimotor deficits were observed with the highest dose of 8-OH-DPAT (by reduced panel press activity), there was no difference across delays or between trial types. We conclude that DMTP requires greater mnemonic processing than DNMTP and is more sensitive to increased $5HT_{1A}$ receptor activation.

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53P DOES NALOXONE HAVE DIFFERING ACTIONS ON REWARD?

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There is evidence that opioid antagonists at low concentrations selectively block the excitatory effects of opioids at the cellular level. Hence, the use of low doses of antagonists may represent a novel approach to attenuating tolerance and dependence associated with opioids (Crain and Shen, 1998). Mesolimbic dopamine pathways are recognised as being important to the expression of opioid mediated reward and dependence (Koob, 1992). The aim of this study therefore was to examine the effect of pretreatment with naloxone in a conditioned place preference paradigm, in conjunction with analysis of dopamine levels in homogenates from two brain regions.

Place conditioning studies used a three compartment apparatus as described by our laboratory (Subhan et al., 1999), and followed an eight day counter-balanced schedule with once a day pairing. Male Wistar rats (150-200g) received two intraperitoneal injections (five minutes apart) 30 minutes prior to conditioning. Subjects were paired in the predetermined preferred compartment following saline vehicle (Sal) administration, and in the least preferred compartment following administration of test drug. Group 1 received two saline (1ml/kg) treatments on each day of pairing (vehicle control). Group 2 received saline pretreatment followed by morphine 2.5mg/kg (positive control). Groups 3 and 4 received naloxone (NLX) pretreatment (1mg/kg and 0.05mg/kg respectively), followed by saline. Place preference was calculated as the change in occupancy time in the initially least preferred compartment before and after conditioning. Nucleus accumbens (NAc) and striatal tissue was dissected on ice 72 hours after the final drug dose. High performance liquid chromatography used a reverse phase ODS packed column and pre-column, with electrochemical detection. Results are summarised in table 1, and are expressed as means ± sem. In place preference studies n=7-8, and in neurochemical studies n=4-5.

Table 1.

Drug treatment (mg/kg i.p)	Preference time(s)	Dopamine levels (ng/g) NAc	Striatum
Sal + Sal	29 ± 38	136 ± 35	2788 ± 57
Sal + Morphine	489 ± 106**	221 ± 57	1826 ± 210**
NLX (1) + Sal	-103 ± 32	140 ± 18	3544 ± 149*
NLX (0.05) + Sal	316 ± 67 *	226 ± 27	3295 ± 219

*p< 0.05; **p< 0.01 one-way ANOVA, Dunnett's analysis

The higher dose of naloxone significantly increased dopamine levels in the striatum but not NAc. The results of the place conditioning study showed contrasting effects of the two doses of naloxone. The lower dose, produced a significant place preference, whilst the higher dose reduced the time spent in the least preferred compartment. It is concluded therefore that low and high doses of naloxone have opposing effects on reward, although this could not be conclusively related to changes in dopamine levels within the nucleus accumbens or striatum.

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54P CHARACTERISATION OF CCK_1 RECEPTORS IN HUMAN GALLBLADDER USING [^{3}H]-L-364,718 AS RADIOLABEL

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We have been developing CCK_1 receptor (CCK_1-R) functional and radioligand binding assays on human gastrointestinal (GI) tissue. Here, we report the development of a binding assay on human gallbladder (GB). This tissue was chosen because it is known to express a high CCK_1-R density. Therefore, it was ideal for optimisation of a muscle membrane preparation for further receptor characterisation in other smooth muscle tissues of the GI tract. Moreover, there is significant variation in the affinity of CCK-R ligands at the human CCK_1 receptor, cloned or otherwise (e.g. Kennedy *et al.*, 1995; Schjoldager *et al.*, 1989). Therefore, having obtained reproducible, high levels of specific binding with [^{3}H]-L-364,718 ([^{3}H]-L), we attempted a quantitative pharmacological characterisation of the receptors using selective CCK-R antagonists. These were chosen on the basis of their structural diversity.

The optimised tissue preparation was as follows. Sections of GB were chopped in buffer A (composition see Harper *et al.*, 1999) and homogenised (Polytron PT-10, 3x10s; setting 12) prior to centrifugation (800xg, 10min at 4°C). Supernatants were pooled and stored (4°C) while the homogenisation and centrifugation steps were repeated twice on the pellet. Combined supernatants were centrifuged (150,000xg, 45min at 4°C) and the pellet re-homogenised (0.32M sucrose in 50mM Tris-HCl, 1g 5ml^{-1}) prior to centrifugation through a sucrose density gradient (1.2, 0.8, 0.32M sucrose in 50mM Tris-HCl; SW28, 39,800xg, 2h at 4°C). Finally, membranes from each interface were added to 500mM Tris-HCl (pH6.9 at 21°C) and centrifuged (150,000xg, 30min at 4°C). The final pellet was re-suspended using a Teflon-in-glass homogeniser.
Total, non-specific (1μM; SR27897) and specific binding of 0.1nM [^{3}H]-L increased with membrane concentration. The specific binding increased linearly with increasing added GB tissue concentration. At added tissue concentrations of 15mg ml^{-1} and 25mg ml^{-1}, 8±2% and 13±2% of the added [^{3}H]-L was bound, respectively (n=3±s.e.mean). At these tissue concentrations the specific binding was 67±2% and 76±4%, respectively. A membrane concentration of 20mg ml^{-1} was used for all subsequent studies. Saturation isotherms were monophasic (pK_D=10.61±0.05, n_H=1.15± 0.02, n=3). In kinetic studies, equilibrium was reached by 15min and remained constant for 180min (k_{+1}=2.26±0.57x10^{9}M^{-1}min^{-1}, k_{-1}=0.033±0.008min^{-1}, n=3). The corresponding pK_D (10.83±0.02) was not significantly different from that estimated by saturation analysis. For competition studies, GB membranes were incubated with [^{3}H]-L (0.1nM) and competing ligand for 150min.

Table 1 pK_I and n_H values for CCK receptor ligands (± s.e.mean)

Compound	pK_I	n_H	n	CCK-R Selectivity
L-364,718 (L)	10.71 ± 0.13	1.04 ± 0.08	4	CCK_1-R
2-NAP	6.33 ± 0.14	0.88 ± 0.13	3	CCK_1-R
SR27897 (S)	10.77 ± 0.07	0.83 ± 0.05	3	CCK_1-R
JB93182	5.22 ± 0.15	1.12 ± 0.31	3	CCK_2-R
PD134,308	6.06 ± 0.06	0.89 ± 0.18	3	CCK_2-R
YM 220	7.81 ± 0.03	0.84 ± 0.04	3	CCK_2-R

The affinity estimate (pK_I) for 2-NAP was consistent with the pK_B estimated previously at CCK_1-Rs in human GB (Hull *et al.*, 1993). L and S affinity estimates were higher than previously reported in both fresh tissue and cloned human CCK_1-R assays (Ulrich *et al.*, 1993; Kennedy *et al.*, 1995; Gigoux *et al.*, 1999). Notwithstanding the high n_H value for [^{3}H]-L, presumably due to depletion of radioligand at concentrations <0.1nM, these data indicate that the GB contains a homogeneous population of CCK_1-Rs. The high pK_I estimates for L and S could be due to post-translational receptor modification or the presence of CCK_1-R isoforms. It seems unlikely that the truncated CCK_1-R isoform, at least, can account for these different pK_I estimates as L-364,718 has been reported to express similar affinity for both isoforms (Kennedy *et al.*, 1995).

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55P ACTIVATION OF NITRIC OXIDE SYNTHASE THROUGH MUSCARINIC ACETYLCHOLINE RECEPTORS IN RAT PAROTID

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Secretory and vasodilatory responses of the parotid gland are regulated by muscarinic acetylcholine receptors. Here we report on the activation of nitric oxide signalling coupled to muscarinic activation and the subsequent secretion of amylase.

Nitric oxide synthase (NOS) activity, assayed with L-[U^{14}C]-arginine as substrate as described by Perez Leiros *et al.* (2000), was dependent on calcium-calmodulin and showed a bell-shaped curve with increasing concentrations of carbachol (NOS activity; pmol/mg wet weight, $x \pm$ SEM: basal: 472 ± 55, carbachol 10^{-6} M: 1035 ± 104 ($p<0.05$); 10^{-5} M: 712 ± 62, n=4). The effect of carbachol was inhibited by 4-DAMP over the whole range of concentrations, while pirenzepine only reversed the effect of 10^{-5} M carbachol, pointing to the participation of both subtypes of receptors.

In support of this, competitive binding assays with pirenzepine in parotid membranes, carried out as in Perez Leiros *et al.* (2000), showed two populations of binding sites (Ki, $x \pm$ SD, high affinity: 26 ± 19 nM, low affinity: 166 ± 89 nM, n=4). In addition, cGMP levels were stimulated by carbachol, although higher concentrations of the agonist were needed compared to NOS activation. Similarly, carbachol-stimulated amylase secretion determined as previously described (Bernfeld P., 1955) appeared partly associated to NOS and guanylyl cyclase activation through M_3 receptors (see Table 1), since it was inhibited by L-NAME, methylene blue and 4-DAMP but not by pirenzepine, which even increased the secretory effect of low concentrations of carbachol.

We can conclude that carbachol stimulates parotid NOS through both M_1 and M_3 muscarinic receptors, with M_3 subtype being predominantly involved in NOS-related amylase secretion.

Table 1

	Amylase (% of release)
Basal	16.8 ± 1.5
Carbachol 5.10^{-5} M	30.7 ± 1.7
Carbachol 5.10^{-5} M + methylene blue	15.0 ± 1.3
Carbachol 5.10^{-5} M + L-NAME	17.3 ± 1.2
Carbachol 5.10^{-5} M + 4-DAMP	17.1 ± 1.5
Carbachol 5.10^{-5} M + pirenzepine	39.6 ± 1.8

Student-Newman-Kaels test after analysis of variance was used. Differences were considered significant at $p<0.05$.

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56P AGONIST POTENCY PROVIDES EVIDENCE FOR FUNCTIONALLY COUPLED MUSCARINIC M_2 RECEPTORS ON MOUSE URINARY BLADDER

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The muscarinic receptors on mouse urinary bladder await full pharmacological characterisation (see Welsh *et al.*, 2000) although contractions of the bladder from a number of other species, measured *in vitro* under standard organ bath conditions, appear to be M_3-receptor mediated despite M_2-receptors being in excess. However, M_2-receptors are negatively coupled to adenylate cyclase and, in anaesthetised rats, an M_2-mediated volume-induced contraction was shown to be blocked by propranolol. Furthermorre, *in vitro*, M_2-mediated contractions were obtained when adenylate cyclase activity was raised (Hegde *et al.*, 1997). Several antagonists exhibit M_2/M_3-selectivity but there are no highly selective agonists, although oxotremorine and oxotremorine-M (oxo-M) are reported to express some selectivity in radioligand binding assays (see Caulfield, 1993). Consistent with antagonist studies, the K_A values reported for oxo-M on isolated, guinea-pig ileum and bladder smooth muscle assays were not different (Ringdahl, 1987), suggesting that the same (M_3) receptor mediated the responses in both tissues. Here, we have compared the effect of several agonists of variable efficacy, 5methylfurmethide (5mef), oxotremorine-M (oxo-M), pilocarpine and McN-A-343, on guinea-pig left atrium, which is reported to contain only the M_2-receptor, and a range of smooth muscle assays.

Tissues were mounted for isometric force recording in 20ml organ baths (Krebs-Henseleit solution gassed with 95%O_2/5%CO_2). Assay temperature and loading force values were as follows: Guinea-pig tracheal rings (37°C, 1g), gastric smooth muscle strip (26°C, 0.5g) and half left atrium (32°C 1g); rat anococcygeus (37°C, 0.5g); mouse urinary bladder (37°C, 2g). The atria were electrically paced (1Hz, 1 ms pulse width, voltage threshold +20%). The data from a single agonist concentration-effect (E/[A]) curve, obtained by cumulative dosing, were fitted to the Hill equation. Mean parameter estimates were compared using one-way ANOVA and values of $P<0.05$ were considered to be significant. Data shown are n=4-8.

Oxo-M and 5mef produced indistinguishable maximum responses in all tissues except the rat anococcygeus where the 5mef maximum response (α) was significantly reduced. Oxo-M was assumed to behave as a full agonist and the maxima for 5mef, pilocarpine and McN-A-343 were expressed as a percentage of the oxo-M α value in each tissue. Pilocarpine and McN-A-343 behaved as partial agonists and the assays where no agonism was observed were those in which oxo-M was least potent as is expected for variation in receptor density and/or receptor-effector coupling efficiency between tissues.

$p[A]_{50} \pm$ s.e.m, and $\alpha \pm$ s.e.m. as % oxo-M maximum (n=4-8)

Tissue	oxo-M	5mef	pilocarpine	McN-A-343
gastric s.m.	7.06±0.13	6.89±0.10 85±16%	6.06±0.08 42±17%	4.85±0.05 17±16%
trachea	7.39±0.06	7.12±0.13 100%	6.29±0.08 90±3%	5.03±0.11 84±1%
anococcy -geus	5.74±0.20	5.62±0.09 71±2%	silent (pA_2=5.5)	silent (pA_2=4.5)
left atrium	7.37±0.14	6.44±0.06 78±22%	5.64±0.07 69±27%	4.23±0.08 33±18%
bladder	6.22±0.05	5.66±0.07 110±17%	5.14±0.09 11±9%	silent

On the gastric s.m., trachea and anococcygeus 5mef was equipotent with oxo-M. However, oxo-M was significantly more potent than 5mef (log potency-ratio = 0.93±0.15) on the reference M_2-receptor left atrium assay. On the urinary bladder, oxo-M was significantly more potent than 5mef (log potency-ratio = 0.56±0.09).

In conclusion, oxo-M displays functional selectivity for the left atrium which cannot be explained by differences in the density or coupling efficiency of a single receptor type between tissues and so appears to be selective for the M_2-receptor. It is also possible that the increased potency of oxo-M over 5mef on the contraction of the bladder assay reflects a contribution of M_2-receptors as though there is basal adenylate cyclase activity in the bladder under the assay conditions used here.

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57P PHARMACOLOGICAL COMPARISON OF THE MUSCARINIC RECEPTORS MEDIATING CONTRACTION OF THE GUINEA-PIG LEFT ATRIUM, GASTRIC SMOOTH MUSCLE AND MOUSE URINARY BLADDER

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Four muscarinic receptor subtypes (M_1-M_4) have been classified operationally. In guinea-pig (GP) heart, negative inotropy is M_2-receptor mediated and only M_2-receptors are detected by radioligand binding or Northern blot. On most smooth muscles, radioligand binding reveals both M_2 and M_3 with M_2 in excess (e.g. rat bladder M_2:M_3 = 9:1, see Eglen & Hegde, 1997 for review). Contractile responses, paradoxically, appear to be M_3-receptor mediated. M_2-receptors are negatively coupled to adenylate cyclase and an M_2 contraction has been shown on guinea-pig ileum when adenylate cyclase activity is raised (Thomas *et al*, 1993). In the absence of highly selective agonists, functional discrimination of M_2- and M_3-receptors relies on selective antagonists like methoctramine (M_2), *p*-F-HHSiD (M_3) and darifenacin (M_3) that display ~30-100 fold selectivity between the two subtypes. Here we describe the functional characterisation of muscarinic receptors on mouse urinary bladder and GP gastric smooth muscle and, for reference, the M_2-receptor GP isolated left atrium assay.

GP (male, Dunkin-Hartley, ~300g) gastric muscle strips (26°C, 0.5g initial loading force), half left atria (32°C, 1g) and mouse (Charles River CD1, 18-28g) urinary bladders (26°C, 2g) were mounted for isometric force recording in 20ml organ baths (Krebs-Henseleit solution gassed with 95%O_2/5%CO_2). Atria were electrically paced (1Hz, 1 ms pulse width, voltage threshold + 20%). Single agonist concentration-effect (E/[A]) curves, obtained by cumulative dosing in the absence and presence of antagonist (60min pre-incubation), were fitted to the Hill equation (n=4-8 for each treatment group). Antagonist activity was quantified by fitting log $[A]_{50}$ data to a derivation of the Schild equation (Black *et al*., 1985) to provide estimates of antagonist affinity (pK_B) and Schild plot slope parameters (b).

Using the non-selective agonist, 5-methylfurmethide, pirenzepine acted as a non-selective, surmountable, antagonist in all three assays. *p*-F-HHSiD, darifenacin and methoctramine also behaved simply on the left atrium and gastric s.m. assays. The pK_B values estimated were as expected for M_2- (atrium) and M_3- (gastric) receptors, respectively. On the mouse bladder, all antagonists behaved simply except darifenacin(*) which produced concentration-dependent decreases in the E/[A] curve maximum. In a receptor protection experiment, pirenzepine was able to reverse the depression seen with darifenacin indicating that it is a muscarinic receptor mediated phenomenon. Notwithstanding this observation, the pK_B values estimated were also consistent with an action at the M_3-receptor.

Antagonist	Assay	pK_B±s.e.m	b±s.e.m
pirenzepine	left atrium	6.71±0.07	0.88±0.09
	gastric s.m.	7.00±0.07	1.12±0.05
	bladder	6.97±0.11	1.11±0.08
*p*FHHSiD	left atrium	6.00±0.09	0.88±0.12
	gastric s.m.	7.57±0.10	0.95±0.07
	bladder	7.50±0.09	1.03±0.09
darifenacin	left atrium	7.20±0.05	1.00±0.04
	gastric s.m.	9.33±0.08	1.15±0.05
	bladder	8.37±0.09*	n.d.
methoctramine	left atrium	7.35±0.08	1.14±0.09
	gastric s.m.	5.40±0.09	1.09±0.23
	bladder	5.16±0.08	0.92±0.12

Using oxo-M on the bladder, a slightly higher pK_B value for methoctramine (5.82±0.09) and a slightly lower pK_B value for *p*-F-HHSiD (7.20±0.07) were estimated. However, collectively, these data indicate that, in common with other smooth muscle assays, mouse bladder and guinea-pig gastric muscle contraction, in the absence of elevated adenylate cyclase activity, is mediated predominantly by M_3-receptors.

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58P NERVE-EVOKED CALCIUM TRANSIENTS IN SYMPATHETIC AXONS OF MOUSE VAS DEFERENS ARE MODULATED BY K_A AND K_V BUT NOT BY K_{Ca}

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We have recently reported the existence of action potential-evoked calcium transients in postganglionic preterminal sympathetic axons (Jackson *et al.*, 2000). It has been suggested that calcium influx during depolarisation may activate potassium channels (K_{Ca}) and facilitate axon repolarisation (Callewaert *et al.*, 1996). To determine the possible function of axonal calcium transients, the effects of potassium channel blockers were therefore investigated.

Experiments were carried out on mouse (Balb/C, 8 - 12 weeks) isolated *vasa deferentia* in which axons had been preloaded with the calcium indicator dye Oregon green 488 BAPTA-1. Calcium transients were detected as changes in fluorescence using laser scanning confocal microscopy (Jackson *et al.*, 2000). Data are expressed as mean ± s.e. mean, relative to control. Statistical significance was determined using the paired t-test.

Iberiotoxin (100 nM), which selectively blocks large conductance K_{Ca}, had no effect on nerve-evoked axonal calcium transients, even at high stimulation frequencies (10 pulses at 1 Hz, 6 ± 9%; 10 Hz, 2 ± 6% and 50 Hz, 12 ± 11%, n = 11 axons; $P > 0.05$, paired t-test). In preliminary studies, apamin (1 μM) also failed to affect the nerve-evoked axonal calcium transient (10 pulses at 10 Hz, 7%, n = 2 axons) suggesting that calcium influx did not activate small conductance K_{Ca}.

To determine if other potassium channels influence the calcium transient, the actions of 4-aminopyridine (4-AP, which blocks delayed rectifiers) was investigated. 4-AP (10 mM) potentiated the calcium transient (10 pulses at 10 Hz, 209 ± 54% increase, n = 3 axons; $P < 0.05$). As 4-AP blocks both K_A and K_V channels, the novel antagonist of transient K_A, pandinustoxin-K (α) and the novel antagonist of slowly inactivating K_V channels, tityustoxin-K (α) (Juhng *et al.*, 1999) were studied. Pandinustoxin-K (α) (10 nM) potentiated the nerve-evoked axonal calcium transient (10 pulses at 1 Hz, 74 ± 44%; 10 Hz, 45 ± 7%, n = 7 axons; $P < 0.05$) as did tityustoxin-K (α) (100 nM) (10 pulses at 1 Hz, 42 ± 28% increase, n = 4 axons and 10 Hz, 33 ± 17% increase, n = 7 axons; $P < 0.05$).

These findings suggest that electrically-evoked calcium transients do not activate K_{Ca} in sympathetic axons, even at high frequencies of stimulation. However, action potential repolarisation appears to be influenced by the fast inactivating K_A and the slower inactivating K_V, as judged by the actions of pandinustoxin-K (α) and tityustoxin-K (α) on the action potential-evoked calcium transient.

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59P INTERACTION OF SEROTONIN AUTORECEPTOR ANTAGONISTS IN THE RAT DORSAL RAPHE NUCLEUS: AN *IN VITRO* FAST CYCLIC VOLTAMMETRY STUDY

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Recent reports in the literature demonstrate that 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptors function as 5-HT autoreceptors in the rat dorsal raphe nucleus (DRN), modulating local 5-HT efflux (Davidson & Stamford, 1995; Starkey & Skingle, 1994). However, there are no studies on the simulataneous blockade of these receptors in the DRN. Therefore, in this study we have investigated the effect of 5-HT_{1B} and 5-HT_{1D} receptor antagonists on 5-HT efflux, alone and in the presence of 5-HT_{1A} receptor antagonists, using the technique of fast cyclic voltammetry.

Rat DRN slices were stimulated (100Hz, 10mA, 0.1ms, 20 pulses every 5 min) to evoke 5-HT efflux. A triangular voltage waveform (-1V to +1.4V) was applied to a carbon fibre microelectrode to oxidise and reduce electroactive substances and generate a current. 5-HT oxidised at 525mV and so the resultant signal was recorded at this voltage. Calibration of the recorded signal deduced that under these stimulation parameters the peak 5-HT efflux was 22±2nM (n=16). Data were expressed as a percentage of the mean of the first 3 control stimulated efflux samples. Statistical analysis (ANOVA followed by a least squares post-hoc t-test) was performed on the mean area under the curve (AUC) following drug perfusion.

The 5-HT_{1A} receptor antagonist, WAY 100635 (100nM), and the 5-HT_{1B} receptor antagonist, SB-224289 (100nM), had no effect on 5-HT efflux from the DRN. The 5-$HT_{1B/1D}$ receptor antagonist, GR 127935 (100nM), produced a small decrease in 5-HT efflux (P=0.06) with a mean AUC of 94±5 % of control (n=3). This may indicate a small degree of partial agonism at one of the autoreceptors. In contrast, the 5-HT_{1D} receptor antagonist, BRL 15572 (100nM), produced a significant increase in 5-HT efflux (P<0.05), with a mean AUC of 123±4% of control (n=4).

Co-perfusion of WAY 100635 and SB-224289 increased 5-HT efflux, with mean AUC of 140±10 % of control (n=4; P<0.05). In addition, WAY 100635 reversed the small inhibition of 5-HT efflux observed with GR 127935 (mean AUC of 109±9 % of control; n=3) but had no effect on the BRL 15572-induced increase (mean AUC of 126±6 % of control; n=3).

In summary, we confirm that 5-HT efflux within the DRN is under control of 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} autoreceptors. Elevation of 5-HT efflux was greatest on co-perfusion of 5-HT_{1A} and 5-HT_{1B} receptor antagonists. Interestingly, it appears that the effect of 5-HT_{1B} but not 5-HT_{1D} receptor antagonism was limited by 5-HT_{1A} receptor activation. These effects may be explained by differences in the anatomical location of these autoreceptors.

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60P EVIDENCE FOR A FUNCTIONAL PRE-SYNAPTIC IMIDAZOLINE RECEPTOR IN THE MOUSE ISOLATED VAS DEFERENS

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Certain imidazolines (S21663) have been shown to improve glucose tolerance *in vivo* by stimulating insulin secretion from pancreatic β-cells (Wang *et al.* 1996), while others (S22954) produce hyperglycaemia (Slough & Taberner, 1999). Although functional studies have shown that imidazolines can inhibit noradrenaline release in vascular tissue (Gothert & Molderings, 1991), the inhibitory action of rilmenidine on noradrenaline release in the rat vas deferens appeared to be α_2-adrenoceptor (α_2-AR) mediated (Avellar & Markus, 1996). Since the hyperglycaemic action of S22954 could be mediated by α_2-AR activation we have examined the ability of S21663, S22954 and S22068 (1,4-diisopropyl-2-(4,5-dihydro-1-H-imidazol-2-yl)-piperazine) to inhibit electrically-evoked twitches in isolated vas deferens in the presence of the α_2-AR antagonist rauwolscine.

Vasa deferentia were removed from adult CBA/Ca mice (30-36g) and mounted singly under 0.5 g tension in a 3.5 ml bath between two platinum wire electrodes in Mg^{2+}-free Krebs Ringer bicarbonate buffer (pH 7.4) gassed with 95% O_2, 5% CO_2 at 37°C. Isometric responses were recorded following addition of imidazoline compounds, or following electrical stimulation (a train of 3x 0.1 msec 80 V rectangular pulses at 10 Hz, every 10 sec) in the presence of the imidazolines. The imidazoline UK14304 was used as a positive control for α_2-AR agonism. Data are expressed as means ± s.e. mean of n in parentheses.

None of the three imidazolines tested produced direct contraction of the unstimulated vas deferens (up to 10 mM) and all were capable of complete inhibition of electrically evoked twitches. IC_{50} values for S22068, S22954, and S21663 were 1.13 ± 0.08 mM (3), 128 ± 50 nM (7), and 54.9 ± 7.0 μM (4) respectively; UK14304 also produced complete inhibition; IC_{50}: 2.44 ± 0.41 nM (4). The inhibition of the twitch response by S22954 was reversibly blocked by rauwolscine (IC_{50} in presence of 1 μM rauwolscine: 1.04 ± 0.09 μM (3)). Rauwolscine (10 μM) had no effect on the inhibition of twitch caused by S22068 or S21663. In unstimulated tissue, S21663 blocked the contraction evoked by 50 μM noradrenaline; S22954 and S22068 had no effect.

Our results indicate that S22954 possesses α_2-AR agonist activity, whereas S21663 appears to block post-synaptic α_1-AR. S22068 however appears to inhibit evoked noradrenaline release by an alternative, possibly imidazoline-receptor mediated, mechanism.

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61P THE ROLE OF UROTHELIUM-DERIVED INHIBITORY FACTOR IN THE PIG BLADDER NECK

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Bladder urothelium was originally thought to be a thick, protective, inactive barrier for the bladder detrusor. However, recent findings indicate that the urothelium plays an active role in bladder function and in the dome region its presence inhibits contractions to muscarinic stimulation by 40-70% (Hawthorn *et al.*, 1999). The aim of this study was to examine the role of the urothelium in the regulation of smooth muscle contraction in the neck of the urinary bladder of the pig.

Paired longitudinal strips of pig detrusor (approx. 20 x 5mm) were isolated from the bladder neck. The urothelium was carefully removed from one strip of each pair before suspension in 30ml organ baths. The tissues were bathed in Krebs-bicarbonate solution, placed under 1g of tension and maintained at 37°C. (Tissues were gassed with 95% O_2/ 5% CO_2). Cumulative concentration-response curves to either carbachol or phenylephrine were constructed. Some tissues were exposed to a low concentration of carbachol (1μM) during the construction of the phenylephrine concentration - response curve. The results are presented as mean ± s.e.m and comparisons made using Student's t-test.

Carbachol (0.1nM - 1mM) produced concentration dependent contraction of urothelium-denuded muscle strips with a maximum contraction of 4.6 ± 0.7g, and a geometric mean EC_{50} value (with 95% confidence limits) of 3.8(3.5 - 4.2) μM (n = 12). The presence of the urothelium significantly ($P<0.05$) depressed responses by 30% with the maximum contraction falling to 3.2 ± 0.8g, with no change in the potency of carbachol [EC_{50} = 4.0 μM (3.5 – 4.6), (n = 12)].

Phenylephrine (0.16nM - 1.6mM) also produced concentration dependent contractions of the bladder strips with a maximum contraction of 3.9 ± 0.7g, and a geometric mean EC_{50} value of 3.1 (2.4 – 3.9)μM, (n = 10). The presence of the urothelium had no significant effect on responses to phenylephrine, [maximum contraction = 3.3 ± 0.7g, geometric mean EC_{50} = 4.1 (3.3 – 5.0)μM)]. However in the presence of 1μM carbachol, responses of intact strips to phenylephrine were significantly inhibited ($P<0.05$), maximum responses being reduced to 1.59 ± 0.4g, with no change in the potency of phenylephrine [EC_{50} = 5.9 (5.2 – 6.7)μM, (n = 6)].

In conclusion, the removal of the urothelium significantly enhances the contractile responses of longitudinal smooth muscle of the bladder neck to carbachol, but not phenylephrine. These results also indicate the release of a local inhibitory factor from the urothelium in response to carbachol, however the mediator of this inhibition has yet to be identified.

Hawthorn, M.H et al,. (1999) *Br. J. Pharmacol.*,**129**, 416-420

62P MUSCARINIC RECEPTOR SUBTYPE MEDIATING CONTRACTILE RESPONSES OF HUMAN DETRUSOR MUSCLE *IN VITRO*

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Acetylcholine, through an action at postjunctional muscarinic receptors, is the major neurotransmitter controlling bladder smooth muscle contraction and bladder emptying. In the human bladder both the M2 and M3 muscarinic receptor subtypes have been found to be present in the detrusor muscle (Yamaguchi et al., 1996). However, it has been found in several species including man that the M2-receptor predominates (Monferini et al., 1988; Wang et al., 1995). In all species studied to date however, it is the minor population of M3-receptors which mediates contractile responses *in vitro* (Longhurst et al., 1995). The present study attempts to elucidate which muscarinic receptor subtype is involved in mediating contraction of detrusor muscle strips from the human.

Samples of bladder were obtained from 5 patients undergoing either cystectomy for bladder cancer or colposuspension. Of these patients 2 were male (mean age 64.2±2.3 yrs) and 3 were female (mean age 47.3±6.2). Strips of detrusor muscle were cut, the urothelium and serosa removed and the strips suspended in gassed Krebs at 37°C under a resting tension of 1.0g. Contractile responses to carbachol were obtained in the absence and presence of 4-DAMP, methoctramine, oxybutynin, tolterodine and pirenzapine with an equilibration period of 30 minutes.

The M3-selective antagonist, 4-DAMP had a high affinity (apparent pK_B) of 9.94±0.10 (n=4), whilst the M2-selective agent methoctramine had a relatively low affinity of 6.75±0.06 (n=20). These antagonists did not significantly alter the maximum developed tension to carbachol (2.27±0.66g for 3nM 4-DAMP vs. 1.68±0.45g in control and 0.68±0.21g for 10μM methoctramine vs. 1.41±0.56g in control. Oxybutynin, tolterodine and pirenzapine had affinities of 7.64±0.17 (n=15), 8.11±0.12 (n=15) and 6.88±0.16 (n=8) respectively. These antagonists also did not affect maximum developed tension to carbachol (3.51±0.49g for 300nM oxybutynin vs. 3.38±0.46g in control, 1.94±0.73g for 300nM tolterodine vs. 1.68±0.63g in control, and 1.54±0.43g for 30μM pirenzapine vs. 1.56±0.50g in control. The mean affinity values obtained for these antagonists at the human detrusor receptor were correlated with those published for these antagonists at the five cloned muscarinic receptor subtypes. The human detrusor receptor correlated best with the m3 and m5 subtypes with correlation coefficients of 0.72 and 0.82 respectively. The correlation plot for the m3 receptor had a slope close to unity, being 0.89±0.32, with the plot for the m5-receptor being slightly flatter (0.75±0.20). The correlations between the human detrusor receptor and the cloned m1, m2 and m4 receptor subtypes yielded lower correlation coefficients of 0.62, 0.32 and 0.48 with slopes of 0.62±0.28, 0.37±0.32 and 0.46±0.28 respectively.

