225P THE ROLE OF NITRIC OXIDE AND cGMP IN REFLEX- AND DMPP-EVOKED CHANGES IN BLADDER AND URETHRAL PRESSURES IN THE ANAESTHETISED RAT

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It has previously been demonstrated *in vitro* that NANC-induced bladder and urethral relaxations are mediated by nitric oxide (NO; see Andersson *et al.*, 1994). Furthermore this action of NO on urethral smooth muscle is mediated by increases in guanosine 3':5'-cyclic monophosphate (cGMP) formation (Morita *et al.*, 1992). However, the role of the NO system in the control of bladder and urethra *in vivo* remains to be fully elucidated. Therefore experiments were carried out to investigate the effects of N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, and zaprinast, an inhibitor of cGMP specific phospodiesterase types 5 and 6 (Ballard *et al.*, 1998), on reflex- and 1,1-dimethylphenylpiperazinium (DMPP; a ganglionic stimulant)-evoked bladder and urethral responses in anaesthetised rats.

Experiments were performed on spontaneously breathing female Sprague-Dawley rats (200g-320g), anaesthetised with urethane (1.2 g kg^{-1}, i.v.). Simultaneous recordings were made of urinary bladder and urethral perfusion pressures as previously described (Kakizaki et al., 1997). Reflex- and drug-evoked responses (in mmHg) were expressed as percentage changes before and after the administration of antagonists, and compared with saline or 5% triethanolamine (zaprinast vehicle) controls by unpaired Student's *t*-test. All values are means ± s.e.mean.

Reflex-evoked urethral relaxations and associated high frequency oscillations in urethral pressure (Kakizaki et al., 1997) were significantly ($P < 0.05$) attenuated by 74 ± 11% (n=6) and 48 ± 16% (n=5), respectively, by L-NAME (20 mg kg^{-1}, i.v). These effects were reversed after administration of L-arginine (150 mg kg^{-1}, i.v), a NO synthase substrate. In addition, close-arterial administration of DMPP (0.5 mg kg^{-1}) elicited a decrease in urethral pressure that was significantly attenuated by 83 ± 11% after L-NAME treatment, and similarly reversed L-arginine (n=7). Zaprinast (0.6 mg kg^{-1} min^{-1}, i.v., for 30 min) significantly potentiated both reflex- and DMPP-evoked urethral relaxations by 91 ± 29% and 48 ± 19% respectively (n=5), but had no effect on high frequency oscillations in urethral pressure. Reflex- and DMPP-evoked bladder contractions and micturition volume and pressure thresholds were unaffected by all test substances (n=13).

The present results indicate that reflex- and DMPP-evoked urethral relaxations in the anaesthetised rat are principally mediated by NO, via the formation of cGMP. Furthermore, these data suggest bladder responses evoked by the micturition reflex and DMPP do not involve nitrergic neurotransmission.

A.W.is a MRC (collaborative) student with Pfizer UK.

Andersson, K.E. *et al.* (1994) *World. J. Urol,* 12, 274-280.
Ballard, S.A. *et al.* (1998) *J. Urol,* 159(6), 2164-2170.
Kakizaki, H *et al.*(1997) *Am .J. Physiol.*, 272, R1647-R1656.
Morita, T *et al.* (1992) *Urol. Int.*, 49, 191-195.

226P EXPRESSION AND FUNCTIONAL IMPORTANCE OF Kv CHANNELS IN HUMAN BLADDER SMOOTH MUSCLE

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Although delayed rectifier K^+-currents carried by K_V channels regulate action potential shape in many cell types they are not known to be of significance in detrusor smooth muscle cells of the bladder. We investigated K_V channel expression and function in detrusor muscle by tension and patch-clamp recording, and by immunological staining.

Human detrusor muscle was obtained fresh from cystectomy specimens. Detrusor muscle was also obtained from 1.5 kg rabbits injected with a lethal dose of sodium pentobarbitone. Isometric tension was measured from muscle strips bathed in Krebs solution at 37 °C. Whole-cell voltage-clamp was used to measure membrane currents from isolated human detrusor smooth muscle cells with a K^+-based patch pipette solution including 10 mM EGTA. Immunofluorescence was measured from snap-frozen 15-μm cryostat sections of human detrusor labelled with polyclonal antibodies to $K_V1.1$-6 (gifts from H.-G. Knaus; Koch *et al.*, 1997). An antibody to smooth muscle α-actin (Sigma) was used to identify smooth muscle cells. Specificity of antibodies was determined by a standard western blotting protocol. Data are given as mean±s.e.mean.