These data suggest that either or both the M3-muscarinic receptor and the M5-receptor may mediate contractile responses of the human detrusor muscle to muscarinic receptor stimulation *in vitro*. However, the M5 receptor subtype is difficult to distinguish pharmacologically from the M3 receptor subtype. In addition, the M5- receptor subtype could not be immunoprecipitated from human bladder membranes (Wang et al., 1995), which leads us to conclude that it is probably the M3-muscarinic receptor subtype which mediates contraction of the human bladder *in vitro*.

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63P INVESTIGATION OF PROSTANOID EP_4 AND IP_1 SYSTEMS IN ISOLATED BLOOD VESSELS OF PIGLETS

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The 7,7-difluoro prostacyclin analogue AFP-07 is a potent agonist at the classical prostacyclin (IP_1) receptor, based on ligand binding and cyclic AMP formation in a mouse cloned receptor / CHO expression system; AFP-07 also appeared to have considerable affinity for the mouse EP_4-receptor (Chang *et al.*, 1997). We recently reported to the Society (Melbourne, April 2000) that AFP-07 was a potent relaxant of the rabbit isolated saphenous vein, a sensitive EP_4 preparation. However, we were unable to determine whether EP_4 or IP_1-receptors were involved, since the EP_4 antagonist AH 23848 (Coleman *et al.*, 1994) produced similar shifts of the concentration-response curves for PGE_2, AFP-07 and cicaprost (cicaprost is considered to be a specific IP_1 agonist). We now report further investigations on isolated blood vessels from the pig, including the saphenous vein, the archetypal EP_4 preparation (Coleman *et al.*, 1994).

Piglets (Landrace Cross) weighing 2 - 3 kg were asphyxiated with carbon dioxide and lateral saphenous and cranial mesenteric veins and common carotid arteries were excised. Circular muscle tension was recorded from 3 mm rings of vessel suspended in Krebs-Henseleit solution aerated with 95% O_2 / 5% CO_2 in 10 ml organ baths. All preparations were maintained at 37°C and continuously exposed to 1 μM indomethacin and 1 μM GR 32191, a TP-receptor antagonist. Phenylephrine (0.2 - 5 μM) was used to generate tone, against which cumulative doses of prostanoids were tested for relaxing activity ($n \geq 4$). The first sequence was always PGE_2, followed one hour later by PGE_2 or test prostanoid. The third sequence was either PGE_2 or a repeat of the second sequence agonist in the presence of AH23848 or vehicle.

On carotid artery, PGE_2 consistently showed no relaxant effect between 0.1 and 10 nM and at higher concentrations induced contraction. AFP-07 and cicaprost induced full relaxation (IC_{50} 2.3 - 20 and 17 - 30 nM respectively). On cranial mesenteric vein, PGE_2 elicited either no effect (0.1 - 150 nM) or weak relaxation (maximum 50% at 150 nM; IC_{50} 25 nM). AFP-07 and cicaprost again showed high relaxing potency (IC_{50} = 1.5 - 9.5 and 10 - 75 nM respectively).

PGE_2 induced full relaxation of saphenous veins (IC_{50} 0.12 - 0.34 nM). Equi-effective molar ratios (PGE_2 = 1.0) for prostacyclin analogues were: AFP-07 7.8 ± 0.9 (± s.e. mean), cicaprost 27 ± 4, prostacyclin 59 ± 13, iloprost 92 ± 4, taprostene >20,000 (maximum relaxation 30 - 47% at 4.4 μM). AH 23848 at 30 μM shifted the log concentration-response curves to PGE_2, AFP-07 and cicaprost in a parallel rightward manner, giving dose ratios of 8.0 ± 1.5, 2.8 ± 0.3 and 4.9 ± 0.9 respectively (n = 4; corrected for shift of curves on control preparations; all $P<0.01$).

The cranial mesenteric vein and carotid artery data demonstrate the high IP_1 agonist potency of AFP-07 in a vascular setting. It would appear that the pig saphenous vein contains an IP_1 relaxation system based on the high potency of cicaprost: its Kds for mouse cloned IP_1 and EP_4-receptors are 10 nM and >10 μM respectively (Kiriyama *et al.*, 1997). AH 23848 may thus be blocking both IP_1 and EP_4-receptors. The low selectivity and also low potency of this antagonist mean that it is of limited value in situations where it is desired to discriminate between IP_1 and EP_4-receptors.

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64P ACTIVATION OF A HYPERPOLARIZATION-DEPENDENT CHANNEL BY 8-BROMO-cAMP IN ALZHEIMER MODEL FIBROBLASTS

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We have previously reported the presence of hyperpolarization-activated currents in skin fibroblasts taken from a transgenic mouse model of Alzheimer's disease (AD) (Jones & Wann, 1999). This activation was seen in the control group (wild-type expression of APP), but was not observed in the AD group (overexpression of APP). Hyperpolarization-dependent activation was observed within the narrow range of 10-40 mV hyperpolarized to rest. A number of distinct conductances were identified, the most prevalent of which was a channel with a unitary current of 77 pS. We have shown that this 77 pS channel is blocked by $BaCl_2$ (1 mM). However, the 77 pS channel was unaffected by TEA (10 mM). This is similar to the reported reduction in the I_h current in neurones by $BaCl_2$, and its lack of sensitivity to extracellular TEA (Pape, 1996). The 77 pS channel is not permeable to Na^+, and is blocked by CsCl and $RbCl_2$ (Jones & Wann, 2000). To investigate the modulation of the 77 pS channel, single channel patch-clamp recordings were made on sub-confluent cultures of the fibroblasts, in both the cell-attached and excised patch (inside-out) modes. The bath and recording electrode solutions contained a high K^+solution (140 mM).The 'intracellular' side of the patch was perfused with 1 mM 8-bromo-cAMP (in 140 mM K^+ solution). 8-bromo-cAMP was active in 73% of the patches (n=11), having 2 principal effects. In patches that were active in the cell-attached mode (2/8 patches), the activation of the channel shifted to a more negative potential on excision of the patch (10-20 mV shift). Following perfusion of the patch with 8-bromo-cAMP, the activation of the channel shifted to a more depolarized potential (10-20 mV shift). The channel identified had a higher conductance in the presence of 8-bromo-cAMP. This depolarizing shift has also been documented for the I_f current in cardiac SAN cells (DiFrancesco, 1999). In patches that were inactive in the cell-attached and inside-out modes (6/8 patches), channel activation was induced by 8-bromo-cAMP in the inside-out mode. This channel was found to have a mean conductance of 105 pS (n=3) (figure 1). The 8-bromo-cAMP-induced activation was reversed slowly on washing.

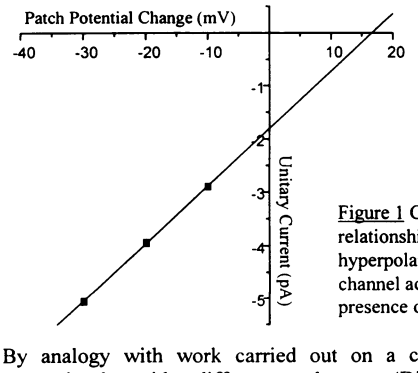

Figure 1 Current-voltage relationship for an hyperpolarization-dependent channel activated in the presence of 8-bromo-cAMP.

By analogy with work carried out on a channel with similar properties, but with a different conductance (DiFrancesco 1999), we tentatively suggest that the action of 8-bromo-cAMP on this hyperpolarization-dependent channel does not require cAMP-dependent phosphorylation of the channel.

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65P BLOCKADE OF NOS UNCOVERS A MORE POTENT VASOCONSTRICTOR RESPONSE TO BIG-ENDOTHELIN IN THE PULMONARY CIRCULATION OF RABBITS WITH PULMONARY HYPERTENSION

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Endothelins have been implicated as potential mediators in pulmonary hypertension (PHT). We have previously shown that endothelin-1 (ET-1) only has pulmonary vasoconstrictor properties in rabbits following blockade of nitric oxide synthase (NOS) and that the response to ET-1 following NOS inhibition was enhanced in rabbits with PHT. These results suggested that NO production masked the effect of exogenous ET-1 and that there was an increased basal NO in rabbits with PHT (Deuchar *et al*, 1999). Using a model of PHT secondary to coronary artery ligation we aimed to investigate the effect of big ET (bET) on the pulmonary arterial pressure (PAP) as this is not broken down to ET-1 by endothelin converting enzyme (ECE) until it has crossed the endothelial barrier. Big ET thereby avoids contact with the ET_{B1} receptors (Uhlig *et al*, 1995) that are located on the endothelium and involved in the NO cascade.

8 weeks after coronary artery ligation or sham operation in male New Zealand White rabbits (n=24, weighing 2.5-3.5kg) anaesthesia was induced with 0.2-0.4ml kg^{-1} midazolam and maintained following intubation by nitrous oxide, oxygen (1:1 ratio) and 1% halothane. PAP was monitored via a catheter inserted into the pulmonary artery through the right jugular vein as previously described (Deuchar *et al*, 1998). The ratio of right ventricular weight / final body weight (g kg^{-1} f.b.w) was used as a measure of right ventricular hypertrophy (RVH). Big ET (0.1-2.0nmol kg^{-1}) was administered cumulatively through the femoral vein in the presence and absence of the NOS inhibitor L-NAME (30μmol min^{-1} i.v. infusion), n=6 in each group. Student's two sampled unpaired *t* tests were applied for comparisons between the ligated and sham operated groups and paired *t* tests were used for analyzing responses within groups. A value of $P < 0.05$ was taken as statistically significant. Data is presented as mean ± s.e.m.

Mean PAP was elevated in the coronary artery ligated rabbits (17.3 ± 0.8mmHg, n=12, cf. 12.7 ± 0.4mmHg, n=12, sham operated controls, $P<0.001$) and there was also evidence for the development of RVH in this group (0.75 ± 0.04 cf. 0.46 ± 0.01g kg^{-1} f.b.w in sham operated controls, $P<0.001$) confirming the development of PHT. Big ET alone, produced a significant pressor response in both groups with a trend towards the response being greater in rabbits with PHT (an increase of 4.8 ± 0.9mmHg, n=6, cf. 3.3 ± 0.6mmHg, n=6,in the controls). In the presence of L-NAME, the response curve to bET was shifted to the left in rabbits with PHT, the ED_{50} being significantly less than those in the shams (0.29 ± 0.08nmol kg^{-1}, n=6 cf. 0.52 ± 0.08nmol kg^{-1}, n=6, $P<0.05$).

In conclusion the pressor response to bET alone, further supports a role for the endothelial ET_{B1} receptors in masking the pulmonary pressor effect of exogenous ET-1 in both groups of rabbits. The increased potency of bET in the presence of L-NAME in the rabbits with PHT may suggest either an increased activity of ECE or an upregulation of the underlying ET_A or ET_{B2} vasoconstrictor receptors on the vascular smooth muscle.

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66P SB209670, A MIXED ENDOTHELIN RECEPTOR ANTAGONIST, BLOCKS THE GREATER PULMONARY PRESSOR RESPONSE TO ET-1 FOLLOWING L-NAME IN RABBITS WITH PULMONARY HYPERTENSION

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We have previously shown that blocking nitric oxide (NO) production with the NO synthase inhibitor L-NAME, uncovers a pulmonary pressor response to endothelin-1 (ET-1) which was greater in rabbits with pulmonary hypertension (PHT) when compared to non-pulmonary hypertensive controls (Deuchar *et al*, 1999). The aim of this study was to investigate the effect of the mixed ET_A/ET_B receptor antagonist (SB209670) on pulmonary arterial pressure (PAP) and the responses to L-NAME and ET-1 in rabbits with PHT.

Experiments were performed 8 weeks following coronary artery ligation or sham operation in male New Zealand White rabbits (n=24, weighing 2.5-3.5kg). Anaesthesia was induced with 0.2-0.4ml kg^{-1} midazolam and maintained following intubation by nitrous oxide, oxygen (1:1 ratio) and 1% halothane. PAP was monitored via a catheter inserted into the pulmonary artery via the right jugular vein as previously described (Deuchar *et al*, 1998). The ratio of right ventricular weight / final body weight (g kg^{-1} f.b.w) was used as a measure of right ventricular hypertrophy (RVH). ET-1 dose response curves (0.001nmol - 4.0nmols kg^{-1}, i.v.) were obtained following 25 minutes L-NAME infusion (30μmol min^{-1}, i.v.) with or without prior administration of SB209670 (10mg kg^{-1}, i.v. bolus) 20 minutes earlier. Student's two sampled unpaired *t* tests were applied for comparisons between the ligated and sham operated groups and paired *t* tests were used for analyzing responses within groups. A value of $P < 0.05$ was taken as statistically significant. Data is presented as mean values ± s.e.m.

Coronary ligated rabbits had higher mean PAP (17.2 ± 0.4, n=13 cf. 13.3 ± 0.3mmHg in the controls, n=14, $P<0.01$) and had evidence for the development of RVH (0.69 ± 0.02 cf. 0.51 ± 0.02g kg^{-1} f.b.w, $P<0.01$) confirming the development of PHT. The pulmonary pressor response to L-NAME alone was greater in rabbits with PHT (an increase of 10.7 ± 1.1mmHg, n=7; PHT rabbits cf. 4.3 ± 1.8mmHg, n=7; controls, $P<0.01$) and the response to ET-1 was greater (10.2 ± 2.3mmHg, n=7; cf. 4.9 ± 1.0mmHg, n=7; controls, $P<0.05$). SB209670 alone had no effect on PAP in the controls, (n=7) but caused a significant fall in PAP in rabbits with PHT (a decrease of 0.9 ± 0.2mmHg, n=6, $P<0.01$). Following SB209670 the effect of L-NAME was reduced in rabbits with PHT (an increase of 2.3 ± 0.5mmHg, n=6, $P<0.001$) but not in sham operated controls (an increase of 2.4 ± 0.6mmHg, n=7). The previously uncovered pulmonary response to ET-1 following L-NAME, was now abolished in both groups of rabbits.

The ability of SB209670 to partially reduce the increased PAP and block the increased response to ET-1 after L-NAME in the PHT rabbits supports a pathophysiological role for ET-1 in this model. The decreased response to L-NAME in the PHT rabbits following SB209670 provides further evidence of an increased NO activity possibly the result of an upregulation of endothelial ET_{B1} receptors (Uhlig *et al*, 1995) in this model.

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67P REGULATION OF NO SYNTHASE BY PHOSPHORYLATION AND Ca^{2+}/CaM IN CARDIAC FIBROBLASTS

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When examining the capacity of isolated cells from the rat heart to produce cyclic GMP (cGMP), we discovered that agonists that stimulate phosphoinositide hydrolysis and mobilize intracellular Ca^{2+} do not stimulate cGMP synthesis in the cardiac fibroblasts (Meszaros *et al.*, 2000; Villegas & Brunton, 1996). However, the fibroblasts respond to nitroprusside with an elevation in cGMP, indicating that they express the soluble (NO-sensitive) guanylyl cyclase (GC) (Villegas & Brunton, 1996). We have examined the conditions under which hormone-sensitive production of cGMP by the soluble GC may occur in isolated cardiac fibroblasts.

Soluble GC is activated by NO, which is produced by NO synthase (Balligand & Cannon, 1997). By RT-PCR and western blot analysis, we find that the fibroblasts constitutively express the endothelial isoform of NO synthase (eNOS). However, agents that stimulate PI hydrolysis and elevate intracellular Ca^{2+} do not result in production of NO (control: 0.3±0.1 mM Nitrite, 1 μM ANG II: 0.3±0.2 mM, 10 μM UTP: 0.4±0.2 mM, $p>0.5$, n=4).

Only a combination of agents that simultaneously elevates cyclic AMP and Ca^{2+} stimulates production of NO in the intact fibroblast (control: 0.3±0.1 mM, 1 μM isoprenaline (ISO) + 1 μM ANG II: 1.2±0.1 mM and 1 μM ISO + 10 μM UTP: 1.8±0.4 mM, $p<0.001$, n=4); this stimulation is blocked by 1 mM L-NMMA and 1 μM BAPTA-AM. PGE_2 (10 μM) and forskolin (10 μM) can substitute for isoprenaline (control: 0.8 ±0.2 mM, forskolin + UTP: 2.6±0.3 mM, PGE_2 + UTP: 1.4±0.3 mM, $p<0.005$, n=4), indicating that specific receptor activation is not required; rather, an increase in cellular cAMP is required. 10 μM H-89, a protein kinase A inhibitor, blocks the stimulation (control: 0.5±0.1 mM, ANG II + ISO + H-89: 0.5±0.2 mM, $p<0.05$, n=4), whereas pre-treatment with 1 μM okadaic acid, a phosphatase inhibitor, enhances activity of eNOS *in vivo* by 30±5%, suggesting that a phosphorylation-dephosphorylation mechanism is involved ($p<0.05$, n=4).

In cell extracts, NO synthase is partially activated in the presence of Ca^{2+}/calmodulin, whereas addition of PKA (1 μg) and ATP (100 μM) enhances activity by 4±0.6 fold ($p<0.01$, n=4); this activation is blocked by Ca^{2+} chelation. Activated eNOS can be inactivated by exposure to phosphatases and re-activated by the addition of PKA. [^{32}P]-labeled eNOS can be immunoprecipitated from fibroblasts treated with isoprenaline and ANG II.

It appears that eNOS in cardiac fibroblasts is only slightly activated by Ca^{2+}/calmodulin; to be fully activated it must also be phosphorylated. Thus, eNOS integrates signals from two second messenger pathways, the cAMP-PKA pathway and the Ca^{2+}/calmodulin pathway.

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68P HOMOCYSTEINE INHIBITS NO FORMATION BY REDUCING L-ARGININE TRANSPORT

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Hyperhomocysteinemia is associated with vascular endothelial cell dysfunction and increased risk for atherosclerosis and atherothrombosis. Free radical formation during homocysteine (HCY) auto-oxidation has been implicated in its vascular toxicity (Loscalzo *et al.*, 1996). It also has been reported that hyperhomocysteinemia impairs endothelium-dependent vasodilation (Lentz *et al.*, 1996). Our previous studies showed that HCY (5mM) reduces acetylcholine-induced relaxation of rat aortic strips. Acetylcholine-induced vasorelaxation in rat aorta is known to depend mainly on the nitric oxide (NO) production from its substrate L-arginine. Therefore, we hypothesized that HCY toxicity involves altered NO production.

We tested this hypothesis with experiments using cultured bovine aortic endothelial cells (BAECs). In order to determine the effects of HCY on NOS activity, we adapted an *in vitro* NOS assay method for use with living BAECs. For this analysis, BAECs were incubated with HCY for various times and assayed for conversion of ^{3}H-arginine to ^{3}H-citrulline. In the last 15 min, buffer containing 10 μM ^{3}H-arginine were added to each well. After the reaction was stopped, ethanol was added to each monolayer. HEPES buffer (pH 5.5) were added after evaporation. Radioactivity in 200 μl of supernatant was counted to determine total arginine uptake. The rest of the supernatant was applied to a resin column. Radioactivity corresponding to ^{3}H-citrulline content in the eluent was measured. eNOS activity was calculated as total amount of ^{3}H-citrulline divided by radioactive arginine transported into cells.

Our results showed that NO production from extracellular arginine was decreased by HCY in a concentration-dependent manner due to a decrease in NOS activity (measured as fractional conversion of ^{3}H-arginine). The maximum reduction of basal NOS activity by HCY was observed at 5 mM HCY for 24 hr (41±6.3%, N=8, $p<0.05$). In addition, HCY-treated cells had lower intracellular levels of the ^{3}H-arginine, indicating that arginine transport is also reduced.

Our group has shown previously that arginine transport in BAECs is mediated predominantly by the y^+ transport system (Ogonowski *et al.*, 2000). To test whether HCY reduces NO formation by reducing arginine availability, we determined effects of HCY on activity and expression of the y^+ transporter. HCY decreased arginine uptake at 24 hr with a maximum reduction of 30±5.5% (N=16, $p<0.01$) from the basal value. Protein expression of the arginine y^+ transporter (CAT-1) was reduced by 28±4% (N=4, $p<0.01$) after 24 hr exposure of BAECs to HCY (5 mM). HCY hyperpolarized BAECs after 6-24 hrs as indicated by increased intracellular content of TPP^+ (33±4.8% to 38±4.3% at 6 & 24 hr for 5 mM HCY), (N=12, $p<0.01$).

In conclusion, 24 hr exposure to HCY decreases NO production in BAECs by reducing arginine uptake and NOS activity. The resulting decrease in NO formation will reduce its beneficial effects. Suppression of arginine transport, even with increased membrane potential, may be related to decreased transport protein expression and/or oxidation of this protein.

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69P ROLE OF L-ARGININE IN THE VASCULAR ACTIONS AND DEVELOPMENT OF TOLERANCE TO NITROGLYCERIN

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The mechanism of GTN's vasodilator actions is generally agreed to involve denitration and release of NO causing vasorelaxation (Salvemini et al., 1992). However, evidence has accumulated to support an additional, endothelium-dependent, mechanism (Malta, 1989). Mechanisms of tolerance to GTN have been intensely investigated and are thought to involve both vascular and humoral mechanisms (Parker & parker, 1998). Our goal was to test the role of nitric oxide synthase (NOS) and its substrate L-arginine in the development of tolerance to nitroglycerine's (GTN) vasodilator actions.

Effects of GTN on NOS activity and NO formation were tested in cultured bovine aortic endothelial cells (BAECs) by an assay for conversion of ^{3}H-arginine to ^{3}H-citrulline and by a photometric assay of NO formation. Tolerance to GTN's vasorelaxing effects was studied by pretreating rat aortic rings with GTN for 2 h and then determining GTN concentration-relaxation curves. Tissue content of L-arginine was assayed before and after the GTN exposure. Effects of GTN on BAECs uptake of L-arginine were also determined. Data were analyzed by ANOVA (N=7/10).

The arginine to citrulline conversion assay showed that GTN stimulated NOS basal activity by 46 ± 18 %, comparable with the acetylcholine (ACh)-treated controls (49 ± 12%). Both effects were blocked by L-NMMA. The photometric assay showed that both GTN and ACh-stimulated NO formation. Both effects were potentiated by L-arginine and inhibited by L-NAME. L-NAME inhibited ACh responses ~80% compared with ~40% for the GTN responses. This is consistent with the known action of GTN as a NO donor. Pre-treatment of rat aorta with GTN caused substantial tolerance to GTN's vasodilating effects as evidenced by a 38-fold rightward shift of the concentration-relaxation curve. Addition of L-arginine to the pre-treatment medium substantially inhibited this effect, reducing the rightward shift to 4.4 fold of control values. D-arginine did not alter GTN tolerance. GTN tolerance was associated with a 40% reduction in L-arginine tissue levels. GTN had a biphasic effect on BAEC uptake of L-arginine, stimulating uptake at 5 min and 15 min (+24 & +40 %), and suppressing uptake after 1 h and 4 h (-22 & -35 %).

In summary, acute GTN treatment stimulates endothelial NOS activity and increases cellular uptake of L-arginine. Prolonged GTN exposure reduces GTN's vasodilator actions, decreases L-arginine tissue levels and depresses BAECs uptake of L-arginine. Supplementation of L-arginine reduces the development of GTN tolerance. These data indicate that GTN tolerance depends in part on activation of the NOS pathway.

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70P ENDOTHELIAL NITRIC OXIDE SYNTHASE IS A SITE OF SUPEROXIDE SYNTHESIS IN ENDOTHELIAL CELLS TREATED WITH NITROGLYCERIN

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Previous studies have shown superoxide ($O_2^{\cdot-}$) production by endothelial cells (EC) treated with a Ca^{++}-iontophore or bradykinin (Katusic and Vanhoutte, 1989) and nitroglycerin (GTN), a nitric oxide (NO) donor (Dikalov *et al.*, 1998). When the supply of L-arg to eNOS is limited, eNOS utilizes oxygen as its pricipal substrate and produces $O_2^{\cdot-}$ (Pritchard *et al.*,1995, Ogonowski *et al.*, 2000). The purpose of this study was to test the hypothesis that GTN stimulates endothelial nitric oxide synthase (eNOS) to increase superoxide ($O_2^{\cdot-}$) synthesis in EC.

Production of $O_2^{\cdot-}$ by bovine aortic EC (passages 3-5) was determined by spectrophotometrically measuring superoxide dismutase-inhibited reduction of ferricytochrome C to ferrocytochrome C. Cells were incubated in buffer without L-arginine (L-arg). Production of $O_2^{\cdot-}$ was measured using EC either untreated or treated with L-NAME or L-arg alone or following treatment with GTN (10^{-6} M for 60 min), alone or with GTN after pretreatment with nitro-L-arginine methyl ester (L-NAME), L-arg or their inactive enantiomers, D-NAME or D-arg (all 5 X 10^{-4} M) (n=6-7/group). Data were analyzed by ANOVA.

Treatment with L-NAME alone produced a reduction in $O_2^{\cdot-}$ levels from 59 ± 11 to 17.8 ± 4 pmol min^{-1} $(10^6$ cells$)^{-1}$ (units). Treatment with L-arg alone had no effect. Treatment of cells with GTN (10^{-6} M) increased $O_2^{\cdot-}$ production from 69 ± 19 to 155 ± 24 units. This effect was prevented by pretreatment with either L-NAME or L-arg, and was unaffected by D-NAME or D-arg. A lower concentration of GTN (10^{-7} M) resulted in greater $O_2^{\cdot-}$ production than GTN (10^{-6} M), probably because of less NO donation from GTN and less peroxynitrite formation. This effect was inhibited by L-NAME and L-arg. The NO-donor, DPTA-NONOate, unlike GTN, did not have a significant effect on $O_2^{\cdot-}$ production.

Our findings of eNOS as a site of $O_2^{\cdot-}$ production in response to GTN are general agreement with those of another recent study (Münzel et al., 2000). In addition, we demonstrate for the first time that GTN stimulation of $O_2^{\cdot-}$ production can be prevented by L-arg supplementation, indicating that the effect occurs as a result of reduced L-arg availability to eNOS. Given that tolerance to GTN may be caused by $O_2^{\cdot-}$ production (Münzel et al., 2000), our previous report of reversal of nitrate tolerance with L-arginine (Kaesemeyer et al., 1997) is consistent with our present findings. In conclusion, eNOS is a site of $O_2^{\cdot-}$ synthesis in EC activated by GTN. L-arginine may be useful in preventing tolerance to GTN.

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71P MECHANISMS OF SODIUM NITROPRUSSIDE-INDUCED VASODILATION IN PULMONARY AND MESENTERIC ARTERIES FROM NEONATAL PIGLETS

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Both inhaled nitric oxide (NO) and NO donors such as sodium nitroprusside (SNP) are used in the treatment of persistent pulmonary hypertension of the newborn (Clark et al., 2000). These agents act mainly through the activation of the soluble guanylate cyclase (sGC) and the subsequent increase in cGMP levels (Cogolludo et al., 1999). The relaxation induced by cGMP may involve a decrease in intracellular calcium concentration ($[Ca^{2+}]_i$) through the activation of different mechanisms, but they have not been well characterised in neonatal pulmonary arteries (PA). In the present study, we have compared the mechanisms involved in SNP-induced relaxation and $[Ca^{2+}]_i$ reduction in neonatal PA and mesenteric arteries (MA).

PA and MA (2-3 mm in length, 0.5-1.5 mm of internal diameter) from neonatal piglets were isolated for isometric force recording or after loading with fura-2 for simultaneous recordings of $[Ca^{2+}]_i$ and contractile force (Pérez-Vizcaíno et al., 1999).

SNP ($10^{-8} - 3 \times 10^{-5}$ M) fully relaxed noradrenaline (NA)-contracted PA and MA ($pD_2 = 6.66 \pm 0.06$, n = 14 and 6.74 ± 0.14, n = 9, respectively). The sGC inhibitor ODQ markedly inhibited the relaxation induced by SNP. The inhibition of the Na^+/K^+-ATPase by the exposure to a K^+-free medium or to ouabain (10^{-6}M) shifted to the right the curve to SNP in MA ($pD_2 = 5.89 \pm 0.27$ and 5.82 ± 0.13, respectively, $P < 0.05$) but not in PA. The exposure to 80 mM KCl and nifedipine (10^{-6}M) to inhibit K^+ and L-type Ca^{2+} channel conductances, respectively, did not attenuate the relaxation induced by SNP in both vessels. However, the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (Thap, 2×10^{-6}M) markedly shifted the concentration-response curve to SNP ($pD_2 = 5.7 \pm 0.19$, $P < 0.01$ and 5.89 ± 0.19, $P < 0.05$, in PA and MA, respectively). In MA, the combination of K^+-free solution plus Thap further inhibited the relaxation induced by SNP ($pD_2 = 5.27 \pm 0.19$, $P < 0.01$ and $E_{max} = 82 \pm 10\%$ of the contractile response, $P < 0.01$). In fura 2-loaded arteries SNP produced a parallel concentration-dependent reduction in $[Ca^{2+}]_i$ and relaxation in both arteries. Thap in PA and Thap plus ouabain in MA elicited a stronger inhibitory action on the decrease in $[Ca^{2+}]_i$ than on the relaxation induced by SNP, indicating the existence of Ca^{2+}-independent mechanisms in SNP-induced relaxation. The activation of the Na^+/K^+-ATPase by the cumulative addition of KCl after the incubation in a K^+-free medium reduced $[Ca^{2+}]_i$ with similar pD_2 and E_{max} values in PA and MA, whereas relaxed PA with much less efficacy than MA ($E_{max} = 47 \pm 10$ vs $96 \pm 2\%$, $P < 0.05$).

In conclusion, SNP reduces $[Ca^{2+}]_i$ and causes relaxation mainly through the activation of SERCA in PA and of SERCA and Na^+/K^+-ATPase in MA. However, Ca^{2+}-independent mechanisms also seem to contribute to SNP-induced effects. The reduced relaxant response induced by Na^+/K^+-ATPase activation in PA could explain the lack of contribution of this pump to SNP-induced relaxation in these arteries.

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72P DECREASED ENDOTHELIAL NITRIC OXIDE SYNTHASE EXPRESSION AT LOW SHEAR STRESS REGIONS OF THE ARTERIAL VASCULATURE

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One of the factors known to induce endothelial nitric oxide synthase (eNOS) expression is shear stress (Kanai *et al.*, 1995). Thus whenever shear stress is disturbed, it would be expected that eNOS would be reduced. We therefore investigated whether the reduced shear stress at the carotid artery bifurcation and an aortic/intercostal artery junction would cause regions of low eNOS expression.

The common carotid artery bifurcations were excised from female Yorkshire White pigs (25-30kg) (method as used by Giddings *et al.*, 1997) and segments (approximately 5mm long) of external or internal carotid artery embedded with the common carotid (control) in optimum cutting temperature compound (OCT) (BDH, UK). Pig thoracic aortae were obtained from a local abattoir, cleaned of connective tissue and the site immediately proximal and distal to an aortic/intercostal artery junction embedded in OCT along with aorta that was located away from the junction (control). Transverse (carotid) and longitudinal (aortic/intercostal) histological sections (10 μm) from each segment were incubated with a 1/50 dilution of anti-eNOS monoclonal antibody (Transduction Laboratories, USA) followed by a rhodamine-conjugated second antibody (Calbiochem, UK). Measurements were then taken from the sections using an immunofluorescent microscope (Leica BMRB, Germany) and analysed using the Image-Pro Plus 4.0 software package. Data are expressed as the mean±s.e.mean. Data were compared using ANOVA followed by the Student Newman-Keuls post test for between group comparisons. Differences were considered significant at the $p<0.05$ level. In the external carotid artery just distal to its bifurcation with the common carotid artery, eNOS expression on the high shear, flow divider side of the artery was not significantly different from the control (91.6±9.48% cf. 100.0±6.51% for control, n=12). However, the site opposite the flow divider, a region of low shear stress, showed a significant ($p<0.001$) reduction of eNOS expression (25.0±4.10% cf. control, n=12). In the internal carotid artery the trend was repeated. On the flow divider side of the artery, eNOS expression was not significantly different from the control (87.1±10.88% cf. 100.0±13.86% for control, n=12) but the site opposite the flow divider showed significantly ($p<0.001$) less eNOS expression (32.5±4.47% cf. control, n=12). In the aortic endothelium immediately proximal and distal to the junction with an intercostal artery, expression of eNOS was 43.1±9.71% and 42.4±13.04% respectively that of the control (100.0±20.10%, $p \leq 0.05$, both n=19).