3,4 diaminopyridine (3,4-DAP, 1 mM) is an inhibitor of many K_V channels. It increased the frequency of spontaneous contractions and elevated the base-line tension of human (n=3) or rabbit (n=5) detrusor strips; in rabbit strips, spontaneous contractions occurred at 4.45±0.66 min^{-1} in control conditions and at 9.25±0.71 min^{-1} in the presence of 3,4-DAP (n=5; $P<0.01$ by Student's *t*-test). K_{ATP} channels are also blocked by 3,4-DAP but glibenclamide (1 μM), a specific inhibitor of K_{ATP} channels, had no effect on tension and did not modify the response to 3,4-DAP in rabbit strips (n=4). Square depolarising voltage steps from a –60 mV holding potential elicited a time-dependent outward current that was sometimes preceded by a fast net inward current. Inhibition of the inward current by 0.1 mM Cd^{2+} did not inhibit the outward current (n=4). Voltage-dependent outward current was elicited by depolarisation positive to –40 mV. 3,4-DAP (1 mM) inhibited the total net outward current at –10 mV by 29.2±4.3 % (n=4). Positive results were obtained for $K_V1.3$- and $K_V1.6$-targetted antibodies, which labelled human detrusor smooth muscle cells in the absence but not presence of antigenic peptide. Western blotting indicated specific labelling of a 56 kDa protein by anti-$K_V1.6$, and an 85 kDa protein by anti-$K_V1.3$ in human detrusor.

Delayed rectifier K^+-current that activates in a physiologically important voltage range has been observed in human detrusor smooth muscle cells. $K_V1.3$ and $K_V1.6$ channel subunits were detected and thus may carry part of the K^+-current. K_V channels appear to be functionally important in rabbit and human detrusor muscle.

Koch, R.O., Wanner, S.G., Koschak, A. et al. (1997). *J. Biol. Chem.* 272(44), 27577-27581.

227P MUTATIONS OF CONSERVED EXTRACELLULAR CYSTEINE RESIDUES IN THE INWARDLY RECTIFYING POTASSIUM CHANNEL Kir2.3

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The inwardly rectifying potassium channel Kir2.3 possesses highly conserved extracellular cysteine residues at positions 113 and 145. In this study we investigated whether these residues are involved in trafficking of the Kir2.3 channel to the plasma membrane, perhaps via the formation of an essential disulphide bond. We have previously shown (Bannister *et al.* 1998) that these cysteine residues are necessary for channel function.

Cysteines at positions 113, 140 and 145 were mutated individually to serine by site-directed mutagenesis (Kunkel *et al.*, 1987). FLAG-tagged constructs at the C terminus of wild type and mutant Kir2.3 channels were made by inserting a FLAG-tag sequence before the stop codon of the Kir2.3 insert. All mutations were confirmed by dideoxy sequencing. cRNA was transcribed in vitro using the T7 promoter and *BamH*I-linearised DNA, injected into *Xenopus* oocytes (1 ng), and two-electrode voltage-clamp recordings carried out 24-48 hours later to study channel function. Oocytes were also fixed in methanol at this time, and frozen sections prepared. The location of the expressed protein was investigated using an M2 FLAG probe primary antibody and FITC-conjugated secondary antibody, followed by examinion using confocal microscopy. In separate experiments, cDNA was transcribed and translated *in vitro* using the rabbit reticulate lysate system with ^{35}S-methionine as label. Labelled proteins were separated on 8% SDS-Page gels, and bands visualized by autoradiography.

As for untagged channels (Bannister *et al.*, 1998), the FLAG-tagged mutants C113S and C145S did not show observable currents in voltage-clamp experiments, while wild type FLAG currents were similar to untagged wild type currents. Confocal immunofluorescent images of sections of oocytes showed specific fluorescent labelling of the membrane in sections from oocytes which had been injected with Kir2.3 cRNA for wild type-FLAG, C113S-FLAG, C140S-FLAG and C145S-FLAG channels. Uninjected control oocytes showed no fluorescent labelling of the membrane, despite the membrane itself being clearly visible in the corresponding light field images.

In vitro translation-transcription of wild type cDNA gave a single heavily labelled band at 59 KDa which is the expected size of a monomer of the Kir2.3 channel. No bands were seen at 118 kDa or 236 kDa, the expected sizes of a dimer or tetramer respectively. A band of the same molecular weight was also seen in the presence of microsomal membranes. Under reducing conditions, in the presence of 2-mercaptoethanol (1.25%), a single band of 59 Kda was again observed. Under non-reducing conditions, in the presence of Cu^{2+} (10 μM), again no bands other than the single band at 59 kDa could be detected. Similar results were obtained for the same experiments using mutants C113S, C140S and C145S.

The results show that mutation of the highly conserved cysteine residues C113 and C145 in Kir2.3 channels prevents function of the channel but yet mutant channels are detected in the oocyte membrane, indicating normal trafficking to the membrane. The *in vitro* translation experiments showed no evidence of linkage between subunits under a variety of conditions, making the presence of inter-subunit disulphide bonds unlikely.

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BANNISTER JPA, YOUNG BA, SIVAPRASADARAO A & WRAY D (1998) *British Journal of Pharmacology* **123**, 37P.
KUNKEL TA, ROBERTS JD & ZAKOUR RA (1987) *Methods in Enzymology* **154**, 367-382.

228P THE ROLE OF 5-HT$_{1A}$ RECEPTORS AND α_1-ADRENOCEPTORS IN THE CONTROL OF THE "MICTURITION REFLEX" IN THE ANAESTHETIZED MALE RAT

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Blockade of central 5-HT$_{1A}$ receptors with WAY-100635 has been demonstrated to attenuate reflex activation of the parasympathetic outflow to the heart (Dando *et al.*, 1998) and the lungs (Bootle *et al.*, 1998). The question, therefore, arises as to whether blockade of these receptors interferes with the micturition reflex. In addition, experiments using doxazosin, a non-subtype-selective α_1-adrenoceptor antagonist, have implicated a role for α_1-adrenoceptors in the central control of bladder function (Ramage & Wyllie, 1995). The aim of the present study was to examine the effects of WAY-100635 and doxazosin on the micturition reflex in anaesthetized rats.

Experiments were carried out in spontaneously-breathing male Sprague Dawley rats (290-390g) anaesthetized with urethane (1.2 g kg^{-1} i.v.). "Micturition reflexes" were evoked by distension of the urinary bladder with saline infusion (0.05 ml min^{-1}). Intraluminal bladder and urethral perfusion pressures were recorded as described by Kakizaki *et al.* (1997). The pressure threshold, the amplitude and duration of the bladder contractions and associated urethral responses were measured. Changes (%) after test drug (i.v.) were compared with vehicle (0.04 M) lactic acid controls using unpaired Student's *t*-test.

Distension–induced bladder contractions were accompanied by contractions of the urethra. WAY-100635 (0.1 & 0.3 mg kg^{-1}; n = 4) significantly (P<0.05) increased the pressure threshold (0.1 mg kg^{-1}; 96 ± 21%, mean ± s.e.mean) and, at the higher dose, significantly attenuated baseline urethral pressure (23 ± 3%) and the amplitude of the reflex urethral contractions (46 ± 6%). The 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (0.3 mg kg^{-1}; n = 6) significantly reduced the pressure threshold (16 ± 6%) but had no effect on the amplitude of the bladder or urethral contractions. Doxazosin (0.1-1 mg kg^{-1}; n = 5-6) had no effect on any of the bladder variables, although 2 mg kg^{-1} significantly increased the duration (235 ± 58%) of the contractions. In contrast, doxazosin (0.1 mg kg^{-1}) caused a maximum and significant fall in baseline urethral pressure of 17 ± 3% and, at a dose of 0.5 mg kg^{-1}, background activity and reflex urethral contractions (63 ± 7%) were significantly attenuated. All doses of doxazosin induced bursts of high frequency, high amplitude oscillations in urethral pressure that were unrelated to contractions of the bladder.

These data indicate that 5-HT$_{1A}$ receptors also play a role in the reflex activation of the parasympathetic outflow to the bladder, while blockade of α_1-adrenoceptors inhibits urethral contractions. Blockade of α_1-adrenoceptors also induces spontaneous bursts of activity in the urethra, which represent external urethral sphincter activity (Kakizaki *et al.*, 1997)

Bootle D.J. *et al.* (1998). *Neuropharmacol.*, 37, 243-250.
Dando S.B. *et al.* (1998). *Br. J. Pharmacol.*, 125, 409-417.
Kakizaki H. *et al.* (1997). *Am. J. Physiol.*, 272, R1647-1656.
Ramage A.G. & Wyllie M.G. (1995). *Eur. J. Pharmacol.*, 294, 645-650.