These data suggest that the low levels of shear stress observed at arterial bifurcations and junctions result in a reduction of eNOS expression. This is consistent with *in vivo* observations that show localised endothelial dysfunction and atherosclerotic plaque formation, both indicators of low eNOS activity, at these areas of low shear stress.

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73P CHRONIC TREATMENT WITH SIMVASTATIN INCREASES CONTRACTILITY OF RABBIT ISOLATED AORTIC RINGS TO PHENYLEPHRINE BY A MECHANISM NOT INVOLVING NITRIC OXIDE

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The beneficial effects of 'statin' therapy in coronary heart disease and stroke occur sooner and exceed those expected from the observed decrease in low-density lipoprotein cholesterol (Aengevaeren, 1999; Vaughan & Delanty, 1999). Additional mechanisms of action involving improved endothelial function mediated via increased activity of endothelial nitric oxide, decreases in inducible nitric oxide synthase, and decreased production of endothelin-1 have been proposed (Hernandez-Perera *et al.*, 1998; Alfon *et al.*, 1999). We studied the potential role of nitric oxide in changes in basal and agonist-induced responses of rabbit aortic rings produced by 24 hour pre-exposure to activated simvastatin.

Aortas from male New Zealand white rabbits were incubated for 24 hours at 37°C in oxygenated, supplemented medium-199 with and without addition of simvastatin 10^{-5}M. Isometric tension was recorded from freshly cut aortic rings mounted under 2g tension at 37°C in oxygenated Krebs solution. After 1 hour, the integrity of the endothelium was determined by observing >25% relaxation to acetylcholine (ACh) 10^{-6}M after contraction to phenylephrine (PE) $2x10^{-6}$M. The endothelium was removed from some tissues by rubbing the surface of the lumen. Cumulative dose-response curves were constructed to PE 10^{-9}-10^{-4}M, and to ACh 10^{-9}-10^{-5}M in the presence of PE $2x10^{-6}$M. Curves were repeated after addition of haemoglobin 10^{-6}M or Nw-nitro-L-arginine methyl ester (L-NAME) $3x10^{-4}$M.

Simvastatin produced both a significant ($p<0.05$, t-test after non-linear regression) 23±5% endothelium-dependent increase in maximum response to PE ($n^3 10$) and a 20±7% greater maximum relaxation to ACh. Addition of haemoglobin ($n^3 6$) caused no change in resting tension and reduced the relaxation to ACh by 78-80%. It caused significant ($p<0.05$) increases in maximum response to PE of 28±3% in control and 19±2% in simvastatin-treated tissues. L-NAME ($n^3 5$) caused no change in resting tension but converted ACh-induced relaxation into constrictor responses. The maximum contraction to PE after L-NAME was increased significantly ($p<0.05$) by 33±8% in control and 28±3% in simvastatin-treated tissues. There were no significant differences in time-matched control tissues.

The data demonstrate that pre-exposure to simvastatin produces an endothelium-dependent increase in contractile response to PE in rabbit aorta. The differential between control and simvastatin-treated tissue was maintained during treatment with either haemoglobin or L-NAME that reduce nitric oxide-mediated effects. The increased responsiveness to PE produced by simvastatin does not involve basal or agonist-induced changes in nitric oxide release.

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74P ANANDAMIDE-INDUCED VASORELAXATION IS PARTIALLY SENSITIVE TO INHIBITION OF Na^+/K^+-ATPases IN THE RAT ISOLATED MESENTERIC BED

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The mechanisms of vasorelaxation to the endocannabinoid anandamide are presently unclear (Randall & Kendall, 1998). However, it has recently been shown that anandamide may act via myoendothelial gap junctions (Chaytor *et al.*, 1999). We have now examined the effects of the gap junction inhibitors carbenoxolone and palmitoleic acid and the gap junction and Na^+/K^+-ATPase inhibitors ouabain and 18α-glycyrrhetinic acid (18α-GA; Terasawa *et al.*, 1992) on anandamide-induced vasorelaxation in rat mesenteric vessels.

Male Wistar rats (250-350g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1}, i.p.) and exsanguinated. The mesenteric arterial bed was isolated and perfused with oxygenated Krebs-Henseleit buffer containing both indomethacin (10μM) and L-NAME (300μM) to inhibit prostanoid and NO synthesis respectively. After 20min equilibration, methoxamine was added to increase perfusion pressure. Concentration-response curves to anandamide (10nM-10μM) were constructed both in the absence and presence of ouabain (1mM), 18α-GA (100μM), carbenoxolone (100μM) and palmitoleic acid (50μM). In some cases, the endothelium was removed by 3min perfusion with distilled water. Data were compared by ANOVA with Bonferroni's *post hoc* test.

Anandamide induced concentration-dependent relaxations (EC_{50}=0.67±0.14μM; R_{max}=91.1±1.5%; n=6). Relaxations to anandamide were unaffected in the presence of either carbenoxolone (EC_{50}=0.71±0.09μM; R_{max}=80.7±6.3%; n=7) or palmitoleic acid (EC_{50}=0.48±0.08μM; R_{max}=90.5±2.4%; n=6). In the presence of 18α-GA, relaxations to anandamide were significantly ($P<0.001$) inhibited (EC_{50}=1.1±0.2μM; R_{max}=40.9±5.8%; n=6). In the presence of ouabain, relaxations to anandamide were also significantly ($P<0.001$) inhibited (EC_{50}=1.4±0.3μM; R_{max}=50.4±6.5%; n=6). Removal of the endothelium had a modest inhibitory ($P<0.05$) effect on responses to anandamide (EC_{50}=1.3±0.1μM; R_{max}=82.6±2.5%; n=5). In the absence of the endothelium, 18α-GA significantly inhibited ($P<0.001$) responses to anandamide (EC_{50}=1.1±0.1μM; R_{max}=37.7±3.7%; n=6).

The results of the present study show that whilst responses to anandamide are attenuated by the gap junction and Na^+/K^+-ATPase inhibitors ouabain and 18α-GA, they are unaffected by the specific gap junction inhibitors carbenoxolone and palmitoleic acid. However, in large mesenteric vessels from the rabbit, Chaytor *et al.* (1999) reported that a selective gap junction inhibitor attenuated endothelium-dependent relaxations to anandamide. Taken together, these findings point to species/regional differences in the vascular actions of anandamide. In rat mesenteric vessels, relaxation to anandamide appears to occur, at least in part, via non-endothelial Na^+/K^+-ATPases.

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75P THE ROLE OF THE ENDOTHELIUM AND ARACHIDONIC ACID METABOLITES IN THE VASORELAXANT ACTIONS OF ANANDAMIDE IN SHEEP CORONARY ARTERIES

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The endocannabinoid, anandamide, relaxes vascular smooth muscle and has recently been proposed as an endothelium-derived hyperpolarising factor, EDHF (Randall & Kendall, 1998). However, its precise mechanism of vasorelaxation remains as yet unclear. Thus, it is not clear to what extent, if at all, the vascular endothelium, cannabinoid (CB) receptors and/or vanilloid receptors are involved in its vascular actions (Randall & Kendall, 1998; Pratt *et al.*, 1998; Zygmunt *et al.*, 1999). We sought, therefore, to establish whether anandamide relaxes sheep coronary arteries by acting directly on the vascular smooth muscle or indirectly *via* the endothelium or a subsequent dilatory metabolite(s).

A pair of rings were removed from sheep circumflex coronary arteries and mounted under 2 g resting tension in a 10 ml tissue bath containing Krebs-Henseleit solution pre-warmed to 37°C and equilibrated with 95% O_2 and 5% CO_2. Isometric tension was monitored *via* a force displacement transducer coupled to a Grass 79D polygraph. One of each pair of rings was left endothelium intact and untreated (control), whereas the other ring was either endothelium denuded or pre-treated for 30 min with either the selective CB_1 receptor antagonist, SR141716A (3 μM) (Rinaldi-Carmona *et al.*, 1994), the NO synthase inhibitor, L-NAME (100 μM), the anandamide amidohydrolase inhibitor, phenylmethylsulfonylfluoride (PMSF; 200 μM), or the cyclo-oxygenase inhibitor, indomethacin (3 & 10 μM). Rings were then precontracted to the thromboxane-A_2 mimetic, U46619 (2-10 μM) and subsequently relaxed by cumulative addition of anandamide (0.01-30 μM). Mean sensitivity (pEC_{50} values) and % maximal relaxation (R_{max}) to anandamide in control and treated rings were compared using Student's *t*-test or ANOVA and Dunnett's *post-hoc* test, as appropriate.

Anandamide (0.01-30 μM) induced concentration-dependent relaxation of U46619-evoked precontractions in rings with intact endothelium, with a mean pEC_{50} of 6.06±0.1 and R_{max} of 95.6±2.5% (n=7). These effects were slow in onset. Removal of the endothelium reduced this effect, decreasing the mean pEC_{50} value for anandamide to 5.06±0.12 ($P<0.002$; n=7) without affecting R_{max} (86.8±4; $P>0.05$). By contrast, pre-treatment with the selective CB_1 receptor antagonist, SR141716A (3 μM), or the NO synthase inhibitor, L-NAME (100 μM), did not modify anandamide-induced relaxations. Mean pEC_{50} and R_{max} values for anandamide in SR141716A (3 μM) treated rings were 6.16± 0.12 and 85.9±5.6%, compared with 5.96±0.2 and 96.2±3.7%, respectively, in control rings ($P>0.05$; n=5). Similarly, the mean pEC_{50} and R_{max} values for anandamide in L-NAME (100 μM) treated rings (5.65±0.2 and 94.3±6.9%) were not different from those in control rings (5.70±0.2 and 90.9±4.3%; $P>0.05$; n=5).

On the other hand, pre-treatment with the anandamide amidohydrolase inhibitor, PMSF (200 μM), or cyclo-oxygenase inhibitor, indomethacin (3 & 10 μM), significantly attenuated anandamide-induced relaxations. Thus, pre-treatment with PMSF (200 μM) markedly reduced R_{max} for anandamide from 96.9±2.2% in control rings to 7.8±16.7% ($P<0.003$; n=6). Similarly, R_{max} for anandamide was reduced from 91.4±6.4% in control rings to 32.5±15% and 15.2±10.5%, respectively, in indomethacin (3 & 10 μM) treated rings ($P<0.01$; n=4-8).

These findings suggest that the relaxant actions of anandamide in the sheep coronary artery (i) are mediated, at least in part, through the endothelium and (ii) require prior cellular uptake and conversion of anandamide to a vasodilatory arachidonic acid metabolite(s), most probably a prostanoid.

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76P POTASSIUM AND NORADRENALINE RESPONSES IN ISOLATED POPLITEA PREPARATIONS FROM PATIENTS WITH SERIOUS PERIPHERAL OCCLUSIVE ARTERIOPATHY

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Peripheral occlusive arteriopathy (POA) represents one of the most important problems in cardiovascular disease, because these patients do not usually respond to pharmacological treatments. Endarterectomy has sometimes been used to mechanically remove atheroma plaques from the arteriopathic vessels. This technique could improve blood flow but may also damage vascular smooth muscle cells. At the present time there are no controlled studies to compare the responses of isolated arteries from patients suffering from POA with the corresponding ones in healthy arterial tissue. Nor do any studies exist to characterize the responses of the isolated endarterectomized arteries from these patients.

This study has been carried out in order to characterize the responses to KCl and noradrenaline (NA) in isolated poplitea preparations, obtained after leg amputations from 60 to 90-year-old men and women suffering from serious POA. The same responses were also characterized in the endarterectomized arterial tissue obtained from these patients. As a reference, isolated arterial preparations were used from non-arteriopathic vascular tissue obtained following traumatic leg amputations or surgery where it was necessary to resect a certain percentage of healthy vessel. In all cases the samples were obtained with the patients' consent and the permission of the Hospital's Clinical Research Ethics Committee. After the surgical operation, the arterial samples were stored in a refrigerator at 4° C. After 12-36 hours they were cut into rings of about 5 mm and mounted in organ baths containing Krebs-Henseleit of the following composition (mM): NaCl 118.2; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 6.25; and glucose 10. They were maintained at 37° C, and constantly bubbled with 95% O_2 and 5% CO_2. We evaluated the concentration-dependent contractions elicited by KCl (15 mM-75 mM) and NA (10^{-7}-10^{-4} M) in these preparations. We obtained only one dose-response curve for each ring. The results are always expressed as mean values ± s.e. mean for at least five patients samples and were analysed by one-way ANOVA. The Bonferroni test was used and the differences were considered significant when $p<0.05$. The value for each patient was obtained by carrying out at least three homogeneous experiments on the sample and taking an average.

The vasoconstrictor responses to KCl and NA were significantly greater in control preparations than in preparations from patients suffering from POA. The responses in the endarterectomized preparations from these patients were similar to those obtained in the non-endarterectomized ones (Table 1). We can therefore conclude that the poplitea arteries from patients with severe POA respond very poorly to KCl and NA and moreover these responses do not improve when an endarterectomy is carried out on these arteries.

TABLE 1. Maximal effect (g) of KCl and NA in different human poplitea preparations.

	Control	Arteriopathic	Endarterectomized
KCl	5.30±0.78	1.21±0.40[a]	0.68±0.04[a]
NA	3.39±0.48	0.28±0.05[a]	0.51±0.23[a]

[a]$p<0.001$ vs control by Bonferroni test.

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77P MECHANISMS OF POTASSIUM-INDUCED VASORELAXATION IN THE RAT AORTA

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The mechanisms of vasorelaxation to K^+ have recently received interest following the proposal that endothelium-derived K^+ may represent an endothelium-derived hyperpolarizing factor (EDHF) (Edwards *et al.*, 1998). In this respect it was proposed that endothelium-derived K^+ induces relaxation via the Na^+/K^+-ATPase and inwardly rectifying K-channels. We have previously shown in the rat mesentery that K^+-relaxation is endothelium-dependent, partly mediated by prostanoids and may also involve gap junctional communication (Harris *et al.*, 1999). The mechanisms of K^+-relaxation have now been investigated in the rat aorta.

Male Wistar rats (250-350g) were anaesthetized with sodium pentobarbitone (60mg kg^{-1}, i.p.) and exsanguinated. Thoracic aortae were removed and placed in oxygenated Krebs-Henseleit, prior to mounting as 2-3mm rings for isometric recording. Once mounted the rings were placed under 1g tension and allowed to equilibrate for 1h. Methoxamine (100μM) was then added as a priming dose to contract the preparation for 10min. The methoxamine was then washed out and in some experiments the buffer was replaced with K^+-free Krebs. The rings were then recontracted with methoxamine (100μM) and solutions of KCl were added cumulatively to the organ bath to achieve concentrations of 1-25mM. The effects of the cyclo-oxygenase inhibitor, indomethacin (10μM); the NO synthase inhibitor, L-NAME (300μM); the gap junction inhibitor, carbenoxolone (100μM), and ouabain (1mM) were investigated. All data were compared by ANOVA.

In the presence of normal buffer, addition of KCl (7-25mM) did not cause relaxation of precontracted rings and caused a contraction of 19.0±5.8% (mean±s.e.mean, n=8) occurring at 8mM. By contrast in K^+-free buffer, KCl caused relaxation of tone with a maximum relaxation (R_{max})=207±36% at 8mM (n=8). In the presence of indomethacin the relaxations to KCl were attenuated (R_{max}=103±7%, P<0.01, n=7) and a contraction was uncovered from 12-25mM, with an increase in tone of 77.3±7.4% at 25mM. The additional presence of L-NAME had no further inhibitory effects (n=7). The presence of ouabain abolished relaxations to KCl (n=6). Carbenoxolone markedly attenuated relaxations to KCl with R_{max}=110±8% occurring at 5mM (n=8) and a contraction was uncovered between 20-25mM K^+ with an increase in tone of 52.0±12.9% at 25mM.

The ability of K^+ to only cause relaxation in K^+-free buffer is compatible with the findings of Andersson *et al.* (2000) and questions the physiological role of K^+ as an endothelium-derived relaxant. The relaxations to K^+ were partly sensitive to indomethacin, which is in agreement with our previous suggestion that K^+ acts, in part, via the release of prostanoids (Harris *et al.*, 1999). The responses to K^+ were also sensitive to gap junction inhibition, suggesting that the relaxations are dependent on direct cellular coupling. Ouabain opposed the responses to K^+, consistent with either a role for gap junctions (Schirmacher *et al.*, 1996) or Na^+/K^+-ATPase activation. In summary, K^+-relaxation is suppressed by physiological levels of K^+ and is transduced by a variety of mechanisms which may include prostanoids and gap junctional communication.

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78P PHARMACOLOGICAL EXAMINATION OF VASOCONSTRICTOR α_2 ADRENOCEPTORS OF THE PORCINE ISOLATED SPLENIC ARTERY

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In our original report on α-adrenoeptors of the porcine isolated splenic artery we noted that the selective α_2-adrenoceptor agonist UK-14304 produced only small contractions, even though α_2-adrenoceptor binding sites were detectable (Wright *et al.*, 1995). Barbieri and coworkers (1998) proposed that these receptors were functionally 'silent', since the selective agonist BHT-920 failed to elicit a response even in the presence of an ancillary vasoconstrictor, phenylephrine. However, we recently reported that large contractions to UK-14304 can be uncovered in the presence of a combination of forskolin and U46619 (Roberts *et al.*, 1999). We have extended these observations to other selective α_2-adrenoceptors agonists and attempted to determine the receptor subtype involved.

Segments of the porcine isolated splenic artery (4mm long; i.d. 4mm) were prepared for isometric tension recording as previously described (Roberts *et al*, 1999). Following the attainment of reproducible responses to 60mM KCl, preparations were exposed to 0.1μM prazosin (to inhibit α_1-adrenoceptors) followed by increasing concentrations of putative selective α_2-adrenoceptors agonists in the presence or absence of a combinaton of forskolin and U46619 (residual tone equivalent to < 10% of that to 60mM KCl). In a separate series of experiments, the effect of UK-14304 was examined in the presence and absence of 0.1μM phentolamine and 0.1μM rauwolscine. The maximum response to the agonists have been compared to that elicited by 60 mM KCl (E_{max}) and the potency expressed as the negative logarithm of the concentration producing 50% of the maximum response (-log EC_{50}).

In control preparations, none of the agonists, UK-14304 (1nM-3μM), clonidine (10nM-10μM), xylazine (10nM-30μM) and rilmenidine (10nM-30μM) produced a contractions greater than 10% of the response to 60 mM KCl (n=6-8). In each case the responses were abolished by 1μM RX-811059, a selective α_2-adrenoceptor antagonist (Wright *et al.*, 1995). In the presence of U46619 and forskolin, responses to the agonists were increased 2-5-fold, with UK-14304 significantly larger than rilmendine (Table 1) and approximately 30-fold more potent. RX-811059 (1μM) caused a greater than 70% inhibition of the response to maximally effective concentrations of the agonists. Rauwolscine (pK_B > 9) and RX-811059 (pK_B 9.02±0.24, n=8) were 25-fold more potent than phentolamine (pK_B 7.60±0.31, n=8) as antagonists of UK-14304-induced contractions.

Table 1: Mean E_{max} and -log EC_{50} values for putative agonists in the splenic artery (forskolin and U46619 present, n=10-13).

	UK-14304	Clonidine	Xylazine	Rilmenidine
E_{max}	42.6±6.0	35.0±2.6	50.8±7.9	17.3±4.1
-log EC_{50}	7.05±0.09	5.43±0.13	5.52±0.08	5.61±0.10

These results confirm the presence of functional α_2-adrenoceptors in the porcine isolated splenic artery that, in the presence of forskolin and U46619, can be activated by both full and partial agonists. Based on the rank order of potency of the antagonists against UK-14304-induced contractions (rauwolscine>phentolamine>>prazosin) the receptor appears to belong to the α_{2A}-adrenoceptor subtype.

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79P A STUDY OF THE EFFECT OF CO-ACTIVATION OF ADENYLYL CYCLASE ON α_2 ADRENOCEPTOR-MEDIATED RESPONSES OF THE PORCINE ISOLATED TAIL AND CORONARY ARTERIES

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Porcine blood vessels possess both vascular and endothelial α_2-adrenoceptors that mediate contraction and relaxation, respectively (Roberts *et al.*, 1998; Bockman *et al*, 1993). While constrictor responses to α_2-adrenoceptors can be augmented by a combination of U46619, a thromboxane-mimetic, and forskolin (Roberts *et al.*, 1998; 1999), the effect of co-activation of adenylyl cyclase on endothelium-dependent α_2-adrenoceptor relaxation is not known. We have compared the effect of forskolin and the ß-adrenoceptor agonist isoprenaline on α_2-adrenoceptor responses of the isolated tail and coronary artery.

Segments (4 mm long) of the pig tail (i.d. <1mm) and coronary artery (i.d. 4mm) were prepared for isometric tension recording in a 20ml organ baths containing Krebs-Henseleit solution (37°C, 95% O_2/5% CO_2; 0.1µM prazosin) and placed under 5 g wt. and 10 g wt., respectively. Preparations were stimulated with 60 mM KCl until reproducible responses were obtained. The tail artery was exposed to UK-14304 (1nM - 3µM) in the absence and presence of residual vasoconstrictor tone produced by U46619 relaxed with either forskolin (0.1µM) or isoprenaline (1µM). For the coronary artery, UK-14304 was examined against vasoconstrictor tone (60% of 60mM KCl) produced by U46619 with or without either forskolin (0.1 µM) or isoprenaline (0.03µM). UK-14304 was also examined in endothelium-denuded segments (coronary artery only) and in the presence of 1µM RX-811059, an α_2-adrenoceptor antagonist (Roberts *et al.*, 1998). The potency of UK-14304 was determined as the -log EC_{50}, while the maximum effect (E_{max}) given as a percentage of the response to 60mM KCl (tail artery) or U46619-induced tone (coronary artery). Differences between the control and treatment values were compared by ANOVA followed by a Dunnett's test.

Under control conditions, UK-14304 elicited small contractions of the tail artery (Table 1). Following exposure to 10-20nM U46619, which produced a large contraction (60% of 60mM KCl), and subsequent relaxation with either isoprenaline or forskolin (to < 10% of 60mM KCl), UK-14304 produced large, concentration-dependent contractions (Table 1). In the coronary artery UK-14304 produced concentration-dependent relaxations of U46619-induced contractions (Table 1). UK-14304 failed to cause a relaxation in endothelium-denuded preparations. As shown in Table 1, relaxations to UK-14304 were not significantly altered by the presence of either forskolin or isoprenaline. RX-811059 (1µM) abolished responses to UK-14304 in both preparations.

Table 1: Mean -log EC_{50} and E_{max} values for UK-14304 in the porcine isolated tail and coronary arteries (n= 6-10).

	Tail Artery (Contraction)		Coronary Artery (Relaxation)	
	-log EC_{50}	E_{max}	-log EC_{50}	E_{max}
Control	<7	5.4±1.7		
U46619 (1)			7.00±0.11	30.1±5.4
U46619/For'	7.93±0.09	66.0±4.7*	7.08±0.16	40.2±6.8
U46619 (2)			7.54±0.16	54.0±5.2
U46619/Isop'	7.99±0.36	58.1±6.1*	7.50±0.09	62.4±8.3

* - denotes a statistically significant difference ($p<0.05$).

These results demonstrate that while vascular α_2-adrenoceptors are potentiated by exposure to U46619 and an activator of adenylyl cyclase, a similar interaction does not occur at the level of the endothelium.

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80P PHARMACOLOGICAL EVIDENCE FOR PRE- AND POST-JUNCTIONAL α_2-ADRENOCEPTORS IN THE PORCINE ISOLATED RECTAL ARTERY

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In the cardiovascular system of the pig α_2-adrenoceptors are widely distributed and the density can vary from 120-500 fmol.mg-1 protein between vascular beds (Wright *et al.* 1995). In a number of large arteries, however, including those of the mesenteric circulation, α_2-adrenoceptor-mediated contractions are only detectable in the presence of an ancillary vasoconstrictor agent (Nielsen *et al.* 1991; Roberts *et al.* 1998). In light of these findings we have examined the density and function of α_2-adrenoceptors in the porcine isolated rectal artery.

Porcine mesenteric tissue, from the descending colon to the anus, and aortae were obtained from a local abattoir and transported to the laboratory in Krebs-Henesleit solution at 4°C. P_2 membranes of the both arteries were prepared and saturation analysis using [3H]-RX821002 performed, as previously described (Wright *et al.* 1995). Five mm segments of the rectal artery were prepared for isometric tension recordings by the method described by (Roberts *et al.*, 1998). Following the attainment of reproducible responses to 60 mM KCl, the effect of noradrenaline was determined in the absence or presence of 0.3µM prazosin, 1µM RX811059 (a selective α_2-adrenoceptor antagonist) or a combination of both agents. The effect of UK14304, a selective α_2-adrenoceptor agonist, was also examined in absence and presence of both U46619, a thromboxane-mimetic, and forskolin (which caused contractile tone equivalent to <10% of 60mM KCl); 0.3µM prazosin was present throughout. In addition, the effect of UK14304 (0.1µM) on electrically-evoked contractions (8Hz, 2 sec every 10 min) was also investigated.

[3H]-RX821002 binding to membranes of the rectal artery was only weakly displaced by 10µM RX811059; non-specific binding was greater that 70% of the total binding at 1nM [3H]-RX821002 with total specific binding estimated at < 20 fmol.mg-1 protein (n=3). In a single experiment on aortic membranes the specific binding of 1nM [3H]-RX821002 was greater than 95% of the total binding.

Noradrenaline (0.1-30µM) elicited concentration-dependent contractions of the rectal artery (10.6±0.4 g wt, at 30µM, n=6). Prazosin (0.3µM) caused a 50-fold rightward shift of the concentration response curve and reduced the maximum by 49.8±6.6% (n=6). RX811059 (1µM) failed to alter responses to noradrenaline either alone or in combination with 0.3µM prazosin (n=6). UK14304 (0.01-3µM) failed to elicit a contraction in the presence of 0.3µM prazosin (n=7). However, following exposure to U46619 (10-50nM) and then forskolin (0.1-1µM), UK14304 (0.1-3µM) caused concentration-dependent contractions. The response to 3µM UK14304, equivalent to 34.8±6.5% of that to 30µM noradrenaline (n=7), was abolished by 1µM RX811059 (n=7). Electrically stimulation (8Hz, 2 sec) of the rectal artery caused reproducible contractions (0.84±0.25 g wt, n=7) which were abolished by both 0.1µM UK14304 (n=7) and 0.3µM tetrodotoxin (n=4). In preparations exposed to 0.1µM UK-14304, the addition of 1µM RX-811059 restored neurogenic contractions to 69.6±18.0% of the control response (n=7).

α_1-Adrenoceptors are the principal subtype mediating contractions of the rectal artery to noradrenaline. However, the presence of neuronal and vascular α_2-adrenoceptors, albeit at a density below the limit of detection by radioligand binding, can be demonstrated functionally under appropriate conditions or following pharmacological manipulation.

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81P RESPONSES TO 5-HT IN RAT ISOLATED CEREBRAL RESISTANCE ARTERIES

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Under normal, isometric conditions, 5HT causes contraction of isolated cerebral blood vessels mediated via $5HT_{1B/D}$ receptors (eg Nilsson *et al.*, 1999). However, it has recently been reported that, in pre-contracted canine cerebral vessels, 5HT produces vasorelaxation via $5HT_7$ receptors (Terron & Falcon-Neri, 1999). Here we determined 5HT responses in pressurised resistance arteries that develop intrinsic tone. These permit monitoring of either vasodilator or vasoconstrictor responses under physiological conditions.

Male Long Evans rats (350-450g) were anaesthetised (sodium methohexitone, 40-60 mg/kg, ip) and killed by exsanguination and the brain removed. A segment of the middle cerebral artery (MCA) was dissected, cleaned and set up in a Halpern pressure myograph (Halpern *et al.*, 1984). Over 90 min, vessels developed myogenic tone at a pressure of 60 mmHg. Concentration-response curves were obtained on addition of 5HT, or the $5HT_{1B/1D}$ agonists, 5-nonyloxytryptamine (5-NOT; Glennon *et al.*, 1996) or 5-propoxy-3-(1,2,5,6-tetrahydropyrid-4-yl)pyrrolo[3,2-b]-pyridine (CP-94,253; Koe *et al.*, 1992) to the superfusion medium. In further experiments, responses were obtained to a single concentration of 5HT (1 μM) before and after exposure of the MCA to the $5HT_{1A}$ receptor antagonist, WAY100635 (0.3 μM; Fletcher *et al.*, 1994) or, the $5HT_{1B/D}$ receptor antagonist, GR127935 (0.5 μM; Skingle *et al.*, 1993). Results are mean±s.e.mean. Differences were considered significant if $p<0.05$ (Wilcoxon signed rank test).

At 60 mmHg, MCA segments developed myogenic tone equivalent to a 19.5±3.7% (n=9) reduction in diameter. The mean basal diameter after development of tone was 185±9 μm (n=9). Thereafter, 5HT, 5-NOT and CP-94,253 caused concentration-dependent contractions. The agonists were approximately equipotent (-logEC_{50}; 5HT 6.9±0.2, 5-NOT 6.6±0.2, CP-94,253 6.8±0.2) but relative to 5HT (maximum reduction in diameter 17.6±3.7%, n=7) and 5-NOT (18.7±3.6%, n=6), CP-94,253 was a partial agonist (9.7±2.4%, n=6). In separate experiments, 5HT (1 μM) produced a 21.6±4.7% (n=5) reduction in diameter. This response was reproducible (2nd response 20.9±2.5%). Responses to 5HT were unaffected by WAY100635 (0.3 μM; control 22.1±3.7%, WAY100635 22.6±3.8%, n=5), but were almost completely abolished by GR127935 (0.5 μM; control 18.7±2.1%, GR127935 2.8±1.0%, $p<0.05$, n=6). Even in the presence of GR127935, no vasodilator responses were produced by 5HT.

This study has shown that 5HT, 5-NOT and CP-94,253 produce vasoconstrictor responses in rat cerebral resistance arteries. Relative to 5HT, 5-NOT was a full agonist while CP-94,253 was a partial agonist. Responses to 5HT were apparently mediated by activation of $5HT_{1B/D}$ receptors. In addition, under the present experimental conditions, there was no evidence to suggest the presence of a 5HT-induced vasodilator response in rat cerebral blood vessels.

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82P PHARMACOLOGY OF THE HUMAN α5H105Rβ3γ2s $GABA_A$ RECEPTOR EXPRESSED IN *XENOPUS* OOCYTES

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A single histidine residue in the N-terminal extracellular region of the α1, 2, 3 and 5 subunit of the human $GABA_A$ receptor, which is replaced by an arginine in the benzodiazepine (BZ) resistant α4 and α6 subunits, has been identified as a major determinant for high affinity binding of BZ-site ligands (Benson *et al.*, 1998; Wieland *et al.*, 1991). In the present study the effect of mutating this conserved histidine at position 105 in the α5 subunit to an arginine residue (mutant α5H105Rβ3γ2s) on GABA potency and BZ-site pharmacology has been investigated.

Using two electrode voltage clamp recording on *Xenopus* oocytes in which the subunits α5, α5H105R, and α6 were expressed as αXβ3γ2s, the percentage modulation of the EC_{20} response to GABA by BZ-site ligands (applied at a concentration approx. 100 times their binding affinity on α5β3γ2s) was investigated. The data represents n values of ≥ 3 and is expressed as the mean ± s.e.mean. The Student's *t*-test was used to test significance ($P < 0.05$).