229P DISTRIBUTION OF RENAL ADENOSINE A_1 RECEPTORS: EFFECT OF DIETARY SODIUM CHLORIDE

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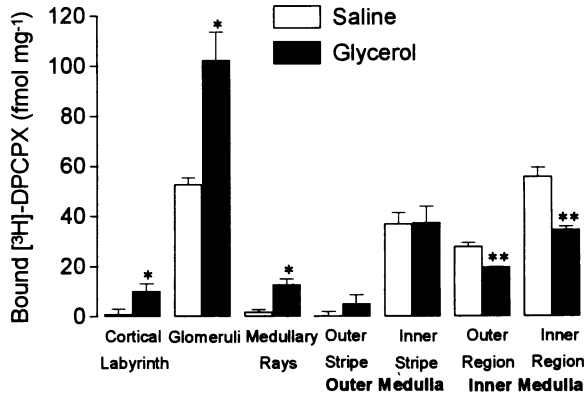
Adenosine is a haemodyamic mediator in acute renal failure (ARF) induced by myohaemoglobinuria (produced by glycerol injection) and renal adenosine A_1 receptor density and mRNA levels are elevated in this form of ARF (Gould *et al.*, 1997). In ARF induced by $HgCl_2$, adenosine does not play a pathophysiological role and A_1 receptor density and mRNA levels are unaltered. The aim of this study was to identify any regional changes in receptor density in the kidneys of rats with ARF induced by either glycerol or $HgCl_2$.

ARF was induced in male Wistar rats (200 - 250g) by either i.m. injection of 50% v/v glycerol in saline (10ml kg^{-1}) or s.c. injection of $HgCl_2$ (2 mg kg^{-1}). Control animals received equivalent saline injections. Following induction of ARF, both kidneys were removed and frozen in isopentane cooled in liquid nitrogen. Kidney sections (20 μm) were incubated for 4h at 4°C in the presence of 0.3 nM [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-DPCPX). Sections were apposed to coverslips coated in nuclear emulsion and left to expose at 4°C for 12 weeks in light proof boxes.

Sixteen hours following the induction of glycerol-induced ARF, there was a 34% ($P<0.01$) increase in labelling in the glomeruli, compared to saline-injected controls; whilst by 48h, glomerular labelling had increased two-fold. In addition, 48h following glycerol injection, significant labelling was now detected in the cortical labyrinth and medullary rays whilst, in both inner and outer regions of the inner medulla, labelling had decreased by 29-38% (Figure 1). The only statistically significant change noted 48h following induction of $HgCl_2$-induced ARF was a 39% decrease ($P<0.01$) in labelling in the inner and outer regions of the inner medulla. Glycerol-induced ARF results in differential expression of renal adenosine A_1 receptors. The increase in density of A_1 receptors in glomeruli and cortical labyrinth may account for the increased renal vasoconstrictor response to adenosine and depressed glomerular filtration rate noted previously in this type of ARF.

Figure 1 Binding of [^{3}H]-DPCPX to kidney regions of rats 48h following induction of ARF with glycerol. Results are given as mean + s.e.mean (n = 3-5). * $P<0.05$, ** $P<0.01$ (Students *t*-test) relative to saline group.



Gould, J., Morton, M.J., Sivaprasadarao, A. *et al.* (1997). *Br. J. Pharmacol.*, 120, 947-953.

230P ADRENAL CHROMAFFIN CELLS OF GUINEA-PIG, BUT NOT RAT, EXPRESS AN ATP-GATED ION CHANNEL SIMILAR TO THE P2X2 PHENOTYPE

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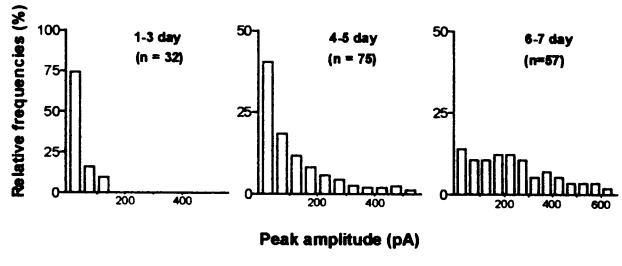
Extracellular ATP stimulates catecholamine release from guinea-pig adrenal chromaffin cells and rat phaeochromocytoma (PC12) cells, mainly through P2X receptors but also P2Y receptors (Illes *et al.*, 1996). We have used patch-clamp techniques to study the pharmacological properties of the P2X receptors in chromaffin cells dissociated from adrenal medullae of immature guinea-pigs and rats (17 days old).