This mutation significantly reduced the GABA potency resulting in a mean EC_{50} value of 13.2μM (pEC_{50} = 4.88 ± 0.05) on α5H105Rβ3γ2s compared to 5.1μM (pEC_{50} 5.29 ± 0.06) on α5β3γ2s ($P < 0.0001$) and 1.1μM (pEC_{50} 5.97 ± 0.04) on α6β3γ2s ($P < 0.0001$).

Diazepam and flunitrazepam which are full agonists on α5β3γ2s and dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and ethyl-β-carboline-3-carboxylate (β-CCE) which are inverse agonists on α5β3γ2s were all inactive on α5H105Rβ3γ2s receptors consistent with an α6β3γ2s-like pharmacology. In contrast, the weak inverse agonist Ro 15-4513, the antagonist flumazenil and the agonist bretazenil on α5β3γ2s, displayed much greater agonism on α5H105Rβ3γ2s than anticipated from their weak agonist effects on α6β3γ2s receptors (Table 1).

To conclude, the H105R mutation confers α6-like pharmacology to some BZ site ligands, however Ro 15-4513, flumazenil and bretazenil display significantly greater agonist activity on α5H105Rβ3γ2s receptors. The data indicates that this histidine residue is not only a key determinant in the affinity of BZ site ligands on α5 $GABA_A$ receptors (Benson *et al.*, 1998), but also plays a major role in determining the efficacy of these ligands.

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Table 1. Modulation of currents evoked by the EC_{20} concentration of GABA on receptors by BZ-site ligands.

Subunits	DMCM (100nM)	β-CCE (3μM)	Ro 15-4513 (100nM)	Flumazenil (100nM)	Bretazenil (100nM)	Diazepam (1μM)	Flunitrazepam (300nM)
α5β3γ2s	-55.4 ± 0.9 %	-15.1 ± 1.9 %	-15.0 ± 2.2 %	-4.9 ± 5.8 %	+12.5 ± 2.7 %	+116.4 ± 12.5%	+147.2 ± 1.7 %
α5H105Rβ3γ2s	+7.5 ± 1.8 %	+10.4 ± 6.1%	+88.5 ± 2.4 %	+72.0 ± 4.2 %	+22.0 ± 3.5 %	+1.8 ± 1.6 %	-4.9 ± 2.9 %
α6β3γ2s	+21.0 ± 12.9	+3.0 ± 2.0 %	+3.0 ± 9.3 %	+13.1 ± 1.8 %	+12.6 ± 6.3 %	+0.5 ± 3.1%	+10.9 ± 4.3 %

83P IDENTIFICATION AND LOCALIZATION OF Na^+K^+ ATPase α-SUBUNITS IN RAT ARTERIES

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Vascular endothelial cells release an "endothelium-derived hyperpolarizing factor" (EDHF), which hyperpolarizes, and thus relaxes, the underlying vascular smooth muscle. Recent studies in rat hepatic and mesenteric arteries identified EDHF as K^+, released from endothelial cells via small- and intermediate-conductance, calcium-sensitive potassium channels. In addition, the resulting local increase in extracellular K^+ concentration was shown to activate smooth muscle cell Na^+, K^+ ATPases and inwardly-rectifying K^+ channels, resulting in hyperpolarization (Edwards *et al.*, 1998; 1999a,b).

The identity of Na^+,K^+ ATPase α-subunits has been investigated in rat arteries using polymerase chain reaction (PCR), immunofluorescence, and Western blot analysis. Rat mesenteric arteries were dissected and divided (n=3). Half of each arterial sample was frozen in liquid nitrogen prior to RNA extraction and reverse transcription of "whole vessel" mRNA. The remainder of each sample was transferred to periodate-lysine-paraformaldehyde fixative for 30 min, followed by cryoprotection in 30% sucrose. Arteries were then embedded in TissueTek® O.C.T.™ compound, and 4μm cryostat sections produced. Immunodetection of Na^+, K^+ ATPase isoforms was achieved using a panel of monoclonal antibodies: anti-α_1, clone M8P1A3 (Affinity Bioreagents), anti-α_2, kindly provided by R. W. Mercer, and anti-α_3, clone XVIF9-G10 (Affinity Bioreagents). A Cy3-conjugated secondary antibody (Jackson ImmunoResearch) was used for fluorescent visualization. Additional tissues were also collected to allow verification of antibody specificity by Western blot analysis.

PCR using gene-specific oligonucleotide primers indicated the presence of α_1-, α_2-, and α_3-subunit mRNA in the whole mesenteric arteries. GAPDH primers were included as a PCR positive control (see Figure 1a for example). In addition, immunofluorescence indicated the presence of α_1-, α_2-, and α_3-subunit protein in the smooth muscle, in agreement with the location proposed by Edwards *et al.* (1998). The specificity of the antibodies was verified by Western blot analysis, which also indicated the presence of α_1-, α_2-, and α_3-subunit protein in vascular tissue (see Figure 1b).

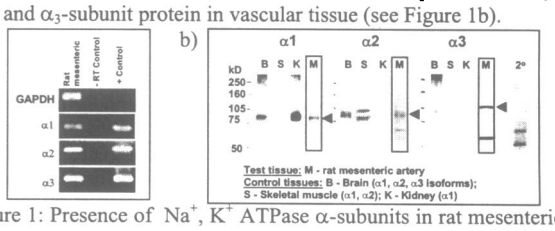

Figure 1: Presence of Na^+, K^+ ATPase α-subunits in rat mesenteric arteries detected using a) PCR and b) Western blot analyses.

Like the EDHF response in rat arteries (Edwards *et al.*, 1999b), the α_2, and α_3 Na^+, K^+ ATPase subunits are inhibited by nanomolar concentrations of ouabain (Blanco & Mercer, 1998). The results of the present study are therefore consistent with the specific involvement of a smooth muscle Na^+, K^+ ATPase containing an α_2- and/or α_3-subunit in mediating the EDHF phenomenon in rat mesenteric arteries (see also Dora & Garland, this meeting).

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84P HUMAN INTERNAL MAMMARY ARTERY POSSESSES A GREATER DENSITY OF THROMBOXANE A_2 RECEPTORS THAN THE CORONARY ARTERY: DIFFERENTIAL DISTRIBUTION IN HUMAN VASCULATURE

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Thromboxane A_2 (TxA_2) is a potent vasoconstrictor with mitogenic properties, which is synthesised from human vascular smooth muscle cells (Mehta & Roberts., 1983). Animal studies and human tissue culture studies have established the presence of TxA_2 receptors in vascular smooth muscle cells (Dorn., 1991). Therefore, the aim of the present study was to establish the differential distribution of TxA_2 receptors on arteries and veins of normal human vessels, and their alteration with disease.

Following optimisation of binding conditions, 30 μm cryostat sections were pre-incubated in assay buffer containing 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, pH 7.4. Saturation studies were performed using the thromboxane receptor agonist, [^{125}I]-BOP (Morinelli et al., 1989). Non-specific binding was defined using 1 μM SQ29,548 (Monshizadegan et al., 1992). Following a 30 min incubation sections were washed in 50 mM Tris, and the radioactivity retained was measured by γ-counting or the sections were apposed to film for 4 days for development. Developed images were quantified using computer assisted densitometry where receptor density was measured in amol/mm^2. Values of affinity (K_D) were compared using the Mann Whitney *U*-test, and receptor density (B_{max}) were compared using unpaired student's t-test with a significance value of $p<0.05$.

[^{125}I]-BOP bound specifically and in a time dependent manner reaching equilibrium by 30 min in human vascular tissue. Mono-phasic competition curve with a K_D of 7.09 ± 0.27 nM was obtained for SQ29,548 and a two site fit was preferred for UK-147,535 (Dack et al., 1998) with corresponding K_D values of 0.29 ± 0.16 nM for the high affinity site and 145 ± 5.07 nM for the low affinity site in human coronary arteries (n=3). Losartan displaced [^{125}I]-BOP binding in coronary artery with a K_D of 4.2 μM. This is of significance as approximately 1 μM concentration is reached following an oral dose of losartan in humans (Munafo et al., 1992). Compared to non-diseased coronary arteries and normal saphenous veins, a significant increase in receptor density was observed in atherosclerotic coronary arteries and saphenous vein grafts on both the medial and the proliferated intimal smooth muscle layer.

Table 1. Ligand affinity and receptor density in human vascular tissue. Values represent mean ± s.e.mean, (n=3-4). *$p<0.05$ compared to coronary artery. Hill slopes were close to unity.

Tissue	K_D (nM)	B_{max} (fmol/mg protein)
Coronary artery	0.12 ± 0.03	2.68 ± 0.80
Saphenous vein	0.38 ± 0.15	6.10 ± 2.16
Internal mammary artery	0.35 ± 0.05	9.80 ± 1.67*

These findings highlight the differences in receptor density that exists in human vascular tissue with TxA_2 and signifies the increase in receptor density observed in diseased vessels, thus enabling TxA_2 to be even more detrimental in vascular pathology.

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85P CHARACTERIZATION OF [^{125}I]-(Pyr^{1}) APELIN-13, THE PUTATIVE LIGAND FOR THE APJ ORPHAN RECEPTOR IN HUMAN TISSUE

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The 13 amino acid biologically active peptide, apelin (Tatemoto et al., 1998) was recently identified as the endogenous ligand for the orphan G protein-coupled receptor, APJ (Tatemoto et al., 1998). This putative receptor protein shares close identity with the angiotensin type 1 (AT_1) receptor (O'Dowd et al., 1993). Animal studies have established the presence of the APJ receptor in brain tissue and the apelin peptide to have hypotensive properties (Lee et al., 2000). Therefore the aim of this study was to characterise a radiolabelled ligand to this putative receptor in human heart tissue.

30 μm cryostat section of non-diseased human left ventricle and right atrium were obtained with approval from the local ethics committee. Following optimisation of binding conditions, sections were pre-incubated in 20 mM HEPES, 1 mM EDTA, 0.3% BSA and a mixture of protease inhibitors and the final incubation carried out in buffer containing 50 mM HEPES, 1 mM EDTA, 100 mM NaCl, 10 mM KCL, 5 mM $MgCl_2$, at pH 7.4. Following a 30 min incubation period the sections were rinsed in ice cold buffer (50 mM Tris) and radioactivity retained was measured by γ-counting. Saturation binding experiments were performed using increasing concentrations (0.01-1.nM) of [^{125}I]-(Pyr^{1}) Apelin-13. Non-specific binding was determined using 1 μM (Pyr^{1})-Apelin-13. Data were analysed using the iterative curve fitting programme LIGAND (Munson & Rodbard., 1980), where ligand affinity (K_D) and receptor density (B_{max}) were compared to a one site and two site model .

[^{125}I]-(Pyr^{1}) Apelin-13 bound to sections of human left ventricle in a time dependent manner reaching equilibrium at 30 min.

Table 1. Ligand affinity and receptor density of [^{125}I]-(Pyr^{1}) Apelin-13 binding to human heart tissue. values represent mean ± s.e.mean, (n=3-4) with Hill slopes close to unity.

Tissue	K_D (nM)	Receptor Density (fmol/mg protein)
Left ventricle	0.30 ± 0.10	6.17 ± 0.77
Right atrium	0.34 ± 0.18	4.89 ± 1.43

[^{125}I]-(Pyr^{1}) Apelin-13 bound with sub-nanomolar affinity to both the human left ventricle and right atrium, without any significant difference in binding affinity and receptor density in the two different chambers.

These results demonstrate for the first time the binding of [^{125}I]-(Pyr^{1}) Apelin-13, the putative ligand for the APJ orphan receptor in human heart tissue. The functional importance of this receptor in human tissue remains to be elucidated.

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86P ENDOTHELIAL VASOCONSTRICTOR FACTORS COUNTERACT THE EFFECT OF HIGH EXTRACELLULAR CALCIUM IN SPONTANEOUSLY HYPERTENSIVE RAT AORTA

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It has been known for some time that an increase in extracellular Ca^{2+} causes a decrease in the contractile activity of rat aorta (Webb et al., 1978). Nevertheless, the increase in extracellular Ca^{2+} potentiates the responses to KCl and α_1-adrenoceptor agonists in the aorta of spontaneously hypertensive rats (SHR) (Ortega et al., 1998). Since the hypertensive process in SHR could be associated with the release of endothelial vasoconstrictor factors (mainly cyclooxygenase-dependent endoperoxides and endothelin-1) (Vanhoutte et al. 1996), we have studied the contractile responses to KCl, methoxamine (MTX) and phenylephrine (PHE) in different aorta ring preparations (intact, de-endothelized, 10^{-5} M indomethacin-treated and 10^{-6} M CGS-27830-treated) from 20-week-old male SHR, varying the extracellular Ca^{2+} concentration (2.5, 5 and 10 mM). CGS-27830 is a potent nonpeptide endothelin receptor antagonist (Mugrage et al., 1993). The preparations were suspended in organ baths at 37° C and constantly bubbled with 95% O_2 and 5% CO_2. A low-bicarbonate physiological salt solution (pH 7.3) was used to prevent Ca^{2+} precipitation. The preparations were mounted with a resting tension of 2 g and allowed to equilibrate with 2.5 mM Ca^{2+} in the medium for a 90-min period. KCl (30 mM and 80 mM) contractions and MTX and PHE dose-response curves were obtained with the different Ca^{2+} concentrations in the organ bath according to the protocol previously used for the isolated SHR aorta (Ortega et al., 1998). The response to 80 mM KCl in the 2.5 mM Ca^{2+} solution was always taken as 100, serving to quantify the remaining responses. Results are expressed as mean values ± s.e. mean for 5-6 experiments. The data obtained in the 2.5 mM Ca^{2+} solution were used as controls and Student's t-test was used for comparison of the means (*$p<0.05$; **$p<0.01$; ***$p<0.001$). In the endothelium-intact preparations, the increase in extracellular Ca^{2+} concentration potentiated MTX contractions and caused no change in KCl and PHE contractions. In the de-endothelized preparations, the increase in Ca^{2+} concentration was associated with a reduction in KCl and MTX contractions and with unchanged PHE contractions. In the indomethacin- and CGS-27830-treated preparations, we observed a decrease in all the contractile responses when extracellular Ca^{2+} concentration increased (Table 1). The differences between the results obtained with MTX and those obtained with PHE should be investigated further, but in any case this research indicates that, in the hypertensive arteries, endothelium-derived contractile factors counteract the relaxant effect of high extracellular Ca^{2+} concentrations.

TABLE 1. Maximal effect of KCl, MTX and PHE in different SHR aorta preparations varying the extracellular Ca^{2+} concentration.

	mM extracellular Ca^{2+} concentration		
KCl	**2.5**	**5**	**10**
Intact	**100.0±0.0**	**97.6±6.5**	**98.7±5.8**
De-endothelized	**100.0±0.0**	**79.0±3.8***	**77.7±4.8***
Indomethacin-treated	**100.0±0.0**	**77.2±3.9***	**55.6±4.5****
CGS-27830-treated	**100.0±0.0**	**73.7±4.7***	**59.1±3.9****
MTX			
Intact	**60.4±3.9**	**82.3±6.1***	**84.1±3.5****
De-endothelized	**75.5±5.5**	**49.6±3.2***	**43.1±9.2***
Indomethacin-treated	**82.4±6.2**	**61.9±2.0***	**49.8±8.8***
CGS-27830-treated	**92.6±3.8**	**64.5±8.6***	**53.3±5.8****
PHE			
Intact	**77.1±1.4**	**75.3±5.8**	**81.4±6.2**
De-endothelized	**81.7±4.7**	**75.1±5.9**	**71.5±3.9**
Indomethacin-treated	**90.2±4.7**	**68.0±2.0***	**54.9±8.9*****
CGS-27830-treated	**87.3±2.6**	**49.2±8.0***	**52.7±1.4***

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87P SYNTHESIS AND PRELIMINARY *IN VIVO* CHARACTERISATION OF [^{18}F]-ET-1, A PET RADIOLIGAND FOR THE ENDOTHELIN RECEPTOR

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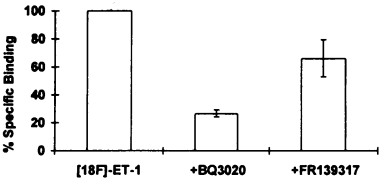
The 21 amino acid peptide endothelin-1 (ET-1) plays an important role in maintaining vascular tone in humans by action on its two receptors ET_A and ET_B. Alteration in ET function has been suggested to play a role in a number of human vascular diseases (Miyauchi & Masaki, 1999). With positron emission tomography (PET), a non-invasive technique for imaging and quantifying receptor-bound radioligands *in vivo* with high sensitivity, function of normal and diseased tissue can be studied. The aim of this work was to develop a positron emitting radioligand as a means to study the significance of the ET receptor *in vivo* in normal and diseased tissue using PET.

ET-1 was labelled according to the method previously described (Johnström *et al.*, 2000) using the Bolton-Hunter type reagent *N*-succinimidyl 4-[^{18}F]fluorobenzoate. A total radiochemical yield starting from [^{18}F]-fluoride of 7.2±1.5% was obtained in 211±7 min (n=6). The isolated radioactive product had a radiochemical purity of >95%. The identity of [^{18}F]-ET-1 was confirmed by co-elution on HPLC with a synthesised reference of (4-fluorobenzoyl)-ET-1 (identity confirmed by mass spectroscopy).

[^{18}F]-ET-1 was characterised using *in vitro* binding assays. Sections of human tissue were incubated at 23°C with either 1 nM [^{18}F]-ET-1 in HEPES buffer for increasing time periods (0-120 min) (association experiment) or with increasing concentrations of [^{18}F]-ET-1 (50 pM - 2.5 nM, saturation experiment) for 90 min. Non-specific binding was defined using 1 μM unlabelled ET-1.

Binding of [^{18}F]-ET-1 to human heart was time-dependent with an observed association rate constant (k_{obs}) of 0.045±0.004 min^{-1} at 23°C. Half-time for association ($t_{1/2}$) was 17 min. [^{18}F]-ET-1 binds to human heart tissue with a Hill slope close to unity and a K_D=0.43±0.05 nM and a B_{max}=27.8±2.1 fmol/mg protein (n=4). Using quantitative phosphor imaging, a higher density of ET receptors was visualised in the medulla compared to the cortex of the kidney as previously shown with [^{125}I]-ET-1 (Karet & Davenport, 1993). [^{18}F]-ET-1 binding to human kidney tissue was inhibited by BQ3020 (ET_B selective) and FR139317 (ET_A selective) (Davenport & Masaki, 1998) with levels comparable to the ratio of ET_A/ET_B receptors previously reported for human kidney (Karet & Davenport, 1993) (Figure 1).

Figure 1. Inhibition of [^{18}F]-ET-1 binding to human kidney (medulla+cortex) by 1μM concentrations of BQ3020 and FR139317 (n=4).



In conclusion, ET-1 has been labelled with fluorine-18 in good radiochemical yields. Preliminary *in vitro* characterisation indicates that [^{18}F]-ET-1 has affinity for the ET receptor and might prove to be a potential radioligand for PET.

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88P VASODILATOR EFFECT OF HUMAN UROTENSIN-II ON HUMAN PULMONARY AND RESISTANCE ARTERIES

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Urotensin (U-II) is a cyclic peptide consisting of 12 amino acids isolated from the caudal neurosecretory system of fish, and recently cloned in man (hU-II) (Bern & Lederis, 1969; Ames et al., 1999). HU-II is a potent vasoconstrictor of rat thoracic aorta, large systemic arteries from various species, and the main pulmonary arteries of the rat (Ames et al., 1999; MacLean et al, 2000). No vasoconstriction was induced by hU-II in rat or human small muscular pulmonary arteries (MacLean et al., 2000). Here, studies were under taken to compare the vasodilator effect of hU-II in small muscular resistance arteries of the pulmonary and systemic systems.

The systemic small muscular resistance arteries (SRA's) were dissected from abdominal adipose tissue biopsies obtained from patients undergoing hernia repair (~200μm i.d.). The pulmonary small muscular resistance arteries (PRA's) were dissected from macroscopically normal samples of lung biopsy tissue removed along with bronchial carcinomas (~200μm i.d.). The two sets of vessels were set up in myography baths in Krebs solution gased with 16%O_2; 5%CO_2; and balance N_2 at 37°C. The endothelial integrity was assessed according to ACh-induced relaxation after pre-constriction to 5-HT. Only vessels with an intact endothelium were used in the study. The vessels were pre-constricted with ET-1 then cumulative concentration response curves were constructed to hU-II (5-10 minutes between each dose) . The ET-1-induced pre-constriction in each vessel was calculated as a % of response to 50mM KCl. In the PRA's the pre-constriction with ET-1 was 146 ± 46%. The fall of the concurrent ET-1 time controls was 23 ± 11%, calculated as % ET-1-induced tone. In the SRA's the pre-constriction with ET-1 was 190 ± 23%. The fall of the concurrent ET-1 time controls was 43±16%, calculated as % of ET-1-induced tone. The maximum vasodilator responses (E_{max}) were calculated as % of ET-1-induced tone.

The *p*IC_{50}'s were calculated from each individual experiment using a BBC microcomputer graphical interpolation. These results are shown in Table 1. The hU-II-induced relaxation in both tissue groups was compared to their respective ET-1 time controls.

Table 1. Vasodilator response to hU-II in preconstricted vessels (as % ET-1-induced tone) and *p*IC_{50}'s in human pulmonary (PRA's) and systemic (SRA's) small muscular resistance arteries. n= number of subjects.

	E_{max}	*p*IC_{50}	n
PRA's	81±8%**	10.37±0.48	6
SRA's	96±4%*	10.28±0.68	4

Statistical analysis was done by using an unpaired Student's T-test. $P < 0.01$**; $P < 0.05$*.

This study shows that hU-II is a potent vasodilator peptide in small muscular arteries of the human pulmonary and systemic circulations. Pre-constriction of these vessels with an intact endothelium revealed a vasodilator action induced by hU-II in both vascular systems. It is not yet clear whether this vasodilatation is endothelium-dependent or -independent, and the 2nd messenger mechanism mediating this relaxation has still to be elucidated.
In conclusion, this is the first report to show that hU-II is a potent vasodilator of human resistance arteries.

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89P LOCALISATION OF BINDING SITES FOR HUMAN [^{125}I]-UROTENSIN II (U-II), THE NOVEL ORPHAN RECEPTOR LIGAND, IN HUMAN AND RAT CNS AND PERIPHERAL TISSUES

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Human urotensin II (Coulouarn *et al* 1998) has recently been identified as a ligand for a novel G-protein-coupled receptor with high sequence homology to the rat orphan receptor GPR-14 (Ames et al 1999). We have compared the distribution of this receptor in human and rat CNS and peripheral tissues using a new radioligand, [^{125}I]-U-II.

Autoradiography was carried out on cryostat sections (30μm) of rat thoracic aorta, spinal cord and brain and human tissues including coronary artery, left ventricle, kidney and skeletal muscle. Following optimisation of binding conditions, sections were pre-incubated for 1 hr in 20mM Tris-HCl buffer, pH7.4 containing 5mM $MgCl_2$ and 0.2%BSA then incubated with 0.25 nM [^{125}I]-U-II (Amersham Pharmacia Biotech) for 1 hour. Sections were washed for 10min in 50mM Tris-HCl, pH 7.4 at 4°C, dried and apposed, with standards, to radiation sensitive film for 3 days. The resulting autoradiograms were analysed using computer-assisted densitometry.

In rat tissues low density of specific binding (22.5±6 amol mm^{-2}) localised to smooth muscle of aorta. Moderate levels (85±14 amol mm^{-2}) were found in dorsal root ganglion of spinal cord with highest binding density observed in the abducens nucleus of brain (140±14 amol mm^{-2}). In human tissues low levels were determined in the smooth muscle layer of coronary artery (14.6±3 amol mm^{-2}), left ventricle (9.4±4 amol mm $^{-2}$) and kidney cortex (14.5±4 amol mm^{-2}) with moderate levels in skeletal muscle (31.9±9 amol mm^{-2}) (Figure 1).

Our localisation of [^{125}I]-U-II binding to rat spinal cord and abducens nucleus corresponds to that reported for pre-pro U-II mRNA by Coulouarn and colleagues (1999).

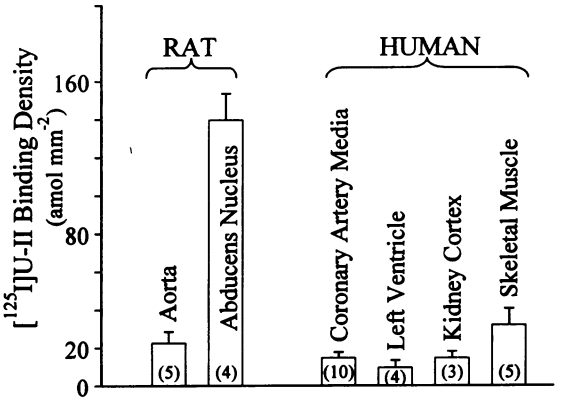

Figure 1. Density of [^{125}I]-U-II binding in rat and human tissues. n-values in parentheses.

We find expression of receptor protein in human vascular smooth muscle, kidney and skeletal muscle which correlate with the expression of human GPR-14 mRNA reported by Ames et al (1999). This group detected highest levels of GPR-14 mRNA in human heart, however we detected only low levels of receptor protein expression in this tissue.

These data are the first report of the putative UT-II receptor in human tissues. The importance of this receptor in human physiology remains to be elucidated.

Supported by grants from the British Heart Foundation.

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90P PREVENTION OF ISOLATION-INDUCED HYPERTENSION WITH A NON-PEPTIDE κ-OPIOID RECEPTOR AGONIST

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Previous research in this laboratory has shown that hypertension in the spontaneous hypertensive rat (SHR) appears to correlate to insufficient production of hippocampal dynorphins (Wang *et al.*, 1994) and that blood pressure could be reduced by intrahippocampal administration of dynorphins and non-peptide kappa agonists (Wang and Ingenito, 1994). The purpose of the present study was to investigate whether kappa agonists could prevent the development of hypertension in a different hypertensive model: the isolated male rat model of hypertension (IHR).

Isolation of young male Sprague Dawley rats (100-125 g) for 5-7 days in standard rat cages caused an increase in systolic blood pressure from a mean of 132 to 184 mmHg (n=6, P<0.005) as determined by the tail-cuff method. The blood pressures of rats grouped 3 per cage remained stable. Rats were anesthetized with methohexital sodium (Brevital, 70 mg/Kg, i.p.) and stereotaxically implanted with permanent bilateral stainless steel guide cannulas (22 gauge, length 11 mm) in the hippocampus (from bregma; AP = -4.0 mm, M L = ± 1.6 mm, DV = -3.0 mm, in the flat head position). After the surgery and a 5 day recovery period, each animal received the non-peptide *kappa* agonist U62,066E, (Spiradoline, Upjohn) or drug vehicle bilaterally into the hippocampus for 3 days prior to and during the 7 day isolation or grouping period. Animals treated with U62,066E did not develop hypertension as compared to isolated animals treated with vehicle (Figure 1). The isolation procedure used in these studies appears to induce anxietal stress, as indicated by a reduced time spent by the rats in the open arms of the elevated plus maze (17 ± 7 s). This time was increased by U62,066E, (75 ± 11 s, n=6, data analyzed using ANOVA with Student-Newman-Keuls post hoc test. P<0.05 considered significant) suggesting that the drug possesses anxiolytic properties and may reduce hypertension in part, by blocking an anxiety/stress component.

After the experiment acute responses to U62,066E were measured in anesthetized animals. All rats had an acute depressor response to U62,066E (a decrease of 27 ± 5 mmHg) which returned to baseline within 30 min. This shows that the hypotensive effect of the drug is not solely due to its anxiolytic properties.

These data strengthen our previous findings that opioids in the hippocampus may be important in restraining increased blood pressure provoked by environmental stimuli such as isolation.

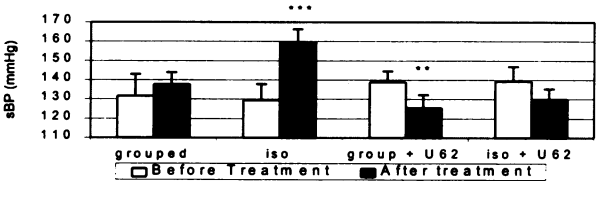

Figure 1. Effects of isolation and grouping, before and after treatment with either 0.2μl of vehicle (saline) or 10nmol/0.2μl per side of U62,066E, n=6. Differences are significant (***P<0.001, **P<0.005) when compared to before treatment values using paired Student's t-test. All groups before treatment are not significantly different according to ANOVA.

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91P REGULATION OF OPIOID RECEPTOR-INDUCED CARDIOPROTECTION BY PROTEIN KINASE C: ISOFORM-SPECIFIC PKC TRANSLOCATION

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It has recently been demonstrated that stimulation of the δ_1-opioid receptor confers cardioprotection to the ischemic myocardium and may serve as a trigger for ischemic preconditioning (IPC)[1]. Since protein kinase C (PKC) has been shown to be an important step in IPC in several species, we examined the role of PKC following δ_1-opioid receptor stimulation in a rat model of myocardial infarction induced by 30 minutes of coronary artery occlusion and 2 hours of reperfusion. Infarct size (IS) was determined by tetrazolium staining and was expressed as a percent of the area at risk (AAR). Male Wistar rats, 350-450 grams, were used for all included protocols. Control animals, subjected to ischemia and reperfusion, had an IS/AAR of 59.9±1.8 (n=9). The δ_1/ δ_2 or δ_1-opioid receptor was stimulated with either [D-Ala^2, D-Leu^5] enkephalin (DADLE) or TAN-67[1], respectively. DADLE (1 or 2 mg/kg) or TAN-67 (10 mg/kg) treatment before ischemia significantly reduced IS/AAR (36.9±3.9, n=9; 36.7±4.7, n=9; and 29.6±3.3, n=8; respectively). The δ_1-opioid receptor antagonist, BNTX, completely abolished TAN-67 induced cardioprotection (58.8±1.9, n=7). Similarly, treatment with the PKC antagonist, chelerythrine (5 mg/kg), completely abolished low-dose DADLE (61.8±3.2, n=6) or TAN-67 (55.4±4.0, n=7) induced cardioprotection, however, it had no effect on IS/AAR in non-opioid treated rats (57.6±5.7, n=6, Figure 1).

Immunofluorescent staining with polyclonal antibodies directed against specific PKC-isoforms was performed in myocardial biopsies obtained in animals after 15 minutes of treatment with saline, chelerythrine, TAN-67, and chelerythrine in the presence of TAN-67. TAN-67 treatment induced translocation of PKC-α to the sarcolemma, PKC-ε to the intercalated disk and mitochondria, and PKC-δ to the mitochondria. PKC translocation was absent in saline and chelerythrine treated animals and was blocked by chelerythrine and BNTX in TAN-67 treated rats. These results suggest a key role for PKC in the cardioprotective effects of opioid receptor stimulation and suggest the involvement of PKC-α, -δ, and -ε in opioid induced cardioprotection.

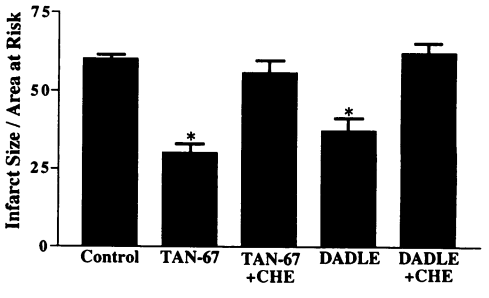

Figure 1. IS/AAR in control and opioid treated animals in the absence or presence of chelerythrine. *p<0.05 vs. Control.

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92P EFFECTS OF ACUTE AND CHRONIC ADMINISTRATION OF NORETHISTERONE ON ISCHAEMIA-INDUCED ARRHYTHMIAS

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It has been reported widely that oestrogens are cardioprotective, with pre-menopausal women having a lower risk of sudden cardiac death than men and post-menopausal women (Wenger *et al.*, 1993). There is little evidence, however, concerning the cardiovascular effects of progestogens. We found previously that norethisterone altered rat platelet aggregation (Fraser & Coker, 1999) and we have now compared the acute and chronic effects of norethisterone on ischaemia-induced arrhythmias.

Female Wistar rats (180-300g) were ovariectomized under Hypnorm/diazepam anaesthesia. After 12 to 16 days, rats (240-360g) were used either for acute experiments, or treated with norethisterone (0.01, 0.03 or 0.1 mg kg^{-1}) or vehicle (sesame oil) s.c. daily for 7 days. For the acute experiments rats received a single s.c. injection of either norethisterone (0.03, 0.1 or 0.3 mg kg^{-1}) or vehicle 20 min prior to anaesthesia. Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.). The trachea was canulated to permit artificial ventilation. Carotid arterial blood pressure and a Lead I ECG were recorded. A left thoracotomy was performed and a ligature was placed around the left coronary artery. After 10 min stabilisation the ligature was tied. Arrhythmias occurring in the following 25 min were quantified as ventricular premature beats (VPBs), ventricular tachycardia (VT) or ventricular fibrillation (VF) according to Walker *et al.* (1988).

Neither acute nor chronic treatment with norethisterone altered the incidences of VT, VF or the mortality significantly. The total number of VPBs was reduced after acute administration of the two higher doses of norethisterone (Figure 1a). However, no changes in the total number of VPBs were seen following chronic administration of norethisterone (Figure 1b).

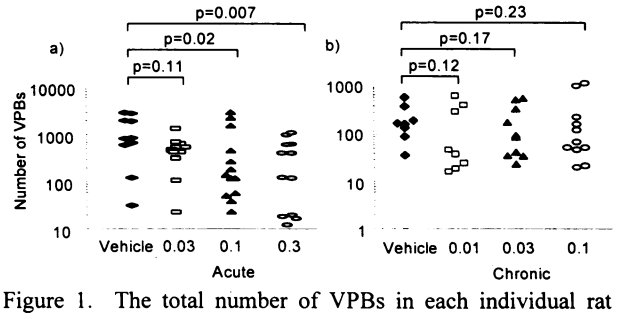

Figure 1. The total number of VPBs in each individual rat receiving a) acute and b) chronic norethisterone. Statistical comparisons, Kruskal Wallis test. (n=10-13 per group).

These results indicate that norethisterone has acute antiarrhythmic activity, but that this is not maintained when similar doses are given once daily for 7 days. Previously, chronic norethisterone had significant effects on platelet aggregation (Fraser & Coker, 1999) suggesting that the dosing schedule was adequate. The lack of activity observed here after chronic norethisterone administration may indicate that some endogenous adaptive response has negated the antiarrhythmic action of acute administration of norethisterone.

Supported by the British Heart Foundation (PG/97084).

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93P COMPARISON OF ERYTHROMYCIN, TERIKALANT AND CLOFILIUM IN AN *IN* VIVO MODEL OF TORSADE DE POINTES

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Erythromycin, a macrolide antibiotic, blocks the rapid component of the delayed rectifier potassium current (Antzelevitch *et al.*, 1996), whereas terikalant, a novel antiarrhythmic agent, inhibits multiple potassium channels (Jurkiewicz *et al.*, 1996). The proarrhythmic effects of these drugs have not been examined in *in vivo* experimental conditions before. Thus, we have compared the proarrhythmic effects of erythromycin and terikalant to that of clofilium, another multiple potassium channel blocker (Li *et al.*, 1996), in an *in vivo* model of torsade de pointes (TdP).

Male NZW rabbits (2.6-3.1kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.v.) and artificially ventilated with room air via a tracheal cannula. ECGs (leads I, II and III), arterial blood pressure and left ventricular pressure were recorded. After a sternal split and pericardiotomy, an electrode was positioned on the epicardial surface of the left ventricle to record monophasic action potentials (Epi MAP). Rabbits were assigned randomly to receive three i.v. infusion rates of erythromycin (133, 400 and 1330 nmol kg^{-1} min^{-1}, n=8), terikalant (2.5, 7.5 and 25 nmol kg^{-1} min^{-1}, n=10), clofilium (20, 60 and 200 nmol kg^{-1} min^{-1}, n=10), or vehicle (0.81% NaCl solution, n=8). Each drug infusion was administered for 19 min. Five min prior to each drug infusion, phenylephrine infusion was started and given i.v. for 24 min in increasing rates (i.e. 75, 150, 225 and 300 nmol kg^{-1} min^{-1} for 15, 3, 3 and 3 min, respectively). All three 24 min dosing cycles were followed by a 10 min drug free interval.

Only clofilium and terikalant evoked TdP. None of the drugs altered blood pressure and heart rate before exerting their maximal proarrhythmic effect, which was during the 2nd infusion. However, clofilium had already prolonged significantly Epi MAP by that time, while terikalant only tended to do so then (Table 1).

Table 1. Incidence of TdP at any time, and mean ± s.e. mean values for mean arterial blood pressure (MBP) heart rate (HR) and Epi MAP duration 10 min after commencing infusion of the second dose of vehicle (Control), erythromycin (Eryth), terikalant (Terik) or clofilium (Clof).

	Control	Eryth	Terik	Clof
TdP (%)	0	0	20	60$^{\#}$
MBP (mmHg)	89 ± 5	87 ± 3	84 ± 3	82 ± 5
HR (beats min^{-1})	206 ± 9	213 ± 6	199 ± 6	203 ± 11
Epi MAP (ms)	188 ± 7	181 ± 5	205 ± 6	227±10*

$^{\#}P<0.05$ compared to control, Fisher's exact test. *$P<0.05$ compared to control and erythromycin, Kruskal-Wallis test.

These data indicate that despite being given in high doses erythromycin did not prolong action potential duration or cause torsade de pointes. Terikalant did not prolong action potential duration to the same extent as clofilium and was less proarrhythmic than clofilium in this anaesthetized rabbit model of torsade de pointes.

Supported by the British Heart Foundation (PG/96100).

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94P A NEW APPROACH TO THE HEART PROTECTION ANTISENSE TO ANGIOTENSIN CONVERTING ENZYME mRNA

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Angiotensin converting enzyme (ACE), an important component of the renin-angiotensin system, plays a major role in many cardiovascular pathophysiological conditions, such as hypertension, heart failure and myocardial ischemia. Currently used ACE inhibitors have some adverse effects and non-compliance problem. Our lab has been developing an antisense approach to inhibit the renin-angiotensin system at the gene expression level (Phillips *et al.*, 1997, Mohuczy *et al.*, 1999). In this study, we aimed to design antisense oligodeoxynucleotide (AS-ODN) directed at ACE mRNA, to check tissue distribution of AS after iv injection and to test AS for heart protection in ischemia- reperfusion (I/R) injury model.

We used epifluorescent and confocal laser-scanning microscopy, immunohistochemistry, isolated perfused heart procedure and Western blotting. The animals were male mice 127SV/C57BL6, 20 g, n=2 and male Sprague-Dawley rats, 200-250 g, n=8 per group. Solutions: Antisense 5'-ATTTCGTGGTGGG-3' phosphorothioate in sterile 5% dextrose 0.4 mg/ml, inverted control (INV) 5'-GGGTGGTGCTTTA-3' phosphorothioate in sterile 5% dextrose 0.4 mg/ml. Both ODNs were mixed 1:3 (w/w) with liposomes N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and dioleoyl phosphatidylethanolamine (DOPE) (1:1, w/w, 2 mg/ml total lipids) and injected iv 0.04 mg/mouse and 0.2 mg/rat. Captopril dose 5 mg/kg in sterile saline. Anaesthesia was induced using sodium pentobarbital 40 mg/kg ip. Isolated perfused heart function recordings: coronary perfusion pressure (CPP), left ventricular end-diastolic (LVEDP) and systolic pressures (LVSP) were measured using the method of Neely & Rovetto, 1975.

Based on the published by Koike *et al.* (1994) rat ACE cDNA sequence, the AS-ODN has been designed as 13-mer directed to the second zinc-binding domain (2998-3010). Tissue distribution of phosphorothioated, fluorescein isothiocyanate (FITC)-labeled AS mixed with liposomes, 4h after iv injection, showed its presence in the lung, spleen, liver and heart, but absence in the brain. Immunostaining of the parallel heart slices with anti-sarcomeric alpha-actinin antibody indicated AS localization within cardiomyocytes.

ACE AS injected iv to the Sprague-Dawley rats 24 h before excising the heart, which was then subjected to 25 min ischemia and 30 min reperfusion, preserved heart function. Increase in CPP and LVEDP during reperfusion was attenuated by AS or captopril, as compared to INV or saline treatment (n=8 in each group, $p<0.05$).

Table 1. Heart function measurements before I/R - "0" and after 30 min of reperfusion.

	0	Saline	Captopril	AS	INV
CPP	67.8±1.2	145.6±5.4	112.6±2.6	122.2±2.1	147.9±5.0
LVEDP	6.8±0.3	55.0±3.8	28.9±2.8	33.3±3.4	61.6±7.3
dLVP	59.3±1.4	38.1±3.4	57.1±4.3	57.6±6.1	41.0±2.1

Moreover, ACE protein was not upregulated, like in the captopril-treated group.

We conclude that ACE AS is taken up by the heart tissue and can effectively prevent myocardial dysfunction caused by ischemia-reperfusion injury.

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95P INHIBITION OF CREATINE KINASE ACTIVITY DECREASES THE FUNCTIONAL RECOVERY OF RABBIT MYOCARDIUM AFTER AN ISCHAEMIA-REPERFUSION CHALLENGE

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The creatine kinase/phosphocreatine (CK/PCr) system plays a complex role in myocardial energetics. Iodoacetamide (IAm), has been proposed as an irreversible ihibitor of myocardial CK, reducing the contractile reserve of the rat heart (Avellanal et al. 1998). Using the isolated arterially perfused interventricular septum, we have investigated the effects of CK inhibition on mechanical function in rabbit hearts submitted to global ischaemia/reperfusion challenge.

Following a pentobarbitone overdose (120 mg i.v., plus 2000 IU sodium heparin), septa from male NZW rabbits (2-2,5 kg) were rapidly dissected and perfused in a N_2-environment plus temperature-controlled plastic chamber at a constant flow (2,1 ml/g wet weight per min) with warmed (32°C), and gassed (95% O_2, 5% CO_2) Tyrode's buffer with glucose substituted by pyruvate. Developed tension (DT) induced by electrical stimulation (15V, 1,5 HZ, 5 ms) was recorded (79D Grass polygraph), digitized (PCL-812PG card) and PC stored. Global ischaemia was induced by stopping the flow through the perfusion cannula for 2, 10 or 30 min followed by 30 min buffer reperfusion (Hamman et al., 1995). IAm (Sigma), 0,5mM, was directly dissolved in the buffer, and perfused 15 min before the ischaemia. In 4 experiments IAm was perfused only during reperfusion. Control and IAm-treated groups were compared by the unpaired t-test for each ischaemia period.

In normoxic conditions, perfusion with IAm did not significantly modify the myocardial performance. In contrast, IAm treatment increased the loss of DT induced by 2 min global ischaemia (78,8 ± 1,31 vs 56,6 ± 2,52% for control; p<0,001), and decreased the post-reperfusion DT recovery (64,0 vs 98,0%; figure 1A). This decrease in the recovery was more evident with increased time of ischaemia (25,5 vs 79,3% for 10 min and 8,92 vs 60,8% for 30 min). Moreover, IAm increased 1,71 ± 1,01g the Δ_{max} in resting tension after 10 min ischaemia and 9,63 ± 1,3 vs 3,51 ± 0,90g; p<0,001, for control after 30 min ischaemia (figure 1B). CK activity in the effluent of IAm treated septa after 30 min ischaemia was negligible. No significant effect was shown when IAm was perfused only during reperfusion.

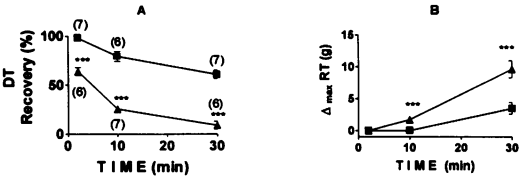

Fig 1:Percentage recovery of developed tension (A) related to its preischaemic value of interventricular septa after different times in ischaemia and 30 min reperfusion, and (B) on the maximal increase in resting tension. Symbols represent: ■ control; ▲ IAm perfused in pre-ischaemia. The figure shows mean ± sem for (n) experiments. *** p<0,001

This study shows that CK inhibition increases and accelerates the rapid DT loss induced by ischaemia suggesting a role for the PCr energy reserve of the myocardium. This also indicates that the CK/PCr system is involved in full mechanical recovery after ischaemia.

Supported by U.C.M grant P.R. 269/98-8172.

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96P HYPOXIA-REOXYGENATION IN COLD-STORED RABBIT AORTA SCAVENGES NITRIC OXIDE BY RELEASING SUPEROXIDE ANIONS

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Endothelial damage in vascular tissue maintained under hypoxic hypothermic conditions must be considered to be a problem in clinical organ transplants and should also be taken into account when establishing the conditions for basic experiments on animal organs. It has in fact been known for some time that hypoxic endothelial cells cause vasoconstriction as a consequence of the release of oxygen-derived free radicals during endothelial superfusion (Rubanyi et al., 1985). The functional alterations in vascular tissues preserved at low temperature and under hypoxic conditions could therefore be related to the decreased nitric oxide (NO) levels when oxygen-derived free radicals react with this mediator. Since acetylcholine (ACh) relaxes the vascular smooth muscle by releasing endothelial NO (Furchgott et al., 1980), in this study we have evaluated the endothelium-dependent relaxations evoked by ACh (10^{-8} M - 10^{-5} M) in the 10^{-6} M noradrenaline (NA)-precontracted rabbit aorta ring preparations mounted in the organ baths (with 4 g tension in Krebs-Henseleit bubbled with 95% O_2 and 5% CO_2 at 37° C) immediately after sacrificing the animals, or mounted after subjecting the tissue to cold (4° C) storage in a refrigerator for 2 hours, 6 hours and 1-3 days. During this period the tissue remained in non-oxygenated Krebs-Henseleit solution. We also evaluated ACh relaxations in some preparations mounted directly after sacrificing the animals and in others mounted after storing the tissue for 1 day, all of which had been alternatively treated with 10^{-4} M L-Arginine (L-Arg) (precursor of NO synthesis), 10^{-3} M vitamin C (Vit C) (free radical scavenger) or 150 U/ml superoxide dismutase enzyme (SOD) (specific scavenger of superoxide anions). The relaxation of each dose of ACh was calculated by considering the contraction developed by the ring after the preceding NA administration to be 100. The maximal effect (ME) of ACh and the pD_2 (-log of the dose producing 50% of the ME) were obtained for each dose-response curve. Results are expressed as mean values ± s.e. mean for 10-12 experiments and an ANOVA with the Bonferroni test was used for comparison of mean values. When the aortic tissue remained stored in the refrigerator for more than 6 hours, the preparations showed a decrease in ACh responses which was similar when the tissue remained stored for 1-3 days. Nevertheless, when the preparations were previously treated with one of the aforementioned drugs, the effect of ACh was similar in those mounted directly after sacrificing the animal and in those mounted after cold storage of the tissue in hypoxic conditions for 1 day (Table 1). The present results indicate that rabbit aorta tissue maintained in hypoxic and hypothermic conditions releases superoxide anions when it is reoxygenated and that these free radicals destroy NO.

TABLE 1. ME and pD_2 of ACh in different rabbit aorta ring preparations mounted immediately after sacrificing the animal (0 h) or mounted after cold storage of the tissue for 1 day (1 d).

		Ring Treatment		
	Non-treated	L-Arg	Vit C	SOD
ME				
0 h	73.3±3.1	83.5±2.5[b]	70.9±3.2[b]	76.8±6.8[b]
1 d	23.0±5.1[a]	83.5±3.4[b]	76.3±2.1[b]	73.9±6.4[b]
pD_2				
0 h	6.9±0.0	6.9±0.1	7.0±0.1	6.9±0.1
1 d	7.0±0.2	7.0±0.0	7.0±0.1	6.8±0.1

The letters show significant differences (p<0.05) compared to 0 h non-treated (a) and to 1 d non-treated (b).

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97P MECHANISM OF VASCULAR INJURY IN EXPERIMENTAL DIABETIC RETINOPATHY

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Proliferative diabetic retinopathy is a vascular disease that consists of an initial period of hyperglycemia-induced vascular injury followed by proliferation of dysfunctional blood vessels within the retina and vitreous. Our analyses in vivo have shown early increases in levels of endothelial nitric oxide synthase (eNOS) protein and nitric oxide (NO) formation during experimental diabetic retinopathy. These alterations are correlated with nitration of retinal proteins on tyrosine, which is a marker for peroxynitrite formation. Peroxynitrite is a highly reactive oxidant produced by the combination of NO and superoxide anion that has been shown to contribute to cell death and tissue injury in many model systems (Salvemini, 1997). We hypothesize that diabetic retinopathy results from hyperglycemia-induced endothelial cell injury due to increased NO and superoxide formation and peroxynitrite-mediated tyrosine nitration of endothelial proteins. The aim of this study was to test this hypothesis and to determine the effects of hyperglycemia on endothelial cell survival in relation to eNOS protein expression, formation of NO and superoxide anion, and protein nitration on tyrosine.

Primary cultures of bovine retinal endothelial (BRE) cells were maintained for 5 days in conditions of 5 mM glucose (normoglycemia), 25 mM glucose (hyperglycemia) or 25 mM dextran (control for hyperosmolarity). Treatment effects on BRE cell survival were determined by annexin staining and flow cytometry. eNOS protein expression was analyzed using western blot techniques. Formation of NO was evaluated by assaying nitrite levels in the culture media (Misko et al., 1993). Production of superoxide anion was analyzed photometrically (Pritchard et al., 1995). Protein nitration on tyrosine was evaluated using immunolabeling and densitometric imaging techniques. Statistical significance of treatment effects was tested using ANOVA. Values of $p < 0.01$ were considered significant.

These experiments showed that the hyperglycemia treated cultures were 50% more vulnerable to starvation-induced cell death than the controls. Levels of eNOS protein expression in BRE cells exposed to hyperglycemia were 60% higher than levels in the control cells. Formation of NO was also increased by hyperglycemia as indicated by a 2-fold increase in nitrite formation (from 1.3 to 2.5 $\mu M/10^6$ cells). Superoxide formation was also increased by 3-fold (from 2.3 to 6.9 μmole/min/10^6 cells). Immunolabeling analyses showed a 65% increase in levels of proteins nitrated on tyrosine in the hyperglycemia treated cells as compared with controls. Collectively, these results indicate that hyperglycemia reduces BRE cell viability while increasing eNOS protein levels and promoting formation of NO and superoxide anion. The association of these effects with the nitration of BRE cell proteins on tyrosine suggests that hyperglylcemia-induced vascular injury is due in part to peroxynitrite-mediated alterations in intracellular signaling pathways related to cell survival and cell death programs.

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98P CHRONIC ANTIHYPERTENSIVE EFFECTS OF THE BIOFLAVONOID QUERCETIN IN SPONTANEOUSLY HYPERTENSIVE RATS

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Several epidemiological studies have shown a significant inverse association between dietary flavonoids (mainly quercetin) and mortality from coronary heart disease (Yochum et al., 1999). We have analysed the chronic effects of quercetin on blood pressure and vascular reactivity in spontaneously hypertensive (SHR) and normotensive (WKY) rats.

WKY or SHR rats weighing 300-400 g were treated orally with vehicle (control, n = 7 and 10, respectively) or quercetin (10 mg Kg^{-1}, n = 7 and 10, respectively) for 5 weeks. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography and direct femoral artery measurements of blood pressure were carried out in conscious SHR after 24 h recovery from anaesthesia (inhaled ethyl ether as needed to achieve deep anaesthesia). Isolated aortic rings (2-3 mm in length) were mounted for isometric tension recording in Krebs solution (Duarte et al., 1993). The responses to acetylcholine (ACh) or nitroprusside (SNP) were analysed in rings pre-contracted by 10^{-7}M phenylephrine and endothelium-dependent contractions to ACh were tested in 10^{-4}M L-NAME-treated rings. Total 8-iso-$PGF_{2\alpha}$ (a marker of oxidative stress) was measured by an enzyme immunoassay.

Quercetin administration induced a progressive reduction in SBP in SHR without any change in WKY. After 5 weeks, systolic and diastolic arterial blood pressure and heart rate were significantly ($P < 0.05$) reduced in quercetin-treated (180 ± 6 and 130 ± 16 mm Hg and 377 ± 23 beats/min, respectively) as compared to control (219 ± 5 and 170 ± 8 mm Hg and 428 ± 5 beats/min, respectively) conscious SHR. The left ventricular and kidney weight indexes in control SHR (3.1 ± 0.08 and 3.4 ± 0.07 mg g^{-1}, respectively) were significantly ($P < 0.05$) greater than in WKY (2.0 ± 0.05 and 2.8 ± 0.05 mg g^{-1}, respectively). These indices were significantly ($p < 0.05$) reduced in quercetin-treated SHR (2.9 ± 0.1 and 3.1 ± 0.1 mg g^{-1}, respectively) as compared to control SHR. In aortae from WKY and SHR the vasodilator responses induced by SNP or the vasoconstrictor responses induced by noradrenaline or KCl were similar in quercetin- as compared to vehicle-treated rats. Aortae from control SHR showed reduced endothelium-dependent vasodilator responses (E_{max} = 58 ± 5% vs 82 ± 4% in control WKY, $P < 0.05$) and increased endothelium-dependent vasoconstrictor responses (41 ± 10% vs 4 ± 0.4% of 80 mM KCl-induced contraction, $P < 0.01$) induced by ACh. Quercetin-treatment restored the vasodilator (E_{max} = 78 ± 5%) but not the vasoconstrictor response (41 ± 4%) to ACh in SHR rats. The 24 h urinary 8-iso-$PGF_{2\alpha}$ excretion was increased in control SHR (4.4 ± 0.8 vs 2.1 ± 0.4 µg/24 h in WKY, $P < 0.01$) but not in quercetin treated SHR (1.3 ± 0.1 µg/24 h, $P < 0.01$ vs control SHR).

These experiments show that chronic treatment with quercetin reduced the elevated blood pressure and the increase in left ventricular and renal weight indices. In addition, it restored the endothelial-dependent vasodilation and reduced the urinary levels of 8-iso-$PGF_{2\alpha}$ in SHR rats.

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99P EFFECT OF DIETARY CALCIUM AND AMLODIPINE ON THE ARTERIAL BLOOD PRESSURE OF SPONTANEOUSLY HYPERTENSIVE RATS

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It has been shown that the combination of dietary calcium and a calcium antagonist (two agents that would be expected to have opposite effects) could paradoxically have a synergistic effect to decrease the arterial blood pressure (ABP) in hypertensive rats (Pang et al., 1992). Studies carried out in our laboratory showed that a high-calcium diet (Ca 2.5% w/w) caused a decrease in the ABP of spontaneously hypertensive rats (SHR), when compared with control animals fed on a similar semi-synthethic casein diet with a normal calcium content (Ca 1% w/w) (Civantos et al., 1999). In this study, after being weaned at three weeks, male SHR were randomized in four groups of animals. Two of these groups were fed on the Ca 1% w/w diet and another two groups were fed on the Ca 2.5% w/w diet. One of the groups fed on each diet also received amlodipine in the drinking water (1 mg/kg/day) after being weaned. Systolic (SBP) and diastolic (DBP) arterial blood pressure were measured weekly in all the rats from the 6-25 weeks of life by the tail cuff method (Buñag, 1973). The results are expressed as mean values ± s.e. mean for a minimum of 8 rats and were analysed by one-way ANOVA. Differences between groups were assessed by Bonferroni test considering these differences to be significant when $p<0.05$.

The SHR fed on the Ca 1% w/w diet showed a gradual increase in SBP and DBP which reached maximum stable values between weeks 20-25. The Ca 2.5% w/w diet caused a decrease in the ABP and clearly slowed down the development of hypertension in this rat strain. Maximum differences in ABP between the animals fed on the Ca 2.5% w/w diet and those fed on the Ca 1% w/w diet were obtained at 15-19 weeks of life, and therefore, the values at 17 weeks of life could represent the optimum time at which to carry out comparisons. In the SHR fed on the Ca 1% w/w diet amlodipine caused a decrease in ABP only in the 20-25-week-old animals. When amlodipine was administered to the SHR fed on the Ca 2.5% w/w diet the SBP and DBP were lower than the corresponding values obtained in all the other groups when the animals were between 15 and 19 weeks old. Nevertheless, the 20-25-week-old SHR fed on the Ca 2.5% w/w diet which were treated with amlodipine showed higher SBP and DBP values than the animals of the same age in the other groups (see table 1). The present results indicate that calcium antagonists have a synergistic effect with calcium supplements to control ABP, but when the treatment is prolonged, these drugs antagonize the effect of dietary calcium on ABP.

TABLE 1. SBP and DBP (mm Hg) in SHR fed on two diets with a different calcium content and treated or non-treated with amlodipine.

	Non-treated		Amlodipine	
Dietary Ca (w/w)	1%	2.5%	1%	2.5%
17-week-old				
SBP	206±5	176±3[c]	187±5	159±6[c,d,e]
DBP	159±2	134±3	141±8	124±5[a]
20-week old				
SBP	192±3	181±3	178±4[a]	189±4
DBP	147±3	137±2	131±5[a]	149±4[e]
25-week old				
SBP	192±3	181±3	170±5[b]	197±3[d,f]
DBP	151±6	141±3	131±3[a]	159±5[d,f]

Values are means ± s.e. mean for a minimum of 8 rats. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ vs Ca 1% w/w ; [d]$p<0.05$ vs Ca 2.5% w/w; [e]$p<0.01$, [f]$p<0.001$ vs Ca 1% w/w + amlodipine by Bonferroni test.

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100P FOLIC ACID REVERSES METHIONINE-INDUCED ENDOTHELIAL DYSFUNCTION IN RABBIT ISOLATED AORTIC RING PREPARATIONS

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An oral dose of methionine (METH) can be used to induce experimental mild hyperhomocysteinaemia (HHCA, an increase in plasma homocysteine (HC) from approximately 8 to 28μM) in healthy human volunteers (Bellamy et al., 1998). In these studies, the induction of endothelial dysfunction is associated with increases in plasma METH levels from around 10 to over 500μM. In patients with severe HHCA (plasma HC >100μM) and concomitant endothelial dysfunction, folic acid (FA) has been used to lower HC levels. In the present study we have therefore investigated the acute effects of METH and FA, either alone or in combination, on endothelial function in isolated aortic rings from male New Zealand White rabbits (2 to 2.5kg).

2-3mm wide endothelium-intact rings were prepared and mounted in 8ml tissue baths containing Krebs buffer (with 10μM indomethacin) and gassed with 95% CO_2/ 5% O_2 at 37°C for isometric tension recording. A resting tension of 2g was used. All rings were preconstricted with phenylephrine (PE, 1μM) until a reproducible constriction was achieved. On reaching a steady plateau, rings were exposed to acetylcholine (ACh, 1nM to 10μM) followed by washing and re-equilibration. Tissues were then incubated for 3 hours with either Krebs buffer alone (control) or buffer containing METH (500μM) and FA (0.5μg/ml), either alone or in combination. Following this incubation rings were reconstricted with PE (1μM) and then exposed to ACh (1nM to 10μM) as before. After washing, METH and FA were re-added appropriately and tissues re-equilibrated for 15 minutes. Rings were then reconstricted with PE (1μM) followed by exposure to sodium nitroprusside (SNP, 1nM to 10μM). PE-induced increases in tension are expressed in g and relaxation responses expressed as a percentage of the PE-induced constriction.

All data is expressed as mean±s.e.m. (n 3) and maximum relaxation (Rmax) responses compared by ANOVA followed by Student Newman Keuls multiple range test. Significant differences are identified where $p<0.05$. Following the 3 hour incubation with buffer or METH and FA, either alone or in combination, no differences in the response to PE were observed between any of the groups (4.43±0.84, 4.48±1.37, 3.76±0.75 and 4.19±0.57g respectively). However, exposure to METH did cause a significant ($p<0.001$) inhibition of endothelium-dependent relaxation to ACh (Rmax 72.3±1.7 cf. 88.8±1.7% for control). FA alone (Rmax 80.9±1.3%) had no effect on the control ACh response, but significantly ($p<0.01$) reversed the inhibitory effect of METH (Rmax 89.2±6.1%). Following exposure to SNP, no differences in Rmax values were observed between any of the experimental groups (105.8±2.8, 130.8±6.9, 118.0±9.2 & 115.0±8.0% respectively for control, METH, FA and METH & FA). These data demonstrate impaired endothelial function following an acute exposure to METH at a concentration similar to that produced by METH loading in healthy volunteers. Whether the improvement in endothelial function in the presence of FA is due to increased clearance of tissue HC or a direct antioxidant effect of FA remains to be established.

Keuls multiple range test. Significant differences are identified where $p<0.05$.

Following the 3 hour incubation with buffer or METH and FA, either alone or in combination, no differences in the response to PE were observed between any of the groups (4.43±0.84, 4.48±1.37, 3.76±0.75 and 4.19±0.57g respectively). However, exposure to METH did cause a significant ($p<0.001$) inhibition of endothelium-dependent relaxation to ACh (Rmax 72.3±1.7 cf. 88.8±1.7% for control). FA alone (Rmax 80.9±1.3%) had no effect on the control ACh response, but significantly ($p<0.01$) reversed the inhibitory effect of METH (Rmax 89.2±6.1%). Following exposure to SNP, no differences in Rmax values were observed between any of the experimental groups (105.8±2.8, 130.8±6.9, 118.0±9.2 & 115.0±8.0% respectively for control, METH, FA and METH & FA).

These data demonstrate impaired endothelial function following an acute exposure to METH at a concentration similar to that produced by METH loading in healthy volunteers. Whether the improvement in endothelial function in the presence of FA is due to increased clearance of tissue HC or a direct antioxidant effect of FA remains to be established.

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101P ENDOTHELIAL DYSFUNCTION GENERATED BY A PRO-OXIDANT, PRO-DIABETIC CHALLENGE IN THE INSULIN-RESISTANT, OBESE ZUCKER RAT *IN VIVO*

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We have recently made the novel observation that a pro-oxidant challenge with hydroquinone (HQ), a redox cycling agent, in combination with buthionine sulfoximine (BSO), a glutathione depleting agent (see Laight *et al.*, 1999b), provokes the onset of type II diabetes mellitus in a model of established insulin resistance, the obese Zucker rat (Zucker & Antoniades, 1972; Laight *et al.*, 1999a). Since endothelial dysfunction may aggravate *in vivo* insulin resistance by restricting postprandial blood flow to insulin sensitive tissues (Steinberg *et al.*, 1996), we have now investigated endothelium-dependent and nitric oxide (NO)-mediated vascular responses in the obese Zucker rat *in vivo* following this pro-oxidant insult. Hence, vasodepression to acetylcholine (ACh) and the NO donor glyceryl trinitrate (GTN) was examined, together with the vasopressor effect of the NO synthase inhibitor L-NAME as an index of the regulation of mean arterial pressure (MAP) by endogenous NO (Rees *et al.*, 1989).

Male, 12-week old lean and obese Zucker rats were treated daily with HQ+BSO (each at 50 mg kg^{-1} i.p.) or normal saline (2 ml kg^{-1} i.p.) for 7 days as previously described (Laight *et al.*, 1999a). Animals were then anaesthetised with thiopentone sodium (120 mg kg^{-1} i.p.) and vasodepressor responses to bolus dose ACh (0.02-2 μg kg^{-1} i.v.) and GTN (0.1-50 μg kg^{-1} i.v.) established, followed by the vasopressor effect of bolus dose L-NAME (100 mg kg^{-1} i.v.). Data are mean±s.e. mean and were compared using Student's unpaired t test.