Rat chromaffin cells, cultured for 1-7 days, failed to respond to ATP and UTP (300 μM) yet did respond to DMPP (10 μM). Guinea-pig chromaffin cells, under the same conditions, responded to ATP (100 μM) with a rapidly-activating inward current, and also responded to DMPP (10 μM). The amplitude of the ATP-evoked current increased from 81.4±18.7 pA (at 1-3 days) in culture to 255.2±22.1 pA (at 6-7 days), with an increase in the number of cells responding from 22% (at 1-3 days) to 89% (at 6-7 days) (Figure 1). Cell size, inferred from membrane capacitance, did not change during the period cells were maintained in culture. ATP-evoked currents desensitized slowly and had a reversal potential of 2.5±2.7 mV. The EC_{50} for ATP was 43±4 μM (at pH 7.4). The potency order for ATP analogues was: 2-MeSATP>ATP>ADP. Adenosine, AMP, and UTP (up to 1 mM) and α,β-meATP (up to 300 μM) were inactive. Suramin (100 μM) inhibited the current activated by a submaximal concentration of ATP (100 μM) by 51±6%. Cibacron blue (50 μM) also inhibited ATP-responses by 47.2±5.8%. PPADS inhibited ATP-responses with an IC_{50} of 3.2 μM. The ATP concentration-response curve was shifted to the left at pH 6.8 and to the right at pH 8.0, without changing the maximal ATP effect. The EC_{50} for ATP was 19±3 μM (at pH 6.8) and 96±15 μM (at pH 8.0). Zn^{2+} inhibited the response to ATP (100 μM) with an IC_{50} of 48±7 μM.

The expression of ATP-gated cation channels in chromaffin cells is species dependent and, where present, dependent on time in culture. P2X receptors in guinea-pig chromaffin cells exhibit a novel pharmacological profile, of which many properties are shared by the $P2X_2$ receptor, but not Zn^{2+}-inhibition.

Figure 1. Frequency histogram of the amplitude of ATP (100μM)-activated current in guinea-pig chromaffin cells.



Illes, P., Nieber, K., Fröhlich, R. *et al.*(1996). *Ciba Found. Symp.* 198, 110-129.

231P ANTAGONISM OF rP2X$_1$ AND rP2X$_3$ RECEPTORS BY DIINOSINE POLYPHOSPHATES

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P2X receptors are ATP-gated ion channels involved in fast excitatory transmission at autonomic neurons, smooth muscle and secretory epithelia. Of the known P2X subunits in peripheral tissues, there is a paucity of P2X subunit-selective agonists and antagonists. We have tested a new class of P2X antagonist, the diinosine polyphosphates (Ip$_n$I, n = 3,4,5), at P2X$_{1-4}$ receptors to assess their P2X subunit selectivity.

Diadenosine polyphosphates were converted into diinosine polyphosphates (for example, Ap$_5$A into Ip$_5$I) using 5′-adenylic deaminase, then purified by reverse-phase chromatography. Defolliculated *Xenopus* oocytes, injected cytosolically with cRNAs for rat P2X$_{1-4}$ receptors, were studied under voltage-clamp conditions (V_h = -60 to -90 mV) to quantitate Ip$_n$I blockade of ATP-activated inward currents. The Ip$_n$I series selectively blocked ATP-responses at Group 1 P2X receptors, showing greater potency at rP2X$_1$ than rP2X$_3$ receptors, but did not affect ATP-responses at rP2X$_2$ (Group 2) receptors, while ATP-responses were potentiated at rP2X$_4$ (Group 3) receptors (see Table 1). None of the actions of the Ip$_n$I series at rP2X$_{1-4}$ receptors were mimicked by their parent diadenosine polyphosphates (see Brown *et al.*, 1999). The nature of Ip$_n$I blockade of rP2X$_1$ receptors was complex. Submicromolar levels of Ip$_5$I caused a surmountable inhibition of ATP-responses, although pA$_2$ values were concentration-dependent and the Schild plot was non-linear, whereas micromolar levels of Ip$_5$I and Ip$_4$I caused a non-surmountable blockade which reversed slowly after prolonged washout (1-2 h). In contrast, Ip$_n$I blockade of rP2X$_3$ receptors was surmountable, even at micromolar levels, in a seemingly competitive manner (pA$_2$ values: Ip$_4$I, 6.75; Ip$_5$I, 6.27).

Thus, Ip$_n$I compounds are selective antagonists of Group 1 P2X receptors. Ip$_5$I is a potent non-competitive antagonist at rP2X$_1$ receptors, being 1000-fold more selective for rP2X$_1$ than rP2X$_3$ receptors. Ip$_4$I and Ip$_5$I appear to be potent competitive antagonists at rP2X$_3$ receptors. The blocking activity of the Ip$_n$I series, and agonist properties of the Ap$_n$A series (Brown *et al.*, 1999), may help discriminate P2X$_1$ and P2X$_3$ receptors in native tissues.