Basal MAP in the obese Zucker rat (143.2±5.3 mm Hg), which was not significantly different from that in lean animals (132.2±4.3 mm Hg) (P>0.05, n=5-6), was raised by HQ+BSO (P=0.09) such that obese basal MAP became higher relative to the lean Zucker rat after pro-oxidant treatment (obese: 155.0±2.3 mm Hg; lean: 136.6±2.8 mm Hg) (P<0.01, n=4-6). In obese animals, vasodepression to ACh (AUC: 69.4±4.6 units) was reduced by HQ+BSO (AUC: 54.9±4.0 units) (P=0.05, n=4-5); while AUC for vasodepression to GTN (53.2±4.7 units) was not significantly affected by HQ+BSO (46.3±7.4 units) (P>0.05, n=4-5) (Figure 1). Similarly, HQ+BSO reduced AUC for vasodepression to ACh in lean animals from 71.7±4.2 units to 57.1±3.1 units (P<0.02, n=6); while AUC for vasodepression to GTN (50.9±3.8 units) was not significantly affected by HQ+BSO (41.9±3.4 units) (P>0.05, n=6). Furthermore, the rise in MAP due to L-NAME in obese (33.8±2.2 %) and lean (31.7±4.0 %) animals was not influenced by HQ+BSO (obese: 31.0±3.0 %; lean: 35.8±7.3 % (P>0.05, n=4-6).

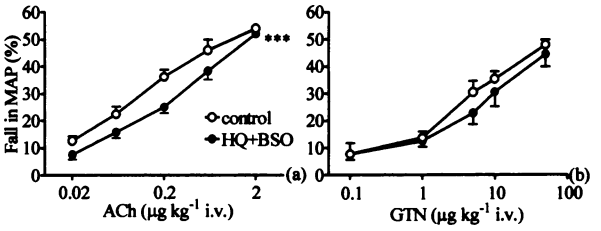

Figure 1. Effects of HQ+BSO on vasodepression to ACh (a) and GTN (b) in the anaesthetised obese Zucker rat. ***P<0.01, 2 way ANOVA

Our data suggest that HQ+BSO generates an impairment in agonist-stimulated, endothelium-dependent vasodilation with little disruption to NO-dependent vasodilator activity in both the lean and obese Zucker rat *in vivo*. This level of endothelial dysfunction, while apparently insufficient to generate significant insulin resistance *de novo* in the lean Zucker rat (Laight *et al.*, 1999a), may nevertheless play a vascular role in the pro-oxidant-mediated progression of established insulin resistance to diabetes mellitus in the obese Zucker rat.

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102P COMPARISON OF THE VASODILATOR ACTIVITIES OF VARIOUS GRAPE AND TEA EXTRACTS

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Recently it has been demonstrated by several authors that red wine, various grape and plant products including tea and tea derivatives, induce vasorelaxation in human and animal models (Andriambeloson *et al.*, 1997, 1998; Fitzpatrick *et al.*, 1995; Huang *et al.*, 1998). Current interest in the proposed beneficial effects of such products in protecting against coronary heart disease (CHD) remains controversial.
The present study was undertaken to determine the possible effects of various grape and tea based extracts on vascular function *in vitro*. Aortic rings from male New Zealand White rabbits (3.5 kg) were set-up at 37°C in organ baths (20ml) filled with Krebs buffer solution and continuously bubbled with 16% O_2, 5% CO_2 and 79% N_2. Vessels were precontracted with phenylephrine (PE, 10^{-7}M). Once a stable plateau had been reached, cumulative concentration response curves for each of the diluted grape and tea extracts (2 x 10^{-4} - 1.4 x 10 $mgml^{-1}$) were obtained.

The present study demonstrates the ability of various grape and tea based extracts to induce significant vasorelaxation in rabbit aortic tissue *in vitro*. All extracts evoked concentration-dependent relaxation in aortic rings. The grape and tea based extracts both produced easily distinguishable concentration response curves. The grape-based extracts were more potent at inducing vasorelaxation than the tea based extracts (Table 1) with the French grape seed extract being the most potent extract and the black tea derivatives, theaflavins extract being the least.
These results show that various extracts of plant origin induced vasorelaxation in rabbit aorta. If such responses occur *in vivo*, they may help to maintain optimal vascular function and contribute to a reduced incidence of CHD.

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Table 1. Maximum % relaxation values (± sem) and threshold concentrations values for relaxation (± sem) obtained with grape and tea extracts in rabbit aorta. *n* = number of animals.

Extract	Max. % Relaxation	Threshold Conc. for Relaxation	*n*
French grape seed	62.6 ± 4	0.4±0 x 10^{-2} $mgml^{-1}$	6
French grape skin	55.0 ± 6	3.4±1 x 10^{-2} $mgml^{-1}$ *	6
Grape skin	63.8 ± 6	0.4±0 x 10^{-2} $mgml^{-1}$	6
Grape powder	58.6 ± 11	3.4±1 x 10^{-2} $mgml^{-1}$*	6
Green tea	40.3 ± 13	35.6±4 x 10^{-2} $mgml^{-1}$***	8
Black tea	35.9 ± 9*	40.0±0 x 10^{-2} $mgml^{-1}$***	7
Theaflavin	28.9 ± 4**	40.0±0 x 10^{-2} $mgml^{-1}$***	3

Statistical analysis was carried out using a Students t-test. *P<005, ** P<0.01, *** P<0.001

103P IDENTIFICATION OF CYTOCHROME P450s CAPABLE OF 1-α-ACETYL-METHADOL (LAAM) AND norLAAM N-DEMETHYLATION

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Introduction: l-alpha-Acetylmethadol (LAAM), an analog of methadone, is used to treat opiate addicts. Its therapeutic efficacy is attributed largely to norLAAM (NL) and dinorLAAM (DL) that are formed by successive cytochrome P450-dependant N-demethylations. Many P450s bind multiple compounds so the possibility for drug interactions exists. To identify drugs that may interfere with LAAM, it is necessary to establish the specific forms of P450s catalyzing the metabolic pathway. A previous study in cDNA expressed human liver microsomes implicated P450 3A4 as the primary pathway (Moody et al., 1997). More recently, cDNA-expressed supersomes™ that provide higher activity, greater specificity, and a broader selection of P450s have become available. The aim of this study was to screen eleven P450 isoforms in vitro to identify those that catalyze the N-demethylation of LAAM and NL.

Methods: Supersomes™ (0.2 mg protein) having cDNA-expressed human P450 enzymes were incubated with either 125 nmol LAAM or NL in 0.5 ml 0.1 M phosphate buffer, 1.0 mM EDTA and 5.0 mM $MgCl_2$ (pH 7.4). Upon adding a NADPH generating system, duplicate samples were incubated at 37°C for zero and either five or thirty minutes. Reactions were terminated by adding 200 µl methanol and transferring to -80°C. NL and DL were measured by GC/MS and normalized to activity per minute.

Results: P450s 3A4, 2B6, and 2C18 all displayed substantial activity (Figure 1). Interestingly, 2C18 was the most active for NL N-demethylation. These results provide a basis for identifying compounds that have potential to interact with LAAM. Previous kinetic studies in human liver microsomes indicate that LAAM N-demethylation follows simple Michaelis-Menton kinetics at low substrate concentrations and complex kinetics at concentrations higher than 100 µM (Nelson et al., submitted). To evaluate the contribution of specific P450s at high and low LAAM concentrations, ongoing studies will investigate LAAM N-demethylation kinetics in supersomes™.

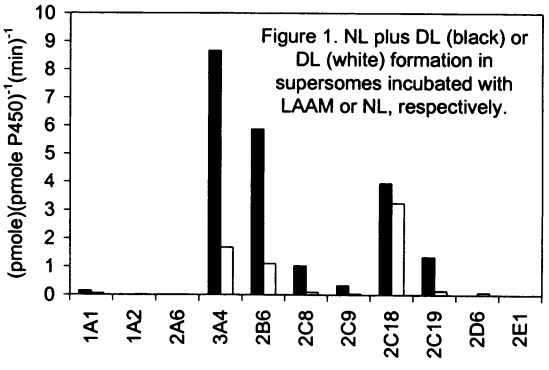

Figure 1. NL plus DL (black) or DL (white) formation in supersomes incubated with LAAM or NL, respectively.

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104P DESTRUCTION OF GLUTATHIONE S-TRANFERASE ZETA PROTEIN BY DICHLOROACETIC ACID TREATMENT

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Controversy surrounds the drug dichloroacetic acid (DCA). Its pharmacological effect of lowering lactic acid has been used in the experimental treatment of children with congenital lactic acidosis, a generally fatal disorder (Stacpoole *et al.*, 1998). Since chronic administration of high doses of DCA in the drinking water of rodents leads to hepatic cancer, by as yet undetermined mechanisms, there are concerns about its human toxicology. Although DCA is not widely used as a drug, chlorinated drinking water contains trace concentrations of DCA and related acids. The first step in DCA biotransformation is conversion to glyoxylate in a reaction catalysed by the glutathione-dependent enzyme, maleylacetoacetate isomerase, also known as glutathione S-transferase zeta (GSTz) (James *et al.*, 1997, Tong *et al.*, 1998). A potential adverse interaction with tyrosine metabolism was suspected when it was shown that oral doses of DCA to rats resulted in a dose-dependent rise in the excretion of the tyrosine catabolite, maleylacetone, a decarboxylation product of the natural substrate for GSTz, maleylacetoacetate (Cornett *et al.*, 1999). Metabolism of DCA to glyoxylate was also impaired. In the present study the amount of GSTz enzyme in hepatic cytosol fraction was examined following oral doses of DCA.

Rats were dosed by oral gavage with one or five daily doses of sodium DCA, 4 to 1000 mg/kg, then held in metabolism cages. Urine was collected daily for measurement of maleylacetone excretion. Twenty-four hours after the last dose, the rats were killed and livers removed for preparation of cytosol. The cytosolic activity of GSTz was measured with DCA as substrate, and the amount of immunoreactive GSTz enzyme determined by Western immunoblot. A chicken anti-human GSTz, courtesy of M. Grompe, was the primary antibody. Immunoreactive bands were detected by chemiluminescence and visualised by fluorography. Bands were quantitated by computerised scanning densitometry.

We found a dose-dependent increase in the daily total urinary maleylacetone excretion from 0.048 ± 0.016 mg/kg before treatment to 6 and 15 times this amount two to three days after treatment with 50 or 1000 mg DCA/kg respectively. There was a dose-dependent decrease in hepatic cytosolic GSTz activity with DCA as reported in Cornett *et al.*, 1999, and a corresponding dose-dependent reduction in the cytosolic GSTz immunoreactive band. Doses greater than 200 mg/kg for one or 5 days resulted in loss of >90% of the GSTz protein in cytosol. A single dose of 4 mg/kg had no effect on activity or protein content, although five doses of 4 mg/kg significantly decreased both to 80 ± 6% of the control values.

These studies showed that high doses of DCA perturb normal tyrosine metabolism by destroying GSTz. This leads to increases in maleylacetoacetate and maleylacetone, both of which are chemically reactive species that may lead to toxicity through a variety of mechanisms. Reasons for the specific loss of GSTz protein are being sought. This work was supported in part by a grant from the US Public Health Service, ES 07375.

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105P INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION IN LIPOPOLYSACCHARIDE-TREATED CULTURED SMOOTH MUSCLE CELLS FROM RAT MESENTERIC LYMPHATIC VESSELS

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Inducible nitric oxide synthase (iNOS) expression and vasodilator nitric oxide (NO) production, activated in blood vessel smooth muscle cells by bacterial lipopolysaccharide (LPS) and immune response cytokines, may contribute to the profound hypotension and poor blood perfusion of vital organs seen in patients with septic shock (Hecker *et al.*, 1999; Kirkebøen & Strand, 1999). Whether similar mechanisms alter lymphatic smooth muscle tone and the inherent contractile function is uncertain (Elias *et al.*, 1992). Here we show that iNOS expression is induced by LPS in cultured smooth muscle cells (SMC) from rat mesenteric lymphatic vessels.

Cells from explants of lymphatic vessels (from adult male rats) were grown at 37°C in Medium 199 containing 10% (v/v) foetal bovine serum and antibiotics in 5% CO_2 : 95% air. They were trypsinized at confluence, and cell numbers were increased over further passages to obtain sufficient material for experiments. At third passage, cells were seeded into multi-well tissue culture trays for experiments and for continuation of cell lines. LPS (from *E. coli*, serotype 055:B5) or drugs were added in culture medium to wells of confluent cells. Corresponding control cells received medium alone. After 20h, nitrite in the medium was measured by the Griess reaction to indicate cellular NO production. No drugs affected cell viability under the conditions used. Cells were identified as SMC by their immunofluorescence after binding a murine anti-smooth muscle actin antibody and a fluorescently-labelled anti-mouse IgG. 5.3 ± 0.9 μM nitrite in medium from control cells was increased significantly ($P<0.01$ Student-Newman-Keuls test) to 8.4 ± 1.1, 10.1 ± 1.4 and 13.8 ± 1.8 after 1, 10 and 100 μg/ml LPS treatment, respectively (means ± s.e.m; n=18 cultures from different rats).

Spontaneous production of nitrite by control cells occurred at early passages, but decreased from passage 5 (P5). These nitrite values (μM) were 9.7 ± 0.9 (P3, n=10); 8.9 ± 1.8 (P4, n=6); 5.9 ± 1.5 (P5, n=5); 2.3 ± 0.7 (P6, n=6); 1.8 ± 1.1 (P7, n=4); 1.4 ± 0.8 (P8, n= 5); 1.1 ± 0.6 (P9, n=4) (different at $P<0.0001$ by ANOVA). Western blotting detected some iNOS in protein extracts from control cells at low passage numbers, but greater amounts of iNOS were always found in corresponding LPS-treated (1 μg/ml) cells. Incubation of cells with the directly-binding NOS inhibitor, 1 mM L-nitroarginine methyl ester (L-NAME) prevented 1 μg/ml LPS-induced increases in nitrite (μM) production (control 5.4 ± 1.6; LPS 9.0 ± 2.1; LPS + L-NAME 3.1 ± 1.0; n=10), as also did the protein synthesis inhibitor, cycloheximide (10 μM CHX) (control 4.7 ± 1.2; LPS 8.1 ± 1.1; LPS + CHX 4.4 ± 1.2; n=8). CHX (but not L-NAME) prevented iNOS expression as seen by Western blotting.

Endogenously and exogenously produced NO are known to reduce lymphatic contractility (Von der Weid *et al.* 1996). Our findings that LPS induces iNOS in lymphatic smooth muscle indicates a further means by which NO may affect lymphatic spontaneous contractility and tissue clearance function, suggesting that this could be an important component of the circulatory dysfunction in septic shock.

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106P DIFFERENTIAL REGULATION OF INDUCIBLE TRANSCRIPTION FACTORS C-FOS AND C-JUN mRNA IN SPINAL CORD FOLLOWING SURGICAL INFLAMMATION

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Tissue damage during surgery can induce central sensitization and the development of hypersensitivity post-operatively (Woolf and Chong, 1993). Inducible transcription factors (ITFs), which are rapidly and transiently induced following tissue injury, are thought to underlie neuronal plasticity in the nervous system (Herdegen and Zimmermann, 1995). The present study set out to determine the effects of surgery on expression of ITFs c-fos and c-jun in spinal cord.

Adult female sheep (n = 12) undergoing a midline laparotomy for collection of ova were euthanased at 1 day and 7 days after surgery and spinal cord tissue collected. Spinal cords were also collected from similarly treated (superovulated) non-surgical control animals (n = 7). Lumbar spinal cord segments were processed for c-fos and c-jun mRNA expression using RT-PCR, as described previously (O'Shaughnessy and Murphy, 1993). The distribution of mRNAs for c-fos was examined in lumbar spinal cord by *in situ* hybridization according to the method described by Wisden and Morris (1994). Cell area was measured using a computerised image analysis system (image, v.5.2 NIH). Data were analysed using an ANOVA with post-hoc Tukey's test.

Moderate expression of c-fos and c-jun mRNA was detected by RT-PCR in lumbar spinal cord from control animals. Semi-quantitative RT-PCR revealed a significant increase in c-fos but not c-jun mRNA, 1 day after surgery (four fold increase; $p < 0.01$). By 7 days post-surgery c-fos mRNA expression had returned to control levels. c-fos mRNA hybridization signals were widely distributed in gray matter, particularly throughout the dorsal horn and in ventral horn motoneurons. A significant increase in c-fos mRNA was evident 1 day after surgery, and was localised to laminae I-II and lamina V neurons (Table 1).

	mean silver grains/ cell/ unit area	
	CONTROL	SURGICAL
laminae I-II	5.1 ± 0.6	12.3 ± 1.4*
lamina V	8.8 ± 1.1	16.9 ± 2.4**
motoneurons	8.4 ± 1.2	13.7 ± 1.6

Table 1. Expression of c-fos mRNA in lumbar spinal cord measured using in situ hybridization. Values are mean ± SEM. Significantly different from control: * $p < 0.05$, ** $p < 0.01$.

The transient up-regulation of c-fos mRNA in neurons located in laminae I-II and lamina V (corresponding to the terminal fields of primary afferent nociceptive fibres), suggests that c-fos may contribute to plasticity of function observed following acute surgical inflammation (Welsh and Nolan, 1994).

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107P P2X$_7$ RECEPTOR EXPRESSION AND FUNCTION IN HUMAN THP-1 CELLS

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P2X$_7$ receptors are ATP-gated cation channels widely expressed in immune cells (Surprenant *et al.*, 1996). These receptors can function both as non-selective cation channels and large 'pores' capable of passing molecules of ~800 Da (Surprenant *et al.*, 1996; Hibell *et al.*, 2000). Regulating P2X$_7$ receptor expression is important for immune cell survival. This study investigated changes in P2X$_7$ receptor expression and function in human monocytic THP-1 cells compared to recombinant receptors.

Studies were performed using THP-1 cells with native P2X$_7$ receptors and HEK293 cells stably expressing human P2X$_7$ receptors. In some studies THP-1 cells were used 18 h after treatment with 0.5μM phorbol myristate acetate (PMA). P2X$_7$ receptor expression was studied by Western blotting, following immunoprecipitation of the receptors with a human P2X$_7$ receptor antibody (Buell *et al.*, 1998). Confocal microscopy was used with antibody-labelled cells. P2X$_7$ receptor function was studied by measuring 3'-*O*-(4-benzoylbenzoyl)-ATP (BzATP)-stimulated influx of the fluorescent DNA binding dye YO- PRO-1 (Hibell *et al.*, 2000).

Incubating PMA-treated THP-1 cells with 1000U/ml interferon γ (IFN-γ) for 24 or 48 h produced a dose-related up-regulation of P2X$_7$ receptor expression, assessed by Western blotting. In fixed THP-1 cells the intensity of staining and the proportion of labelled cells increased after IFN-γ treatment and was blocked by 10μg/ml cycloheximide. In both untreated and PMA-treated THP-1 cells BzATP elicited a delayed accumulation of YO-PRO-1 and IFN-γ decreased the delay and increased the rate of YO-PRO-1 accumulation. In untreated THP-1 cells, IFN-γ (24h) reduced the delay (control 30±5 min; IFN-γ 15±5 min; n=4; $p<0.05$ 1 way ANOVA/Dunnett's test) and increased the maximum rate of BzATP-stimulated YO-PRO-1 accumulation ($t_{½}$ values: control 96±4 min; IFN-γ 42±6 min; n=4; $p<0.05$ unpaired Student's t-test) but did not affect the BzATP pEC_{50} value (Control 4.9±0.05; IFN-γ 5.1±0.04; $p>0.05$ unpaired Student's t-test). YO-PRO-1 influx was seen in 53.8±1.4% of PMA/IFN-γ-treated cells after 60 min (n=3) and only 22.4% of PMA-treated cells (n=2). P2X$_7$ receptor expression was much higher in the transfected HEK293 cells than in IFN-γ-treated THP-1 cells (n=5). In these cells, BzATP-stimulated YO-PRO-1 influx occurred in all cells without detectable delay and the maximum rate of influx was more rapid ($t_{1/2}$ =21±6 min; n=5) than in THP-1 cells.

This study has confirmed that P2X$_7$ receptor expression in THP-1 cells can be upregulated using IFN-γ (Humphreys & Dubyak, 1996) with the increase being due to protein synthesis as it was blocked by cycloheximide. Increased receptor expression was associated with a decrease in the time taken for pore formation and an increase in the rate of BzATP-stimulated YO-PRO-1 influx. These results suggest that cells expressing endogenous P2X$_7$ receptors have regulatory processes to control expression of this protein and demonstrate that increases in receptor expression affect the rate of dilation to form pores.

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108P IDENTIFICATION OF A PUTATIVE NOVEL SPLICE VARIANT OF THE PORCINE 5-HT$_{3A}$ RECEPTOR SUBUNIT

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To date, cDNA encoding the 5-HT$_{3A}$ receptor subunit has been isolated from mouse, rat, human, guinea pig and pig (Maricq *et al.*, 1991, Hope *et al.*, 1993, Belelli *et al.*, 1995, Lankiewicz *et al.*, 1998, Fletcher *et al.*, 1999), and a cDNA encoding the human 5-HT$_{3B}$ subunit has recently been described (Davies *et al.*, 1999). The 5-HT$_{3A}$ subunit appears to exist as two splice variants (5-HT$_{3A(a)}$ and 5-HT$_{3A(b)}$) encoded by a single gene in all species (although the longer splice variant of the 5-HT$_3$ receptor has not been detected in the human or pig). The two splice variants differ by the presence or absence of five or six amino acids in the putative large intracellular loop between the third and fourth transmembrane regions, and are generated by an alternative use of 3′ acceptor sites in exon 9 (Werner *et al.*, 1994). The missing stretch contains a putative caesin kinase II phosphorylation site, but to date no obvious functional differences between 5-HT$_{3A(a)}$ and 5-HT$_{3A(b)}$ have been detected. Here we report the identification of a putative novel splice variant of the 5-HT$_{3A}$ subunit.

Total RNA was isolated from pig nodose ganglion tissue (RNA isolation kit, Stratagene) and reverse transcriptase (RT)-PCR performed using oligonucleotide primers (P1 ATCCTCGAGGTGGATGAGAAGAACCAA/GGT and P2 TTCATCGATGGCTGCAGTGGTTA/G/C/TCCCAT) designed to hybridise to previously identified 5-HT$_{3A}$ sequences, and which have already been used successfully in the cloning of a guinea pig 5-HT$_{3A}$ subunit (Lankiewicz *et al.*, 1998). Oligo (dT) primed cDNA and 100 ng of primers P1 and P2 were incubated in buffer (67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20; Bioline) containing 2 mM $MgCl_2$, 0.2 mM of dNTPs (NBL) and 5 U of *Taq* DNA polymerase (Stratagene). 30 cycles (95 °C, 1 min; 60 °C, 1 min; 72 °C, 4 min) were performed. RT-PCR resulted in the amplification of a 968 bp cDNA product, which was identified as the porcine orthologue of the 5-HT$_{3A}$ subunit (Fletcher *et al.*, 1999), and a 514 bp product, which was observed to react with a mouse 5-HT$_{3A}$ probe in Southern blots. This cDNA was thus cloned into the vector pCR Script 2.1 (InVitrogen) and sequenced on both strands.

The product was identified as a variant of the 5-HT$_{3A}$ gene, which lacks exons 4, 5 and 6. It is predicted to have a molecular mass of 39 kDa, and a shortened extracellular N-terminus, without the cys-cys loop characteristic of ligand gated ion channels, nor three of the glycosylation sites seen in the 5-HT$_{3A(a)}$ and 5-HT$_{3A(b)}$ subunits. This protein may correspond to a protein of 37 kDa in the native 5-HT$_3$ receptor purified from porcine cerebral cortex, which gives a reaction with an antiserum directed against the 5-HT$_{3A}$ subunit (n=8, unpublished results). Future expression studies should help identify the functional significance of this variant.

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109P EXTRACELLULAR LYSOPHOSPHOLIPIDS STIMULATE SUPEROXIDE PRODUCTION IN RAT ALVEOLAR MACROPHAGES, POSSIBLY VIA EDG RECEPTORS

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During inflammatory reactions increased levels of extracellular lysophospholipids may occur. Recently, the EDG (endothelial differentiation gene) receptors were identified as a family of G-protein coupled receptors specific for either lysophosphatitic acid or sphingosine 1-phosphate (for review see Racké *et al.*, 2000). Human and rat alveolar macrophages have been shown to express mRNA for multiple EDG receptors (Hornuß *et al.*, 2000; Hammermann *et al.*, 2000). Here, effects of extracellular lysophospholipids on the produtionof superoxide anions (O_2^-) by alveolar macrophages was studied.

Rat alveolar macrophages ($0.5x10^6$ cells $well^{-1}$) obtained by lavage of isolated rat lungs were cultured for 20 h in DME-F12 medium under serum free conditions (Hey *et al.*, 1995). Thereafter, medium was replaced by medium containing 0.5 mg/ml iodonitrotetrazolium (INT) violet and various test substances. The accumulation of INT-formazan during 1 h incubation was determined photometrically as a measure of superoxide anion production. Given are mean values ± s.e.mean of n = 6-9.

In intial experiments it was shown that 4α-phorbol 12-myrestate 13-acetate (PMA, 100 nM) increased INT-formazan formation by 271 ± 56%. N-formyl-Met-Leu-Phe (fMLP, 100 - 1000 nM) enhanced INT-formazan formation maximally by 43 ± 10% at 300 nM and lipopolysacharides (LPS, 0.1 – 10 μg/ml) enhanced it maximally by 61 ± 11% at 10 μg/ml. Lysophosphatitic acid (0.1 - 10 μM) increased INT-formazan formation maximally by 64 ± 23 % at 1 μM. Sphingosine 1-phosphate (0.1 - 10 μM) increased INT-formazan formation maximally by 122 ± 23% at 1 μM, whereas sphingosylphosphorylcholine (0.1 - 10 μM) failed to cause a significant increase.

In conclusion, extracelluar lysophosphatitic acid and sphingosine 1-phosphate can cause activation of superoxide production in rat alveolar macrophages. As rat alveolar macrophages express, both a lysophosphatitic acid specific (EDG2) receptor as well as a sphingosine 1-phosphate specific (EDG5) receptor (Hornuß *et al.*, 2000; Hammermann *et al.*, 2000), these effects might be mediated via these EDG receptors.

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110P A ROLE FOR HI ANTAGONISTS TO FACILITATE WOUND HEALING?

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These experiments were designed to investigate in an *in vitro* model of myofibroblasts, which are also known as contractile fibroblasts (Ryan et al., 1974), if different H_1 antagonists had different contractile effects. This knowledge could therefore lead torational use of such agents in subsequent *in vivo* wound healing experiments.

Rat testicular capsules (n=20) and capsules formed around subcutaneously placed silicone cylinders (n=12), removed 10-42 days after placement in male Hooded Lister rats (weight 250-300 g) were arrangeded *in vitro* for isometric recording. The tissues (rings of 5 mm width) were bathed in 4 ml of aerated Krebs solution under a resting tension of 2 g. Cumulative concentration response curves were established over a 25 minute period for promethazine, pyrilamine, tripelennamine and diphenhydramine (all obtained from Sigma). An initial concentration of 16 mM was used, and theconcentiation was arithmetically increased at 5 minute intervals. Promethazine was considered to be the "standard" and all drug effects were related to this H1 antagonist. Histological and immunocytochemical techniques were used to verify the presence of myofibroblasts in the tissues (Lossing *et al.*, 1993).

All the H1 antagonists used contracted both types of tissues in a concentration-dependent, reproducible manner which could be reversed by washing. In the rat testicular capsule the order of drug effectiveness to produce a submaximal response of 0.5 g change in tension was: promethazine (mean concentration 21mM, standard deviation S.D. 34) > tripelennamine (34 mM, S.D. 2.1) > pyrilamine (50mM. S.D. 4.1) > diphenhydramine (52 mM, S.D. 3.8), $p<0.013$ (2-tailed). Capsules from subcutaneous implants showed a similar order of effectiveness, with the maximal contractile responses occuring between days 10-28. Histology and immunocytochemistry identified a population of myofibroblasts in both the testicular capsule and the silicone-induced capsule. In capsules around silicone cylinders, the myofibroblasts were especially noticeable 10-28 days from the time of implantation. This correlated with the degree of contractile responses of the tissues.

In this study all of the H1 antagonists demonstrated an agonist effect on myofibroblasts to varying degrees, suggesting different receptor stimulation. Given the central role of myofibroblasts in wound healing (Gabbiani *et al.*, 1971), this would suggest that H1 antagonists might expedite the wound healing process. This could be important clinically in modifying wound healing/closure by stimulating contraction of the local tissues via a myofibroblast action.

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111P IS THERE A ROLE FOR PROMETHAZINE IN WOUND HEALING?

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The antihistaminic (H1), antiemetic, sedative and antimotion sickness properties of promethazine in clinical practice are well established (Clark *et al.*, 1978; Brown *et al.*, 1997). Little has been written on its role in wound healing, although there is a suggestion in the literature that promethazine might act as an agonist on the contractile cells of healing wounds which are known as myofibroblasts, cells with a fundamental role in wound contraction and healing (Gabbiani *et al.*, 1976). The aim of this study was to investigate whether promethazine has a role in wound healing via its effects on myofibroblasts, which exist either as constitutive or induced cells.

1. Testicular capsules from 20 male Hooded Lister rats were used to study the effects of promethazine on constitutive myofibroblasts.
2. Induced myofibroblasts were studied from:

(a) capsules around silicone implants in humans
(b) fibroproliferative situations such as Dupuytren's nodules and cords in hands and (c) capsules formed around subcutaneously placed silicone rods in rats (6 male/6 female Hooded Lister, 250-300g, killed by stunning and exsanguination), which were harvested on days 10, 14, 21, 28, 35 and 42, post-implantation.
Rings/strips of capsules and strips from Dupuytren's nodules and cords (width 5mm) were suspended in 4ml aerated Kreb's solution connected to an isometric transducer. A Grass Model 7D Polygraph recorded the contractile response. Promethazine was added at an initial concentration of 16mM. This concentration was added every 5 min, cumulatively for 25 min and then the tissues were washed out. After experimental use the tissues were processed for histological staining of the connective tissue and immunocytochemistry using α-smooth muscle actin and vimentin for the confirmation of the presence of myofibroblasts.

(1) All the tissues contracted to varying degrees. The contractile responses were concentration-dependent, reversible, and reproducible.
(2) There was a positive correlation between the number of myofibroblasts present in the tissues and their contractile response ($p<0.001$, Kruscal Wallis test).
(3) The mean contractile tension after addition of 80mM was: testicular capsule (2.4g)>rat capsule around silicone rod 2 weeks post-implantation (1.8g)>rat capsule around silicone rod 4 weeks post-implantation (1.2g)>human breast capsule around silicone rod 6 weeks post-implantation (0.4g)>Dupuytren's nodule (0.3g)>Dupuytren's cord (no response).
(4) The density of the collagen in the extracellular matrix correlated to the contractile responses obtained.
(5) The tissues were found to be capable of being stimulated for more than 8 hours

This study has demonstrated that promethazine promotes tissue contractility, in a number of different tissues that contain myofibroblasts which have a significant role in wound healing.

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112P ADENOSINE RELEASES HISTAMINE FROM SENSITIZED BUT NOT UNSENSITIZED GUINEA-PIG LUNG MAST CELLS IN THE PRESENCE OF ZILEUTON AND INDOMETHACIN

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The mast cell is believed to play a key role in the pathogenesis of asthma, since released mediators such as histamine, leukotriene C4 and prostaglandin D2 exert a direct spasmogenic activity on airway smooth-muscle (Rossi & Olivieri, 1997). Inhaled adenosine provokes bronchoconstriction in patients with asthma, but not in normal subjects (Feoktistov *et al*, 1998). The mast cell has been linked to this effect since 5'-AMP- or adenosine-induced bronchoconstriction was attenuated by antihistamines or the mast cell stabilizers, sodium cromoglycate and nedocromil (Ng, Polosa & Church, 1990). This study compares effects of adenosine on histamine release from mast cell preparations of sensitized and non-sensitized guinea-pigs.