Table 1. *Activity indices of the Ip$_n$I series.*

Compound	rP2X$_1$ (§)	rP2X$_2$	rP2X$_3$ (§)	rP2X$_4$ (¥)
Ip$_5$I	3.1±0.4 nM	inactive	2.8±0.7 μM	2.0±0.4 nM
Ip$_4$I	560±80 nM	inactive	1.0±0.3 μM	1.7±0.4 μM
Ip$_3$I	>30 μM	inactive	>30 μM	inactive

(mean±s.e.mean, n = 4; §, IC$_{50}$ values; ¥, EC$_{50}$ values)

Brown, S.G., Wildman, S.S., King, B.F. *et al.* (1999) *Br. J. Pharmacol.* 126, 24P.

232P SB-269970 IS A POTENT AND SELECTIVE ANTAGONIST AT HUMAN CLONED AND GUINEA-PIG BRAIN 5-HT$_7$ RECEPTORS

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The 5-HT$_7$ receptor has been cloned from a number of species, including human and guinea pig and is positively coupled to adenylyl cyclase (AC) when expressed in cell lines (eg. Bard *et al.*, 1993). The 5-HT$_7$ receptor is present in brain with the highest density in thalamic and limbic areas (To *et al.*, 1995). Although a 5-HT$_7$-like receptor has been reported to be involved in circadian rhythym control (Lovenberg *et al.*, 1993), the role of 5-HT$_7$ receptors in brain function has not been clearly established, due partly to a lack of suitable selective ligands. However, the selective 5-HT$_7$ receptor antagonist, SB-258719, has recently been used to confirm that 5-HT$_7$ receptors are positively coupled to AC in guinea pig hippocampus (Thomas *et al.*, 1999). Subsequently, the SB-258719 analogue SB-269970-A ((R)-3-(2-(2-(4-Methyl-piperidin-1-yl)ethyl)-pyrrolidine-1-sulfonyl)-phenol) has been synthesised, which displays higher affinity for the human cloned 5-HT$_7$ receptor than SB-258719. In the present study, we report the selectivity profile of SB-269970-A and its antagonist profile at both the human cloned 5-HT$_{7(a)}$ and guinea pig hippocampal 5-HT$_7$ receptor.

AC activity was determined in washed membranes from HEK293 cells stably expressing the human cloned 5-HT$_{7(a)}$ receptor (h5-HT$_{7(a)}$/293) or washed guinea pig hippocampal membranes, by measuring conversion of [α-^{33}P]-ATP to [^{33}P]-cAMP (Thomas *et al.*, 1998). Data are the mean of at least three separate experiments.

In radioligand binding studies using [^{3}H]-5-CT, SB-269970-A displayed high affinity for the human 5-HT$_{7(a)}$ receptor (pK$_i$ 8.9 ± 0.1) and showed at least 100-fold selectivity versus all other 5-HT receptor subtypes tested except the human 5-ht$_{5A}$ receptor (50-fold, pK$_i$ 7.2 ± 0.1). 5-CT stimulated adenylyl cyclase activity in h5-HT$_{7(a)}$/293 membranes by 500 ± 80%, with a pEC$_{50}$ of 7.5 ± 0.1. SB-269970 (tested at 0.03, 0.1, 0.3 and 1μM) produced a concentration-related rightward-shift of the 5-CT response curve with no significant alteration in the maximal response to 5-CT, consistent with competitive antagonism. Schild analysis of the data gave a pA$_2$ of 8.52 ± 0.19 and slope of 0.84 ± 0.06 (95% confidence interval 0.70 - 0.97), which correlates with its binding affinity at the human cloned 5-HT$_{7(a)}$ receptor. In guinea pig hippocampal membranes, 5-CT produced a 21% stimulation of adenylyl cyclase activity with a pEC$_{50}$ of 8.4 ± 0.2. SB-269970 (0.3μM) displayed a similar surmountable antagonist profile to that seen at the human cloned receptor, consistent with competitive antagonism. The estimated pK$_B$ for SB-269970-A was 8.3 ± 0.1, in good agreement with its antagonist potency at the human cloned 5-HT$_{7(a)}$ receptor. SB-269970 (0.3μM) also produced a small inhibition of basal adenylyl cyclase activity in the absence of added 5-CT. This effect is consistent with antagonism of tonic 5-HT$_7$ receptor stimulation due to residual endogenous 5-HT, as reported by Thomas *et al.*, (1999).

In summary, SB-269970 is a potent, selective antagonist at human cloned and guinea pig brain 5-HT$_7$ receptors and represents a valuable compound to aid investigation of the role of 5-HT$_7$ receptors in native tissue function.

Bard J.A., Zgombick, J., Adham, N., *et al.*, (1993). *J. Biol. Chem.*, 268, 23422-23426.
Lovenberg, T.W., Baron, B.M., *et al.*, (1993) *Neuron*, 11, 449-458.
Thomas, D.R., *et al.*, (1998) *Br. J. Pharmacol.*, 124, 1300-1306.
Thomas, D.R., *et al.*., (1999) *Br. J. Pharmacol.*, 128, 158-164.
To Z.P., *et al.*, (1995) *Br. J. Pharmacol.*, 115, 107-116.

233P [^{3}H]-SB-269970: A SELECTIVE ANTAGONIST RADIOLIGAND FOR 5-HT$_7$ RECEPTORS

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The 5-HT$_7$ receptor has been cloned from a number of species, including guinea pig and man (Bard *et al.*, 1993) and is present in brain, particularly in thalamic and limbic areas (To *et al.*, 1995). 5-HT$_7$ receptor binding studies in recombinant systems and brain tissue have commonly used the agonist radioligands [^{3}H]-5-HT or [^{3}H]-5-CT. However, neither is 5-HT$_7$-selective and blocking drugs must be included in brain binding studies to inhibit binding to non-5-HT$_7$ sites. There is, therefore a need for a selective 5-HT$_7$ receptor radioligand. SB-269970, which has been identified as a potent and selective 5-HT$_7$ receptor antagonist (Thomas *et al.*, this Meeting), has been tritiated (Sp. Act. 49 Ci mmol^{-1}) and the profile of [^{3}H]-SB-269970 binding to the human cloned 5-HT$_{7(a)}$ receptor has been investigated in comparison with that for [^{3}H]-5-CT.

Binding studies were carried out using well-washed membranes from HEK293 cells stably expressing the human cloned 5-HT$_{7(a)}$ receptor (h5-HT$_{7(a)}$/293) essentially as described by Thomas *et al.*, (1998).

[^{3}H]-SB-269970 (1nM) showed full association with h5-HT$_7$/293 membranes after 40 min. Specific binding at equilibrium represented >90% of total binding and was fully reversible by addition of 5-HT (10μM), full dissociation occuring by 100 min. The association (k_{+1}) and dissociation (k_{-1}) rate constants were 0.05 nM^{-1} min^{-1} and 0.05 min^{-1} respectively, giving a K_d (k_{-1}/k_{+1}) of 1.0 nM, similar to that derived from saturation analysis (see below). [^{3}H]-SB-269970 (0.1 - 10nM) bound saturably and monophasically to h5-HT$_{7(a)}$/293 membranes, giving a K_d of 1.3 ± 0.1nM. The B_{max} for [^{3}H]-SB-269970 (5.8 ± 0.4 pmoles mg^{-1} protein) was similar to that for [^{3}H]-5-CT (6.2 ± 0.2 pmoles mg^{-1} protein), suggesting that both radioligands labelled the same receptor population. The profile of inhibition of [^{3}H]-SB-269970 (1nM) binding by a range of 5-HT$_7$ receptor agonists and antagonists correlated well with that for [^{3}H]-5-CT (0.5nM) binding (correlation coefficient 0.98) (Table 1). Hill slopes for drug inhibition of both [^{3}H]-SB-269970 and [^{3}H]-5-CT binding were not significantly different to 1 (data not shown), also consistent with binding to a single population of receptors.

Table 1. Inhibition of [^{3}H]-5-CT and [^{3}H]-SB-269970 binding.

	pK_i (± s.e.mean; n ≥3)	
	[^{3}H]-5-CT	[^{3}H]-SB-269970
5-CT	9.13 ± 0.08	8.86 ± 0.15
SB-269970-A	8.85 ± 0.06	8.61 ± 0.10
Methiothepin	8.49 ± 0.14	8.41 ± 0.08
5-HT	8.21 ± 0.08	8.10 ± 0.19
Risperidone	8.14 ± 0.08	7.84 ± 0.10
Mesulergine	7.45 ± 0.17	7.63 ± 0.06
SB-258719	7.47 ± 0.03	7.48 ± 0.02
Clozapine	7.05 ± 0.07	6.83 ± 0.12
8-OH-DPAT	6.58 ± 0.05	6.94 ± 0.08
Sumatriptan	5.82 ± 0.22	5.91 ± 0.07

In summary, [^{3}H]-SB-269970 represents the first selective antagonist radioligand for 5-HT$_7$ receptors and should prove valuable for studies of 5-HT$_7$ receptors in recombinant and native tissues.