Male Dunkin-Hartley guinea-pigs (250-300g) were sensitized to ovalbumin (OA) (10 μg OA and 100 mg $Al(OH)_3$ in saline i.p.). All procedures were started 14-21 days later, mast cells were isolated from guinea pig lung tissue by a modification of a method described by Ali & Pearce (1985). Lungs were chopped for 15 minutes and washed with full HEPES buffer (FHB). The tissue was incubated in FHB (10 ml g^{-1} of tissue) containing collagenase 1a (200 iu ml^{-1}) and bovine serum albumin (1 mg ml^{-1}) in a shaking water bath at 37°C for 90 minutes. The tissue was disrupted mechanically with a syringe, filtered through nylon gauze and the filtrate centrifuged (150 x g for 5 min). The supernatant was discarded and the cell pellet resuspended in FHB (20 ml) and washed again.

Mast cells from sensitized and unsensitized guinea pigs were incubated in buffer or with zileuton (Zil) and indomethacin (Ind) (3 μM) 15 minutes prior to exposure to calcium ionophore A23187 (10^{-7} M) or OA (10 mg ml^{-1}). Cell were also exposed to adenosine (AD) (100μM) either 15 minutes prior to ionophore and OA (t=0) or concurrently(t=15). After 30 min the reaction was terminated with ice cold FHB (600 μl) and centrifuged (200x g for 4 min). The supernatant was poured off and the cell pellet fraction reconstituted with FHB. Histamine release was measured using a glass microfibre analysis (Nolte *et al.* 1987.). Both supernatant and cell pellet histamine levels were examined to give percentage histamine release after subtraction of spontaneous histamine release (n=5-6 animals).

	Unsensitized		Sensitized	
	%Histamine Release (±SEM)			
Releaser		+Ind&Zil		+Ind&Zil
Ionophore	3.94±0.56	2.11±2.36	4.05±0.84	5.72±1.28
+AD t=0	3.87±0.57	1.26±0.81	4.60±0.79	4.58±1.56
+AD t=15	4.69±0.71	4.22±0.39	4.80±0.92	8.66±1.29
Ovalbumin	-0.12±0.39	-5.36±1.01	1.46±0.34	6.48±1.58
+AD t=0	ND	-5.24±1.10	2.24±0.67	7.60±1.66
+AD t=15	ND	-6.12±1.13	2.63±0.75	9.00±3.11
Adenosine	-0.32±0.41	1.98±0.99	0.20±0.12	6.50±1.98

There was a trend for adenosine in conjunction with ionophore and ovalbumin to enhance histamine release from sensitized lung mast cells, although not significantly. Adenosine alone had no significant effect ($P>0.05$) in sensitized or unsensitized lung on histamine release. However in the presence of zileuton and indomethacin there was significant release of histamine by adenosine alone in the sensitized lung. Thus, when the cyclooxygenase and lipooxygenase pathways are blocked by indomethacin and zileuton, the release of histamine by adenosine is enhanced.

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113P EARLY AND LATE PHASE BRONCHOCONSTRICTIONS, AIRWAY HYPERREACTIVITY, CELL INFLUX AND STEROID OR ROLIPRAM SENSITIVITY AFTER INHALED OZONE IN CONSCIOUS GUINEA-PIGS

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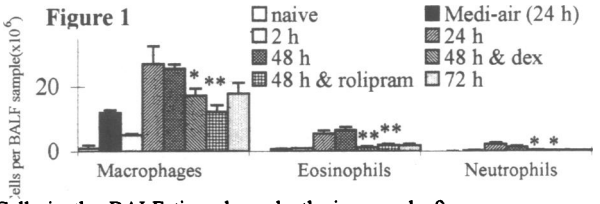
Chronic obstructive pulmonary disease (COPD) and asthma are associated with a reduced airflow (obstruction or early and late phase (LPB) bronchoconstrictions, respectively), dyspnoea, airway influx of leukocytes and airway hyperreactivity (AHR) (Vrugt & Aalberes, 1993). The degree of AHR correlates with the severity of COPD and asthma (Rijcken *et al*, 1997). Corticosteroids and phosphodiesterase 4 (PDE4) inhibitors attenuate AHR and pulmonary cell influx (Torphy *et al.*, 1999). Ozone inhalation also induces leukocyte infiltration and AHR (Murlas *et al.*, 1988). We therefore characterised the PDE4 inhibitor and steroid sensitivity of lung function changes, AHR and cell influx in conscious ozone-challenged guinea pigs, as a potential COPD and asthma model, for further investigation of these inflammatory disorders.

Respiratory rate (breaths.min^{-1}: Br.p.m.) and specific airway conductance (sGaw) were measured in groups (n=6) of conscious Dunkin-Hartley guinea-pigs (male 300-350g) by whole-body plethysmography (Griffiths-Johnson *et al.*, 1988). Baseline (BL) sGaw values were obtained and 30 min later they received a nose-only exposure to a threshold bronchoconstrictor dose of nebulised (0.2 ml.min^{-1}) histamine (Hist: 1mM, 20s) and sGaw was recorded at 0, 5 and 10 min. 24h later the guinea-pigs were box-exposed to 'Medi-air' (0.3-0.4 l.min^{-1}) only, or 'Medi-air' via an ozone generator (2.15+0.05 p.p.m.), for 90 min. Br.p.m and sGaw were measured at 0, 15, 30, 60 min, and hourly thereafter. Airway reactivity to Hist was re-assessed at 2, 24, 48, or 72h after exposure. After assessing airway reactivity, animals were overdosed with pentobarbitone sodium (0.6 mg.100g^{-1}, i.p.), the lungs lavaged (1% EDTA, 1 ml.100g^{-1}, twice) and the cell content of the BALF determined. In other animals, the steroid dexamethasone (dex: 20 mg.kg^{-1}), or the PDE4 inhibitor, rolipram (1 mg.kg^{-1}) were administered (i.p.) 24h and 0.5h prior to ozone exposure and 24 h afterwards.

Ozone caused an increased initial bronchoconstriction (-52.2±6.5 peak % changes from BL sGaw, $P<0.01$) compared to 'Medi-air' exposure (-13.4±3.3%), which was attenuated with dex treatment (-28.2±4%, $P<0.02$). After 2h, sGaw recovered to BL values. At 5h after ozone, a LPB developed ($P<0.04$), coinciding with an increased ($P<0.001$) respiratory rate (-38.0±7.2 and +107.5±28.0 peak % changes from BL sGaw and Br.p.m, respectively), which resolved at 72h. Ozone caused an increased bronchoconstriction to Hist (AHR) at 2 ($P<0.02$), 24 ($P<0.05$) and 48h ($P<0.05$) after exposure (-34.3±9.7, -37.3±16.5 and -11.63±3.7%), compared with before ozone (-2.2±8.4, -7.7±4.5 and +7.4±8.9%, respectively). Reactivity was restored by 72h ($P>0.05$) and unchanged 2h after 'Medi-air' ($P>0.05$). Rolipram and dex inhibited ($P<0.05$) the ozone-induced AHR to Hist at 2h.



Cells in the BALF time-dependently increased after ozone exposure (Figure 1, *$P<0.01$, **$P<0.001$). Rolipram and dex treatment reduced the airways influx of macrophages (51.8 & 32.3% inhibition, respectively), eosinophils (80.9 & 65.1%) neutrophils (75.9 & 70.9%), at 48h.

This study shows for the first time that ozone exposure caused a LPB in conscious guinea-pigs analogous to the asthmatic LPB. In common with asthma and COPD, ozone induced leukocyte infiltrated airways, AHR to Hist and dyspnoea. The AHR and cell influx were rolipram- and steroid-sensitive, but the dyspnoea and LPB were not.

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114P HISTOLOGICAL IDENTIFICATION OF THE EFFECTS OF ADENOSINE AND ANTIGEN ON DEGRANULATION OF MAST CELL SUBTYPES IN SENSITIZED GUINEA-PIG AIRWAYS

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Adenosine and ovalbumen exert bronchoconstrictor responses on sensitized guinea-pig airways (Thorne *et al*, 1996). Ovalbumen binds to mast cell IgE receptors resulting in release of mediators that produce smooth muscle contraction. The mechanism by which adenosine causes its effect is not fully understood, in particular which cell type is involved. This study examines if adenosine affects mast cells and if so which type of mast cell.

Male Dunkin-Hartley guinea-pigs (250-300g) were sensitized to ovalbumen (10μg with $Al_2(OH)_3$ i.p.) and used 14-21 days later. They were killed by cervical dislocation and the trachea removed, cut spirally, divided and superfused with warmed (37°C), gassed (95% O_2, 5% CO_2) Krebs bicarbonate solution for 20 mins. The lungs were removed and separated at the tracheal bifurcation. Lung halves were cannulated at the tracheal bifurcation and perfused with warmed (37°C), gassed (95% O_2, 5% CO_2) Krebs bicarbonate solution for 20 mins. After equilibration either adenosine (Aden.,1mM) or ovalbumen (Oval.,10μg) was added to each half lung. Each tracheal half was infused with either adenosine (300μM) or ovalbumen (10μg). After the maximum bronchoconstriction the tissues were removed. The lungs were divided into outer, middle and inner regions. Cells were then isolated by digestion in Krebs bicarbonate solution containing 500μg/ml collagenase and 1mg/ml bovine serum albumen for 2h and centrifuged at 200G for 6 mins. Using a haemocytometer, total cell counts for each region were made. The suspension was spun onto slides by cytospin. One slide was stained with Leishmans for visualising mast cells. Only intact non-degranulated mast cells were counted. The other slide was stained with alcian blue and counter-stained with safranin O to distinguish mucosal tissue mast cells (MTMC) and connective tissue mast cells (CTMC). Values are expressed as the number of CTMC per 100 MTMC. Comparisons were made between tissues perfused/superfused (control) and those receiving adenosine or ovalbumen. Statistics used a Tukey's multiple comparison test at 95% confidence intervals.

Exposure to adenosine or ovalbumen had no effect ($p>0.05$) on the total number of cells/g in any region of the sensitized or non-sensitized lung or trachea. There was a significant ($p<0.05$) reduction in the proportion of mast cells to non-mast cells in all three regions of the sensitized, but not ($p>0.05$) in the non-sensitized, guinea-pig lung or trachea after an adenosine or ovalbumen challenge. There was no significant difference ($p>0.05$) in the proportion of CTMC to MTMC in any region of the sensitized guinea-pig lungs or trachea after an adenosine or ovalbumen challenge (table 1).

Table 1. Mast cells in sensitized and non-sensitized airways.

	% Mast Cells						No. CTMC/100 MTMC		
	Sensitized			Non-Sensitized			Sensitized		
Region	Control	Aden.	Oval.	Control	Aden.	Oval.	Control	Aden.	Oval.
Inner	4.4±0.8	1.7±0.3	1.4±0.2	3.9±0.3	4.5±0.8	3.8±0.5	102±24	69±17	77±20
Middle	5.8±0.7	1.8±0.4	2.0±0.5	3.6±0.4	4.0±0.3	4.3±0.3	82±19	43±8	63±8
Outer	6.2±0.9	2.1±0.4	1.5±0.2	3.4±0.5	4.5±0.3	4.9±0.3	34±4	55±15	99±40
Trachea	7.7±0.6	2.7±0.8	2.8±0.6	4.5±0.7	5.9±1.3	5.3±0.9	118±25	127±20	180±22

These results show that either adenosine or ovalbumen cause a loss of intact mast cells in sensitized but not in non-sensitized guinea-pig airways. This would explain the bronchoconstriction by adenosine in sensitized animals and asthmatics. Degranulation appears to act equally on both mast cell type.

Thorne, J.R., Danahay, H. & Broadley K.J. (1996). *Eur. J. Pharmacol.* **316,** 263-271.

115P SIMULTANEOUS MEASUREMENT OF ANTI-TUSSIVE AND ANTI-BRONCHOCONSTRICTOR EFFECTS OF NEUROKININ RECEPTOR ANTAGONISTS AGAINST CITRIC ACID-INDUCED COUGH IN GUINEA-PIGS

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Sensory neuropeptide-containing fibres are thought to be implicated in cough and reflex bronchoconstriction (Forsberg et al. 1988). This study investigated the involvement of substance P (SP) and neurokinin A (NKA) receptors in citric acid-induced cough and airway obstruction using a novel selective NK_1 (NKP608) (Lewis et al. 2000) and a selective NK_2 (SR48968) receptor antagonist.

Unanaesthetized, unrestrained male Dunkin Hartley guinea pigs (250-500g) were placed individually in a whole body plethysmograph (Buxco) and exposed to a nebulized aqueous solution of citric acid (0.6 M) for 10 min. The chamber was fitted with a microphone connected to both an external speaker and a computer allowing visualization of sound signal and the number of coughs were counted by an observer. Simultaneously, the degree of airway obstruction due to citric acid exposure was measured using whole body plethysmography (increase in Penh). The airflow changes in the chamber were monitored with a differential pressure transducer connected to a non-invasive airway mechanics analyzer (Biosystem XA software). Both parameters were recorded for 15 min after nebulisation of citric acid. To determine the antitussive and antibronchoconstrictor effects of neurokinin antagonists, NKP608 (0.03-1mgkg^{-1}), SR48968 (10mgkg^{-1}) dosed alone or in combination or vehicle (2%DMSO/17% cremophor EL/81% NaCl) were given orally 2h prior to the citric acid exposure. Data are expressed as mean ± s.e. mean. Statistical significance (ANOVA and post-hoc Tukeys test) was taken as a p-value of less than 0.05.

Exposure of animals, pre-treated with vehicle, to 0.6 M citric acid resulted in a cough response (13 ± 2.8 coughs, n=10) and a gradual development of airway obstruction as measured by Penh. The increase in Penh was still evident during the 5 min post citric acid challenge period. Oral administration of NKP608 (0.03, 0.3 and 1 mgkg^{-1}) 2h prior to citric acid significantly inhibited the number of coughs by 78±5, 73±8 and 79±8%, respectively (n=7-10). In contrast, the increase in Penh was unaffected at all doses. SR48968 (10 mg/kg p.o., -2h) did not inhibit the cough response (7 ± 2.2 coughs) but significantly reduced the increase in Penh by 49±18% (n=10). Animals dosed with NKP608 (1 mg/kg) and SR48968 (10 mg/kg) simultaneously (p.o., -2h) significantly inhibited cough by 91±7% and inhibited the increase in Penh by 74±4% (n=5).

Therefore, in the guinea pig, the NK_1 receptor is responsible for citric acid-induced cough and not involved in the increase in Penh. In contrast, the NK_2 receptor appears to be the predominant receptor responsible for citric acid-induced airway obstruction. Thus, blockade of both NK_1 and NK_2 receptors is needed to suppress the consequences of sensory nerve activation in guinea-pig airways. Data from this model suggest that dual NK_1/NK_2 antagonism would have a greater impact on neuropeptide driven effects in respiratory disease.

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Lewis et al. (2000) *Br. J. Pharmacol.* This meeting

116P ANTI-INFLAMMATORY EFFECT OF AN INDUCIBLE NITRIC OXIDE INHIBITOR IN MURINE ALLERGEN-INDUCED AIRWAY INFLAMMATION: MECHANISM OF ACTION

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Growing evidence demonstrates that inducible nitric oxide (iNOS) is induced in the airways of asthmatic patients. However, the precise role of nitric oxide (NO) in lung inflammation is unknown. This study investigated the effects of the iNOS inhibitor, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine (AMT) (Tracey *et al.*, 1995) in a murine allergen-driven lung inflammation model.

Female Balb/C mice (25-30 g, 6-8 animals per group) were immunised intraperitoneally (i.p.) with 10 µg of ovalbumin (OA) in 0.2 ml of alum on day 0 and 14. On day 21, animals were challenged with aerosolised OA (50 mg ml^{-1}, 20 minutes) or phosphate buffered saline (PBS). AMT (4 mg kg^{-1}) or PBS were given twice daily by i.p. injection (0.1 ml) from day 20 to the day of sacrifice. At specified time points, mice were anaesthetised with pentobarbitone sodium (60 mg kg^{-1}, i.p.). Statistical significance ($P < 0.05$) were determined by an ANOVA. Data are expressed as mean ± s.e. mean.

At day 3 post challenge, OA induced an accumulation of eosinophils (in 10^4 cells ml^{-1}; OA, 27.1 ± 1.8; PBS, 0.1 ± 0.1) and neutrophils (in 10^4 cells ml^{-1}; OA, 1.4 ± 0.3; PBS, 0.02 ± 0.01) in the bronchoalveolar lavage fluid (BALF) which was significantly inhibited by AMT treatment (in 10^4 cells ml^{-1}; eosinophils: 5.7 ± 0.2; neutrophils: 0.5 ± 02). Despite the inhibition of BALF inflammatory cell accumulation, IL-4 (111 ± 27 unit ml^{-1}) and IL-5 (1512 ± 216 unit ml^{-1}) production from purified lung T-cells, activated *in vitro* (Coyle *et al.*, 1996), was significantly increased and IFN-γ (617 ± 130 unit ml^{-1}) production significantly decreased in mice treated with AMT, when compared to the PBS-treated group (in unit ml^{-1}; IL-4, 10 ± 1; IL-5, 949 ± 85; IFN-γ, 1241 ± 280).

As determined by ribonuclease protection assay, 6 hours after the OA challenge, OA induced a significant increase in lung eotaxin, MIP-2, MCP-1 and TCA-3 mRNA expression. OA-induced increase in MIP-2 and MCP-1 were significantly reduced in the lungs of AMT-treated mice, whereas eotaxin and TCA-3 expression were not affected. As determined by ELISA, 6 hours after the OA challenge, AMT treatment significantly inhibited the OA-induced increase in lung protein levels for MCP-1 (in ng ml^{-1}; OA, 2.1 ± 0.1; OA + AMT, 1.4 ± 0.1) and MIP-2 (in ng ml^{-1}; OA, 1.1 ± 0.1; OA + AMT, 0.46 ± 0.02), whereas eotaxin levels were not affected (in ng ml^{-1}; OA, 2.1 ± 0.2; OA + AMT, 1.7 ± 0.2).

Incubation of primary cultures of murine lung fibroblasts with a NO donor, hydroxylamine, for 24 hours, induced a dose-dependent release of MIP-2 and MCP-1 with a maximal effect at 600 µM. At this concentration, hydroxylamine induced a 7- and 3-fold increase over basal levels for MIP-2 (in ng ml^{-1}; basal, 0.08 ± 0.01; stimulated, 0.56 ± 0.05) and MCP-1 (in ng ml^{-1}; basal, 7.3 ± 1.1; stimulated, 19.6 ± 3.4), respectively ($P < 0.05$). Hydroxylamine did not induced eotaxin release.

These results suggest that lung inflammatory cell influx following allergen challenge in mice is dependent on NO produced by iNOS. NO appears to increase lung chemokines expression and thereby to facilitate inflammatory cells influx into the airways.

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117P TIME-DEPENDENT CHANGES IN BRONCHIAL RESPONSIVENESS TO DIRECT AND INDIRECT BRONCHOSPASMOGENS FOLLOWING ALLERGAN CHALLENGE IN ACTIVELY SENSITISED BROWN NORWAY RATS

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Although airway hyperresponsiveness (AHR) is a defining feature of asthma, its mechanistic basis remains poorly understood (O'Connor et al., 1999). We here define the changes in sensitivity to direct (methacholine [MCh] and 5-HT) and indirect (adenosine [Ado] and bradykinin [BK]) bronchospasmogens at different times following allergen challenge in actively sensitised (AS) Brown Norway (BN) rats and relate these to the inflammatory status of the lung.

BN rats (ca. 200 g) were sensitised to ovalbumin (OA) and prepared for recording of blood pressure, heart rate and pulmonary function as previously described (Hannon et al., 1999). In separate animals (n=10 per group), differential cell counts in bronchoalveolar lavage fluid (BALF) were made using an automated cell analysing system (Cobas Helios). Eosinophil peroxidase (EPO) activity and protein levels in BALF were determined using standard photometric assays. For histology, lungs (n=15) were formalin-perfused, sectioned (3 µm) and stained with haematoxylin and eosin or May Grundwald Giemsa.

Bronchoconstrictor responses to i.v. Ado, 5-HT, MCh and BK were established in AS animals 3 or 24 h after i.t. challenge with saline (0.2 ml) or OA (0.3 mg kg^{-1}). 3 h following OA the response to Ado was markedly augmented whereas responses to 5-HT and BK were little changed. At 24 h the sensitivity to Ado, returned to that of control animals and responses to 5-HT and BK were significantly augmented (Figure 1). Responses to MCh (3, 10 & 30 µg kg^{-1} i.v.) were not affected by challenge with OA at either time point. 3 h following OA challenge there were minimal changes in BALF markers of inflammation (Hannon et al., 1999). 24 h following OA challenge, there were marked increases in the numbers of eosinophils (7.7-fold), neutrophils, (16.3-fold), macrophage (2.4-fold) and lymphocytes (5.3-fold) as well as EPO activity (32.9-fold) and protein (4.2-fold) in the BALF. Histological analysis. of lungs taken from saline-challenged rats showed minimal signs of inflammation. 3 h following OA challenge, 11 of 15 lung samples showed some degree of perivascular oedema and polymorphonuclear leukocyte (PMNL) infiltration. 24 h following OA challenge the lungs manifested a severe inflammatory response characterised by extensive multifocal granuloma formation, perivascular PMNL infiltration and oedema.

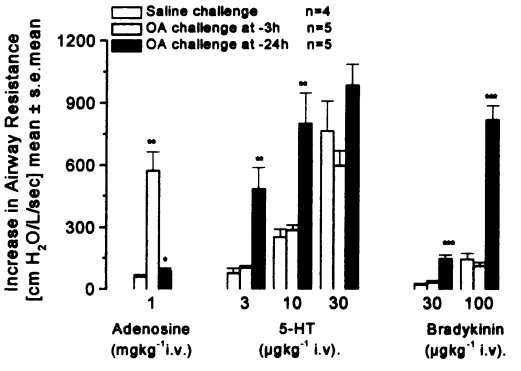

Figure 1: Bronchoconstrictor responses to adenosine, 5-HT and bradykinin in actively sensitised BN rats challenged 3 or 24 h previously with OA or saline. Means ± s.e. mean are presented. *p<0.05, **p<0.01, ***p<0.001 indicates significant difference between saline and OA challenged animals.

Thus, changes in the sensitivity of the airways to bronchospasmogens following allergen challenge in sensitised BN rats varies widely depending on the spasmogen used. In addition, there appears to be no simple relationship between the intensity of lung inflammation and AHR to either direct or indirect acting spasmogens in this model.

Hannon, J.P. et al. (1999). Br. J. Pharmacol., 127, 76P.
O'Connor, B.J. et al.(1999). Trends Pharmacol. Sci., 20, 9-11.

118P PHARMACOLOGY OF NKP608, A NOVEL SELECTIVE NEUROKININ-1 RECEPTOR ANTAGONIST WITH ORAL ACTIVITY

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Tachykinins are sensory neuropeptides released from neurones located in both the central and peripheral nervous systems. Their biological effects are mediated via NK_1, NK_2 and NK_3 receptors which show preference for substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), respectively (Barnes et al. 1997). The present report describes the pharmacological properties of NKP608, (2R,4S)-N-[1-{3,5-Bis(trifluoromethyl)-benzoyl}-2-(4-chloro-benzyl)-4-piperidinyl]-quinoline-4-carboxamide), a novel, selective and non-peptide, NK_1 receptor antagonist.

Affinities to cloned human (hr) NK_1, NK_2 and NK_3 receptors expressed in CHO cells were measured in radioligand binding assays using ^{3}H-Sar^{9}SP, ^{125}I-NKA and ^{125}I-MePhe^{7}NKB, respectively. Tracheal rings from male Dunkin Hartley (DH) guinea-pigs (ca. 500g) were set up for recording isotonic tension changes (Kenakin, 1999). NKP608 was incubated with the tissues for 15 min and Sar^9, Met(O$_2$)^{11}-SP or (βAla^8)-NKA(4-10), were applied cumulatively. The tissues were washed repeatedly during 1h and the agonist concentration-response curves re-established. In vivo, male DH guinea-pigs (ca. 550g) were anaesthetized (phenobarbitone 100mgkg^{-1} and pentobarbitone 30mgkg^{-1} i.p.), mechanically ventilated and the jugular vein was cannulated. Airway resistance was calculated and dose-response curves were constructed to Sar^{9}SP or (Ala^5,β-Ala^8)-α-NKA(4-10). NKP608 or vehicle were given i.v. (5 min) or orally (2 or 12h) prior to the agonist dose-response curve. Mean values ± s.e. mean are presented and significance is defined by p values < 0.05.

NKP608 bound selectively and with high affinity to hrNK_1 receptors (pKi, 8.96±0.02) compared to hrNK_2 and hrNK_3 receptors (pKi values of 6.1±0.01 and 6.25±0.02, respectively). NKP608 was a potent, non surmountable antagonist at the NK_1 receptors of guinea pig trachea (pD'$_2$, 7.7±0.1, n=4). Blockade increased following repeated washing of the tissues during 1 h. At the NK_2 receptor, NKP608 was less potent but the antagonism was surmountable (pA$_2$ 6.0±0.1, n=4) and could be reversed by repeated washing. In vivo, NKP608 (0.1 and 1 mgkg^{-1}, i.v. -5 min) significantly shifted the dose-response curve to Sar^{9}SP 8- and 36-fold to the right, respectively (n=3-4). Orally, NKP608 (0.1 and 1 mgkg^{-1}, -2h), significantly antagonised bronchoconstrictor responses to Sar^{9}SP 5- and 34-fold, respectively (n=3-4). Blockade of NK_1-mediated bronchoconstriction by 1 mgkg^{-1} NKP608 following oral administration, persisted for at least 12 h (27-fold shift, n=5). In contrast, NKP608, at doses up to 10 mgkg^{-1} given p.o. had no effect on bronchoconstrictor responses to (Ala^5,-Ala^8)-NKA(4-10) 2h prior to the agonist (n=5-6).

These data establish NKP608 as a potent and selective antagonist at tachykinin NK_1 receptors both in vitro and in vivo. The long-lasting and potent activity by the oral route renders NKP608 particularly suitable for exploring the role of NK_1 receptors in (patho)physiological functions.

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119P DIFFERENTIAL EFFECT OF THE PROTEIN KINASE C INHIBITORS Go-6976 AND Go-6983 ON SIGNALLING TO THE NUCLEUS FROM THE HUMAN INSULIN RECEPTOR

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We have previously provided evidence for an involvement of protein kinase C μ (PKCμ) in signalling from adenosine A_1-receptors to the nucleus (Hill & Hill, 2000). Since insulin receptor-mediated activation of the MAP kinase cascade is partially sensitive to inhibition by pertussis toxin (Luttrell et al., 1995), we have investigated whether insulin receptor signalling to the nucleus also involves this PKC isoform.

Studies were performed in CHO-TA1 cells, which express the human insulin and adenosine A_1- receptors, (Presland et al., 1998) following transfection with pGL3-basic (Promega) containing the full c-fos promoter (Hill & Hill, 2000). Cells were grown in DMEM-F12 medium containing 10% foetal calf serum and 2mM glutamine, under an atmosphere of 5% CO_2 in humidified air. Cells were serum-starved for 24h prior to agonist stimulation for 5h. C-fos promoter-regulated luciferase activity was then measured using the Packard Luclite™ assay kit following the manufacturer's instructions.

Insulin (log EC_{50} −10.4 ± 0.3; E_{MAX} 21020 ± 4689 relative light units, RLU; mean ± s.e.mean, n=13) and the A_1-agonist N^6-cyclopentyladenosine (CPA; log EC_{50} −8.2 ± 0.7; E_{MAX} 4030 ± 2093 RLU; n=16) both stimulated luciferase expression. The maximal response to insulin was reduced by 58.4 ± 5.8 % (n=4) by 20h treatment with pertussis toxin (100ng/ml), while that to CPA was abolished. The non-selective PKC inhibitor Ro-31-8220 (10μM) completely inhibited the response to insulin (n= 3). Pretreament with 1μM phorbol-12,13-dibutyrate for 20h had no significant inhibitory effect on the concentration-response curve to insulin (n=3), thus ruling out an involvement of those PKC isoforms which are downregulated in CHO-K1 cells by this procedure (i.e. PKCα, PKCδ and PKCε; Hill & Hill, 2000). Incubation with Go-6976 (1μM), an inhibitor of PKCα and PKCμ (Martiny-Baron et al., 1993; Gschwendt et al., 1996), reduced the maximal response to insulin by 66.3 ± 6.6% ($P<0.01$, Student's t-test) without significant effect on the log EC_{50} value (n=3). Go-6976 produced a maximal inhibition of the response to 10nM insulin of 80.3 ± 11.6% (n=6; log IC_{50}= -6.2 ± 0.1). In contrast, 1μM Go-6983, a potent inhibitor of most PKC isoforms apart from PKCμ (Gschwendt et al., 1996), produced no significant change in E_{MAX} or log EC_{50} for insulin (n=3).

These data suggest that Go-6976 and Go-6983 have a differential effect on insulin-stimulated gene expression, which is similar to that previously reported for the adenosine A_1-receptor (Hill & Hill, 2000). The results of this study therefore point to a role of PKCμ in this response to insulin.

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120P INHIBITION OF CREB/CRE-DIRECTED GENE TRANSCRIPTION BY DESMETHYL IMIPRAMINE (DMI) IN A CHO-K1 CELL LINE.

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It has previously been shown that a number of compounds exhibiting antidepressant activity can induce changes in gene transcription. Schwaninger *et al.* (1995) demonstrated inhibition of cyclic AMP response element binding protein (CREB)/cyclic AMP response element (CRE)-directed gene transcription in HIT and PC-12 cells. It was proposed that antidepressant-induced changes in transcription were mediated by inhibition of Ca^{2+} channel function. The present study aimed to investigate the effects of desmethylimipramine (DMI) on CREB/CRE-directed gene transcription in a non-excitable cell type. Here we demonstrate the effect of DMI on β_2-adrenoceptor-mediated gene transcription in CHO cells transfected with the human β_2-adrenoceptor.

A Chinese hamster ovary (CHO-K1) cell line stably transfected with the human β_2-adrenoceptor (β_2-AR) and the secreted placental alkaline phosphatase (SPAP) reporter gene, was used (McDonnell et al., 1998). For SPAP assays, cells were grown in 24 well plates for 24 hours and were then serum starved for a further 24 hours. DMI was added 18 hours prior to agonist addition. CREB/CRE-driven gene transcription was measured as SPAP production, using the SPAP reporter gene assay described by McDonnell *et al.* (1998). The data presented represent the means ± s.e.mean of at least 3 separate experiments

Isoprenaline stimulated SPAP production in a concentration-related fashion (EC_{50} = 3.4 ± 0.4 nM). 18 hour preincubation with 1μM DMI produced no change in basal SPAP production, but produced a small, but significant change in EC_{50} (7.8 ± 0.8 nM; $p<0.001$, unpaired t test). This was accompanied by a 50% decrease in E_{max} (from 10.7 ± 1.5 to 5.4 ± 0.7 μU/ml, $p<0.05$, in the absence and presence of DMI respectively). DMI inhibited the SPAP response to 100nM isoprenaline in a concentration-dependent manner, IC_{50} = 16.7 ± 0.17 μM. Forskolin (1μM) also stimulated SPAP production and this response was inhibited by increasing concentrations of DMI (IC_{50} = 46μM).

The data presented demonstrate that 18h treatment with DMI has an inhibitory effect on β_2-adrenoceptor-mediated CREB/CRE-directed gene transcription in non-excitable CHO-K1 cells. Furthermore, since DMI had a similar effect on forskolin-stimulated gene transcription, it seems likely that this effect occurs at a post-β_2-adrenoceptor level, although the precise locus of action is still unclear.

JKR holds an MRC studentship

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121P ABERRANT CAVEOLIN-1 EXPRESSION IN PSORIASIS: A SIGNALLING HYPOTHESIS

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Caveolin-1 is a 22kd integral membrane protein localised to the cytoplasmic leaflet of smooth coated membrane invaginations termed caveolae. Such structures have been implicated in a diverse number of physiological processes including the compartmentalisation and mediation of signal transduction pathways. Caveolin-1 serves a direct regulatory role in signal transduction events, acting as a 'molecular brake' for selective signalling cascades. Specfically, caveolin-1 functions as a kinase inhibitor by recognising a conserved caveolin-1 binding motif in the catalytic domain of many known kinases. This recognition extends to tyrosine kinase receptors such as the epidermal growth factor receptor (E-GFR) and its down stream effector molecules Ras and, MAP kinase (p42/p44) (Couet et al., 1997). The key molecular events leading to the pathological changes observed in psoriasis remain to be fully elucidated. However, its is proposed that psoriasis a non-malignant hyperproliferative skin disorder occurs as a result of aberrations in signal transduction mechanisms within cells of the epidermis leading to hyperproliferation and abnormal keratinocyte differentiation. Increased activities of EGF-R (King et al 1990), Ras, and MAP kinase (Dimon-Gadal et al 1998) have all been independently reported to be up-regulated in psoriasis and implicated in the molecular basis of it's pathology. Further, signalling down the EGF-R⇒ p42/p44 pathway is associated with the proliferation of many cell types including keratinocytes. Therefore we hypothesise that aberrations in the expression of caveolin-1 may occur in the psoriatic epidermis thus leading to the increased activity of the above signalling pathways thus contributing to the hyperproliferation of epidermal keratinocytes that is observed in psoriasis.

A retrospective immunocytochemical study was undertaken upon archival skin material obtained from the Department of Pathology, University of Wales College of Medicine (UWCM). Ten skin specimens exhibiting normal morpholgy and ten specimens with a clinical diagnosis of psoriasis were selected, all were matched for sex and age. All psoriatic subjects exhibited the pathological features of psoriasis such as elongated retes, increases in the number of mitotic bodies, parakeratosis, marked angiogenesis within the dermis and a profound inflammatory dermal infiltrate. Paraffin wax sections (5μm) were cut, rehydrated and incubated overnight at 4°C with anti-caveolin-1 antibody (1:20). The sections were then probed with a secondary horseradish peroxidase antibody with diaminobenzadine serving as the colormetric substrate. Slides were dehydrated and coverslipped for light microscopic analysis. A specific caveolin-1 immunoperoxidase brown stain could be seen throughout the entire level of the epidermis in all seven normal subjects although stain of greater intensity was noted in the proliferative basal layers and in the uppermost differentiated granular layers. The staining in all normal subjects was of similar intensity. However, a marked reduction in the level of caveolin-1 expression was observed in the epidermis of all psoriatic subjects tested. The down-regulation of caveolin-was particularly evident within the hyper-proliferative basal layer and elongated retes of the psoriatic epidermis. Caveolin-1 expression appeared unaltered in the uppermost more differentiated non-proliferating regions. Interestingly, intense caveolin expression was observed in mitotic bodies of the psoriatic epidermis in all subjects examined. Appropriate isotypic and omission controls demonstrated no staining thus authenticating the above results. Consistent with our hypothesis this study is the first to demonstrate aberrations in the expression of caveolin-1 in psoriasis and may represent a novel therapeutic target for pharmacological intervention.

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122P BLOOD-BRAIN BARRIER PERMEABILITY ASSESSMENT OF STEREOSELECTIVE OPIOID ANALOGUES

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[D-Penicilamine2,5] enkephalin (DPDPE), a delta opioid selective peptide analogue of met-enkephalin, has been shown to enter the brain via a saturable mechanism (Williams *et al.*, 1996). However, the blood-brain barrier (BBB) generally inhibits the passage of peptides from blood to brain via it tight endothelial cell junctions. Here we studied DPDPE, modified by trimethylating the beta-carbon of the phenylalanine (TMP), resulting in four stereoselective conformations: [(2S,3S)-Phe-DPDPE], [(2R,3R)-Phe-DPDPE], [(2R,3S)-Phe-DPDPE], and [(2S,3R)-Phe-DPDPE] (Witt *et al.*, 2000).

Octanol/buffer distribution analysis showed enhanced lipophilicity of all TMP forms, with a 6-fold enhancement associated with (2S,3S)-TMP. *In situ* brain perfusion showed a 1.6-fold ($p < 0.01$) increase in ratio of brain uptake for (2S,3S)-TMP and a 1.5-fold ($p < 0.01$) decrease in uptake for (2R,3R)-TMP. P-glycoprotein affinity was shown *in situ* for the parent and (2S,3S)-TMP ($p < 0.01$). Protein binding capacity of the TMP compounds showed (2R,3S)-TMP and (2S,3R)-TMP with lowest degree of protein binding ($p < 0.01$), and (2S,3S)-TMP and (2R,3R)-TMP with comparable affinities to DPDPE (control). Analgesia, via i.v. administration, showed significantly reduced ($p < 0.01$) end effect and time course for (2R,3R)-TMP, (2R,3S)-TMP, and (2S,3R)-TMP as compared to DPDPE.

Results, determined via ANOVA followed by Newman-Keuls post hoc test, indicate that the methylation of the Phe pharmacophore of DPDPE significantly effects BBB permeability and end analgesic effect, thus demonstrating that topographical modification in a conformationally restricted peptide can significantly modulate potency, lipophilicity, and BBB permeability resulting in a change of bioavailability. This analysis provides insight for future peptide drug design.

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123P CHARACTERISATION OF A YEAST REPORTER ASSAY FOR THE HUMAN ADENOSINE A_1 RECEPTOR

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The yeast *Saccharomyces cerevisiae* utilises a G-protein coupled receptor (GPCR) signalling pathway to regulate pheromone responses and hence mating (Brown *et al.*, 2000). The pheromone response pathway has been adapted to allow measurement of signalling of heterologously expressed GPCRs (Brown *et al.*, 2000), in which pathway activation induces expression of a *lacZ* reporter gene. Such technology provides a simple, inexpensive assay for pharmaceutical screening of ligands for GPCRs. In this study, we have utilised this technology to generate and pharmacologically characterise a yeast β-galactosidase reporter assay for the human adenosine A_1 receptor, using a range of adenosine agonists and antagonists (Sheehan *et al.*, This meeting).

Yeast expressing the human A_1 receptor ($1.6x10^4$ cells/well) were incubated in 384-well plates for 18h at 30°C with compounds and the fluorescent β-galactosidase substrate fluorescein digalactoside (20μM). Assay volume was 80μl. Fluorescence was measured using a Wallac Victor. Data generated in the yeast assay were compared to that from cAMP reporter and [^{35}S]-GTPγS binding assays in CHO-A_1 cells (Sheehan *et al.*, This meeting).

The A_1 receptor was expressed in yeast strains containing one of a range of yeast Gpa1/mammalian G_α chimaeras (Brown *et al.*, 2000). Optimum responses to N^6-ethylcarboxamidoadenosine (NECA) were obtained using the Gpa1/$G_{\alpha 16}$ chimera (data not shown), which was used in all subsequent studies. While agonists were less potent in yeast compared to the CHO cAMP reporter assay (Table 1), there was a good correlation (r = 0.95, slope = 0.90, n=12) between equipotent molar ratios (EMR) in yeast and CHO cell assays. With the exception of GR162900 (intrinsic activity = 0.83 ± 0.20, n=3), all agonists were full agonists in the CHO reporter assay. However, the rank order of intrinsic activity of agonists in yeast was identical to that in the [^{35}S]-GTPγS binding assay in CHO-A_1 membranes (Table 1). The adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) caused concentration-related shifts in concentration-response curves evoked by both NECA and the selective A_1 agonist, GR79326, with pK_B values of 9.03 ± 0.10 and 9.11 ± 0.08, respectively (n=3). Schild slopes were not significantly different from unity ($P < 0.05$). These affinities are similar to those obtained in CHO-A_1 cells (Browning *et al.*, This meeting).

Table 1. Comparison of potency, EMR and intrinsic activity values

	pEC_{50}		EMR value		Intrinsic activity	
	Yeast	CHO reporter	Yeast	CHO reporter	Yeast	[^{35}S]-GTPγS
NECA	7.8	8.8	1.0	1.0	1.00	1.00
GR79236	7.6	8.7	1.7	1.6	0.97	1.05
CGS21680	5.1	6.3	709	591	1.01	n.d.
IB-MECA	6.3	7.4	40	66	0.94	n.d.
GR190178	6.5	7.6	31	15	0.85	0.84
GR161144	6.6	7.6	19	15	0.67	0.55
GR162900	7.2	8.0	5.7	3.0	0.10	0.14

n.d. = not determined. pEC_{50} SEM < 0.20. i.a. SEM < 0.10. n=3-10

We have developed a simple, robust 384-well assay in yeast for adenosine A_1 receptor agonists and antagonists. The pharmacology of both agonists and antagonists was identical to that obtained in a mammalian cell line. This assay will be very useful for pharmaceutical screening for GPCR agonists and antagonists.

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124P THE EFFECT OF RECEPTOR EXPRESSION AND TEMPERATURE ON AGONIST POTENCY AT THE $P2X_7$ RECEPTOR ASSESSED USING AN INDUCIBLE EXPRESSION SYSTEM

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The $P2X_7$ receptor is an ATP-gated cation channel (Surprenant *et al.*, 1996), whose pore can dilate to allow permeation of molecules with MW of < 800Da (large-pore formation). We have generated an inducible-expression system for this receptor to address two issues. First, the potency of 3'-*O*-(4-benzoylbenzoyl)-ATP (BzATP) at endogenous $P2X_7$ receptors of THP-1 cells is less that at the recombinant receptor expressed in HEK293 cells (Kidd *et al.*, 2000). Since receptor expression in THP-1 cells is lower than in HEK293 cells, this suggests that differences in receptor expression may affect potency. Second, at the recombinant receptor, BzATP-stimulated large-pore formation occurs at 4°C (A.D. Michel, unpublished data). However, in those studies it is difficult to exclude the possibility that large-pore formation occurred during passage of the cells when ATP should be released. Both of these issues can be studied further using the ecdysone inducible expression system (No *et al.*, 1996).

Stable cell lines expressing an ecdysone-inducible human $P2X_7$ receptor were established in HEK293 cells. Expression was induced by incubating cells with ecdysone receptor agonists (muristerone and ponasterone produced similar results) for 24-48h. Expression was determined in fixed cells using a human $P2X_7$ receptor monoclonal antibody (mAb, Kidd et al., 2000). $P2X_7$ receptor function was studied electrophysiologically or by measuring BzATP-stimulated influx of the fluorescent, DNA binding dye, ethidium (Michel et al., 2000). Data are the mean±s.e.mean of 5 experiments. Statistical significance was determined using a 1 way ANOVA followed by a post hoc Tukey's test.

$P2X_7$ receptor expression was detected using the mAb 24h after induction with muristerone. Expression was detected in either a proportion of cells or in all cells after 0.1μM and 1μM muristerone, respectively. After 10μM muristerone, the intensity of staining was increased. Studies are in progress to quantify expression levels using the mAb. Following a 48h incubation with ponasterone (1–10μM), BzATP evoked whole cell inward currents and stimulated ethidium accumulation. The maximal rate of BzATP-stimulated ethidium accumulation increased significantly with increasing ponasterone concentration ($t_{1/2}$ values using a maximal concentration of BzATP were 12.7±2.2 and 3.4±0.3 min after 1 and 10μM ponasterone, respectively; $P<0.05$). The pEC_{50} values for BzATP were 5.7±0.08, 5.8±0.07 and 6.0±0.08 after 1, 3 and 10μM ponasterone respectively (values not significantly different; $P>0.05$). Following induction with 10μM ponasterone (48h), BzATP-stimulated ethidium accumulation was detected when cells were subsequently examined at 4°C although the maximal rate of BzATP-stimulated ethidium accumulation ($t_{1/2}$=31+11min) was slower than at 22°C and the BzATP potency lower (pEC_{50} 4.9±0.06). At 37°C the pEC_{50} for BzATP was 6.6±0.12 (significantly different from the values at 22°C and 4°C; $p<0.05$).

This study has shown that transient expression of $P2X_7$ receptors can be achieved by use of the ecdysone-inducible expression system. The system has been used to study the effect of receptor expression level on the function of this receptor and the results suggest that the differences in agonist potency between THP-1 cells and transfected HEK293 cells are not due to differences in receptor expression. Agonist potency at the $P2X_7$ receptor increased with increasing temperature but $P2X_7$ receptor-mediated large-pore formation could be detected at 4°C. This latter observation suggests that large-pore formation does not require recruitment of additional sub-units to existing $P2X_7$ receptors since the mobility of proteins within the membrane would be restricted at 4°C.

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125P INHIBITION OF RAT SOLUBLE CATECHOL-*O*-METHYLTRANSFERASE BY BIA 3-202, A REVERSIBLE TIGHT-BINDING INHIBITOR

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A tight-binding inhibitor exerts an inhibitory effect on an enzyme-catalysed reaction at a concentration comparable to that of the enzyme. Kinetic studies reported a tight-binding behaviour for entacapone (Schultz & Nissinen, 1989) and tolcapone (Borges *et al*, 1997), two new inhibitors of catechol-*O*-methyltransferase (COMT). In the present study a similar tight-binding profile is described for BIA 3-202 (1-[3,4-dihydroxy-5-nitrophenyl]-2-phenyl-ethanone) on rat liver soluble COMT (S-COMT). S-COMT was isolated from saline perfused livers, obtained from pentobarbitone (60 mg kg^{-1}) anaesthetised male Wistar rats 60 days old (Harlan, U.K.). Tissues were homogenised in 5 mM phosphate buffer, pH 7.8, and centrifuged at 15,000 *g* for 20 min at 4°C. The high-speed supernatants (100,000 g for 60 min at 4°C) were used as the soluble fraction of liver COMT. COMT activity was evaluated by the ability to methylate adrenaline to metanephrine in the presence of a saturating concentration (500 µM) of the methyl donor (S-adenosyl-L-methionine) as described by Borges *et al* (1997). Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. A Michaelis-Menten behaviour was observed for S-COMT with K_m and V_{max} values of 412±82 µM and 62±6 nmol mg protein^{-1} h^{-1}, respectively. Reversibility was proved when the inhibitory effect of 100 nM BIA 3-202 (40±1 % reduction) was completely reverted by gel filtration of samples through Sephadex G-25 columns. The reaction velocity of S-COMT in the presence of varying inhibitor and enzyme concentrations (Ackerman-Potter plot) increases progressively with increasing enzyme concentrations. Also, the velocity curve is parallel to the control curve at sufficiently high enzyme concentrations, demonstrating the tight-binding nature of the inhibition. The catalytic number (K_{cat}=4.5±0.4 min^{-1}) and the molar equivalency (M_{eq}=112.2±12.4 nM) of the enzyme were determined from the Ackerman-Potter plot obtained in the absence and the presence of 50 and 100 nM BIA 3-202 (figure 1). These results are in agreement with the finding that IC_{50} values for BIA 3-202 were dependent on the amount of COMT (table 1).

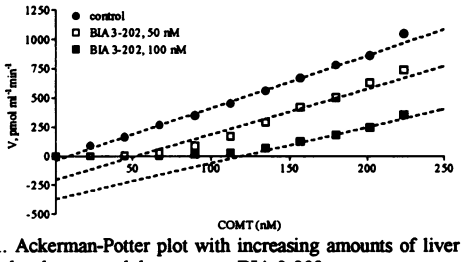

Figure 1. Ackerman-Potter plot with increasing amounts of liver S-COMT in the in the absence and the presence BIA 3-202.

Table 1. IC_{50} values for BIA 3-202 using increasing concentrations of rat liver S-COMT.

S-COMT (nM)	45	112	224	448
IC_{50} (nM)	12 (5, 27)	44 (11, 174)	73 (35, 152)	124 (47, 324)

Due to the tight-binding nature of this compound, IC_{50} values should not be used as indicators of the inhibitory potency. For such a reason, a K_i value (16.5±1.6 nM) was calculated by plotting IC_{50} values obtained using a 20 min pre-incubation period against enzyme concentration and estimating the intercept at the IC_{50} axis. The results obtained in the present study indicate that BIA 3-202 behaves as a reversible tight-binding COMT inhibitor.

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126P INCREASED INTRACELLULAR CONCENTRATION OF P-GLYCOPROTEIN SUBSTRATES IN MULTIDRUG RESISTANT CELLS BY MOLECULES ISOLATED FROM GRAPEFRUIT OIL

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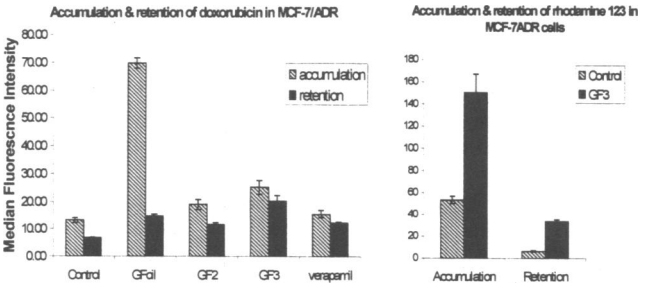
A major problem associated with cancer chemotherapy is the ability of tumor cells to develop resistance to the cytotoxic effects of anti-cancer drugs with unrelated chemical structures and mechanisms of action. This phenomenon is referred to as multidrug resistance (MDR). The best-documented mechanism for MDR is correlated with the expression of P-glycoprotein (P-gp). There is a need for effective agents which may be used to re-sensitize MDR cells to therapeutic agents. Recently, more attention has been paid to the influence of grapefruit (GF) juice on the metabolism of many drugs. It has been reported that GF juice increases the plasma concentrations of orally administrated drugs through inhibition of Cytochrome P450 3A4 [1]. Grapefruit oil is extracted from the peel, and contains some of the GF juice ingredients concentrated up to 3 order of magnitude. The purpose of this work was to study the effect of GF oil and its components on the intracellular kinetics of P-gp substrates, in a selection of resistant and sensitive cancer cell lines. Isolation and purification of the components from GF oil was achieved by column chromatography using silica gel 60. The individual components were identified by ^{1}H and ^{13}C NMR and by mass spectroscopy and confirmed by comparison with the synthesized compounds. Validation of P-gp and MDR1 mRNA expression in the resistant cell lines, KBV1 and MCF-7/ADR, was confirmed by flow cytometric immunofluorescence labeling using MRK16 monoclonal antibody against P-gp; and reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers. Accumulation and retention of intracellular rhodamine 123 and doxorubicin P-gp substrates in drug resistant and sensitive cell lines were examined by flow cytometry.

MCF-7/ADR cells treated with GF oil achieved 5.4 and 2.7 greater intracellular doxorubicin concentration than untreated cells compared to cells treated with high verapamil concentration (40µM) which achieved 1.18 and 1.8 in the [A] and [R] phase, respectively ($P<0.05$, ANOVA). Moreover, MCF-7/ADR treated cells with GF3 extract, achieved 2.9 and 5.3 greater intracellular rhodamine 123 concentration than untreated cells ($P<0.05$, ANOVA).

In conclusion, we isolated some molecules from grapefruit oil that can circumvent multidrug resistance, via increasing the drug transport into the cell.



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127P OXIDATIVE STRESS IMPAIRS INSULIN UPTAKE IN BOVINE AORTIC ENDOTHELIAL CELLS

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Insulin uptake and subsequent transport across the endothelium is a regulated and possibly rate-limiting step in insulin delivery to insulin target tissues (Jansson *et al.*, 1993). Defects in the capacity of the endothelium to promote insulin transport has been reported in animal models of insulin resistance (Miles *et al.*, 1998). As oxidative stress has been linked to insulin resistant disease states and a beneficial effect of anti-oxidant treatment has been documented (Gopaul *et al.*, 1995; Paolisso *et al.*, 1993), we have tested the notion that a pro-oxidant challenge using xanthine oxidase/hypoxanthine (XO/HX) on endothelial cells *in vitro* can alter insulin uptake into the endothelium.

Following 2h exposure to XO/HX (10mU ml^{-1}/1mM), bovine aortic endothelial cells (BAEC) were incubated with monoiodinated human [Tyr^{A14}]-insulin (1.85KBq ml^{-1}; 74TBq $mmol^{-1}$) for 18-20h at 4°C with or without 10^{-5}M insulin. Unbound [Tyr^{A14}]-insulin was washed away and the cells incubated at 37°C for 5-60min. The medium was analyzed for TCA-precipitable and TCA-soluble radioactivity. Cell surface associated [Tyr^{A14}]-insulin was removed by washing the cells with acetic acid (pH 3) and acid-releasable radioactivity quantitated. Cells were solubilized with 1M NaOH. Data are expressed as mean±s.e. mean and statistical difference between means tested by unpaired *Student's* t-test.

Following 15min incubation at 37°C, cell surface associated [Tyr^{A14}]-insulin declined to 10-20% of its initial value (fig.1.a). In contrast, XO/HX (AUC=427±109, n=3) markedly reduced [Tyr^{A14}]-insulin endocytosis relative to control (AUC=1245±209, n=3; P<0.05, fig.1.b). The amount of [Tyr^{A14}]-insulin equilibrating with the cells was not altered by XO/HX exposure. XO/HX treatment (AUC=3460±455, n=3 vs. AUC(control)=2660±354, n=3) elicited a modest increase in the release of non-TCA-precipitable [Tyr^{A14}]-insulin into the medium although this was not statistically significant (fig 1.c). Insulin degradation was most pronounced in control cells (AUC=297±75, n=3 vs. AUC(XO/HX)=115±30, n=3; P=0.09) reaching 11±5% of surface bound [Tyr^{A14}]-insulin after 60min (fig 1.d). Cell viability was unaffected by XO/HX treatment.

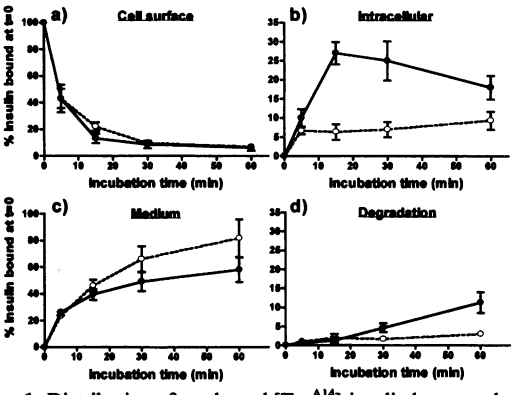

Figure 1: Distribution of pre-bound [Tyr^{A14}]-insulin between the cell surface and intracellular compartments in control (●) and XO/HX treated (O) BAEC. All values are related to ng protein ml^{-1} and expressed as % of control (n=3).

Our data demonstrate that a pro-oxidant insult can significantly diminish insulin internalization in endothelial cells *in vitro*. Since this was not due to differences in total insulin binding, we propose that oxidative stress is able to impair insulin uptake which may be of relevance in the distribution of circulating insulin in disease states associated with oxidative stress, such as type II diabetes.

Gopaul N.K., Änggård E.E., Mallet A.I. *et al.* (1995) *FEBS Letters* 368, 225-229.
Jansson P.E., Fowelin J.P., von Schenck H.P. *et al.*(1993) *Diabetes* 42, 1469-1473.
Miles P.D.G., Li S., Hart M. *et al.* (1998) *J. Clin. Invest.* 101, 202-211.
Paolisso G., D'Amore A., Giugliano D. (1993) *Am. J. Clin. Nutr.* 57, 650-656.

128P DIFFERENTIATION OF Ca^{2+} MOBILIZATION MEDIATED BY PHARMACOLOGICAL AND PHYSIOLOGICAL DOSES OF VITAMIN D_3 IN HOS CELLS IN SUSPENSION CULTURE

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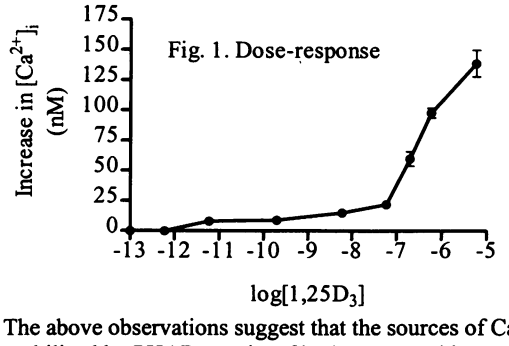
The non-genomic effects of 1,25$(OH)_2$ vitamin D_3 (1,25D_3) on intracellular Ca^{2+} level ($[Ca^{2+}]_i$) have been studied extensively (Civitelli et al. 1990; Kim et al. 1996). However, the dose-response relationship in reference to the mechanisms underlying the Ca^{2+} mobilization has yet to be delineated. Therefore, studies were undertaken to determine pertinent parameters for differentiating the 1,25D_3 dose dependency of the mechanisms by which calcium is mobilized from different compartments.

HOS cells grown in DMEM for 3 days were used. Fura 2/AM loaded cells were suspended in 1 ml of basal salt solution containing 1 mM Ca^{2+}, and real time changes in emission intensities at excitation wave lengths of 340 and 380nm were measured at 1 second intervals in SPEX spectrofluorometer before and after the additions of 1,25D_3 and other reagents.

The dose-response curve showed biphasic $[Ca^{2+}]_i$ response (Fig. 1). The first, physiological phase spans from 10^{-12} M to 10^{-9} M, followed by the second , pharmacological phase, from 10^{-8} M to 10^{-5} M 1,25D_3. Sequestration of extra-cellular Ca^{2+} with 4 mM EGTA reduced the $[Ca^{2+}]_i$ response in the pharmacological dose (PHAD) phase by 25%, whereas the same pretreatment did not affect the response to physiological dose (PHYD). Nifedipine (10^{-5} M) pretreatment of cells reduced the PHAD effect by c.a. 50%, while the same pretreatment did not affect PHYD effect. The optimum pH for $[Ca^{2+}]_i$ response to PHAD was 7.4, and at pH 6.4 or 8.4, the responses were reduced by c.a. 50% of the value at pH 7.4. Unlike the PHAD effect, the response to PHYD at pH 8.4 was significantly higher than that at pH 7.4.



The above observations suggest that the sources of Ca^{2+} mobilized by PHAD consist of both extra- and intra-cellular compartments, while that by PHYD is mainly from intra-cellular storage sites. The findings suggest further that numbers and types of receptors participate in PHAD phase are different from those in PHYD phase.

Supported by NIH grants DK49728 and AR32087

Civitelli et al. (1990). Endocrinology. 127:2253-2262.
Kim et al. (1996). Endocrinology. 137:3649-3658.

129P THE EFFECTS OF PRESSURE ON CELL SIGNALLING

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Pressure has a profound inhibitory effect on the ionotropic receptor for glycine that is entirely consistent with the pharmacologic analysis of the action of pressure *in vivo* (Roberts *et al.*, 1996). However, pharmacological experiments *in vivo* indicate that the neurological processes involving the metabotropic receptors for both dopamine (DA1 & DA2) and serotonin (5-HT1b, 5-HT2c) are also affected by pressure (Kriem *et al.*, 1996; 1998).

The aim of this study was to investigate whether pressure affects intracellular signalling processes.

The effect of pressure on an intracellular pathway involving Gq, PLC and IP3 activation, was studied by expressing receptors for the bacterial protein N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) in *Xenopus* oocytes. Additional experiments tested the effect of pressure on oocytes stimulated with NaF; believed to directly activate Gs and stimulate AC. Oocytes (stage V) were injected with 50nl of mRNA solution (1µg/µl) and maintained overnight in modified Barth's medium containing penicillin (50 U/ml) and streptomycin (50 µg/ml) at 18°C to allow expression of receptors. Prior to use all oocytes were collagenase treated in Ca^{2+}-free Barth's to remove follicular and thecal cells. Receptor function was measured using two-electrode voltage clamp and an apparatus designed to fit in the pressure chamber (Roberts *et al.*, 1996).

Oocytes were perfused with frog Ringer (NaCl 120, KCl 2, CaCl2 2, Hepes 10; mM. pH 7.4) and clamped at -70mV for electrical recordings. Oocytes were stimulated by 30s perfusion with either fMLP (100nM) or NaF (40mM) in Ringer. Recordings were made at atmospheric pressure, and at 5 MPa and 10 MPa with pressure applied using helium.

Stimulation with fMLP produced a biphasic inward current. The fast component reversed at –25mV and was blocked by SITS suggesting it is carried by Cl^-. The slow component showed strong inward rectification, was Ca^{2+} dependent and blocked by Cd^{2+}, TEA and 4AP suggesting activation of a Kir channel. Stimulation with NaF produced a monophasic inward current that showed voltage dependence suggestive of a Na^+ channel. The fast conductance stimulated by fMLP was not affected by pressure; average currents relative to control were 0.69 +/- 0.17 (s.e.m.,n=5) and 0.90 +/- 0.34 (s.e.m., n=5) at 5 and 10 MPa, respectively. However, the slow conductance was inhibited by pressure; average relative currents 0.50 +/- 0.1 (s.e.m., n=5, Student's t-test p ~ 0.05) and 0.62 +/- 0.13 (s.e.m., n=5). In contrast, the slow conductance elicited by NaF is enhanced at pressure; average relative currents 1.54 +/- 0.3 (s.e.m., n=2) and 2.47 (n=1) at 5 and 10 MPa, respectively.

These results suggest that the modification by pressure of dopaminergic and serotonergic processes in vivo may arise from an action of pressure on intracellular signalling pathways and not on receptor-ligand binding or G-protein activation.

Roberts, R.J., *et al.* (1996) *Neurosci. Letts.*, **208**, 125-128.
Kriem, B., *et al.* (1996) *Pharmacol. Biochem. Behavior*, **53**, 257-264.
Kriem, B., *et al.* (1998) *Brain Res.*, **796**, 143-149.

130P AN INTERACTIVE MULTIMEDIA COMPUTER-ASSISTED LEARNING PROGRAM TO TEACH BASIC ANATOMY AND MECHANICAL FUNCTION OF THE RESPIRATORY SYSTEM TO MEDICAL STUDENTS

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The teaching of anatomy to medical students at the University of Edinburgh has undergone significant change in recent years: cadaver dissection has ceased and contact hours have been reduced by 75% with anatomy now being taught within an integrated systems approach. The multimedia program we describe here has been developed to support the teaching of respiratory anatomy and is designed as a resource for student-centred learning and tutorial teaching. The program was developed using Macromedia Director (version 7) for delivery on either PC (minimum specification: Pentium PC, Windows 95/98/NT4, 16 Mb RAM, 10 Mb available HD space, 16 bit colour graphics) or Macintosh (minimum specification Power PC, 16 Mb RAM, OS 7.5, 10 Mb free HD space, 16 bit colour graphics) platforms or potentially the Internet.

The program covers the basic anatomy and mechanical function of the respiratory system and is divided into several sections accessible from a menu:

- Chest Wall - bones (vertebrae, ribs, sternum), intercostal muscles, diaphragm (structure, nerve & blood supply), accessory respiratory muscles, visceral & parietal pleurae.
- Lungs - development of pleural cavity, pleural membranes, lung structure, blood supply, lymphatics, innervation).
- Nose and nasal cavity - structure, innervation, blood supply, sinuses, conchae
- Pharynx - wall, muscles, innervation, blood supply.
- Larynx - epiglottis, cartilages, control of the airway, vocal cords.
- Trachea - structure, blood supply, innervation.
- Bronchi - structure of bronchial tree, innervation.
- Alveoli - structure, cells (macrophages, pneumocytes).
- Breathing - diaphragmatic, animated movements of chest wall, animated radiographs.
- Speech - animated vocal cord movements.

High quality graphics (thumbnails which may be 'zoomed' for greater detail), diagrams, animations and videoclips (e.g. vocal cord movement) are used extensively to explain principles such as how the respiratory muscles and diaphragm interact to cause inspiration and expiration. There are also self-assessment questions attached to each section. Additional features include: a notes function (notes may be created as you work through the package, copied to the clipboard and pasted into a word-processing package or e-mail); an alphabetical index of structures which allow access directly to the relevant screen describing that structure.

The program is aimed at medical students and will occupy them for 3-4 hours of self-directed learning. It may also be suitable for other students e.g. physiotherapy.