Bard, J.A., Zgombick, J., Adham, N., *et al.*, (1993). *J. Biol. Chem.*, 268, 23422-23426.
Thomas, D.R., *et al.* (1998) *Br. J. Pharmacol.*, 124, 1300-1306.
Thomas, D.R., *et al.* (1999) *Br. J. Pharmacol.*, (this meeting).
To, Z.P., *et al.*, (1995) *Br. J. Pharmacol.*, 115, 107-116.

234P THYROXINE CAUSES CELL TYPE-DEPENDENT REGULATION OF THE SEROTONIN AND NORADRENALINE TRANSPORTERS

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There is evidence that there may be a benefit in co-administration of antidepressants and thyroid hormones in depression therapy (Aronson *et al.*, 1996). Serotonin (5-HT) transporter (SERT) and noradrenaline (NA) transporter (NAT) inhibitors are used as antidepressants. NAT in rat brain is upregulated by thyroxine (T4) (Tejani-Butt & Yang, 1994). Hence, the aim of this study was to examine the effects of T4 on regulation of the SERT and NAT in cultured cells that express these transporters (human JAR choriocarcinoma cells and rat lung microvascular endothelial cells (RLMEC) for SERT and rat PC12 phaeochromocytoma cells and human SK-N-SH-SY5Y neuroblastoma cells for NAT).

JAR, RLMEC, PC12 and SK-N-SH-SY5Y cells were cultured in appropriate media and subcultured into 12-well plates. T4 (100 nM, 1 μM or 10 μM T4) was added to the culture medium for 2, 4, 12, 24, 36 or 48 h. The culture medium was then replaced by Krebs-Hepes buffer (± 1 μM paroxetine to inhibit SERT or 1 μM nisoxetine to inhibit NAT to determine non-specific uptake) and the cells were incubated for 15 min at 37°C. [^{3}H]-5-HT or [^{3}H]-NA (10 nM) was then added (and, in kinetic experiments on JAR cells, a total of 250-4000 nM [^{3}H]-5-HT) for 2 min. The cells were washed with ice-cold buffer and, after lysis, their [^{3}H] and protein contents were determined. 5-HT or NA specific uptake values are in fmol/mg protein. Data were analysed by repeated measures analysis of variance followed by Tukey *post hoc t*-tests.

In JAR cells, 100 nM (n=4) or 1 μM (n=4) T4 for 48 h or 10 μM T4 (n=3) for 24, 36 or 48 h significantly increased 5-HT uptake, compared with controls (no T4: 147, s.e.mean 5.7, n=11) ($P< 0.05$). Shorter T4 exposure times did not affect 5-HT uptake in the JAR cells ($P>0.05$). The increases in 5-HT uptake after 48 h exposure were 22% for 100 nM, 28% for 1 μM and 66% for 10 μM T4. Exposure to 10 μM T4 for 24 h significantly increased ($P<0.05$) V_{max} (in pmol/mg protein/min) (T4: 24.2, s.e.mean 2.3, n=4; controls: 15.7, s.e.mean 1.5, n=4), but had no effect on K_m ($P>0.05$), of 5-HT uptake, and 100 nM T4 for 24 h had no effect on K_m or V_{max} ($P>0.05$). In contrast, in RLMEC, exposure to T4 for 24 or 48 h at 100 nM had no effect (n=3-4, $P>0.05$) and at 10 μM caused significant decreases in 5-HT uptake of 22% after 24 h and 19% after 48 h ($P<0.05$), compared with controls (534, s.e.mean 120, n=4; 1845, s.e.mean 119, n=3, respectively).

In PC12 cells, T4 exposure for 24 or 48 h increased NA uptake at 100 nM (by 18% and 16%, respectively, $P<0.05$), 1 μM (by 32% and 21%, respectively, $P<0.01$) and 10 μM (by 40% and 32%, respectively, $P<0.001$), compared with controls (98, s.e.mean 9.0, n=16). In SK-N-SH-SY5Y cells, T4 exposure for 24 or 48 h at 1 or 10 μM had no effect on NA uptake ($P>0.05$).

The results show that T4 upregulates both SERT and NAT, but the effects are cell type-dependent, occurring in JAR cells but not RLMEC for SERT and in PC12 cells but not SK-N-SH-SY5Y cells for NAT. The time course of T4 effects on SERT and the increase in V_{max} with no effect on K_m of 5-HT uptake suggest that the effects may be due to changes in transporter expression.

Tejani-Butt, S.M. & Yang, J. (1994) *Neuroendocrinol.* 59, 235-244.
Aronson, R., Offman, H.J., Joffe, R.T. *et al.* (1996) *Arch. Gen. Psychiatry*, 53, 842-848.

235P THE GABAPENTIN ANALOGUE 3-METHYL-GABAPENTIN BLOCKS BOTH STATIC AND DYNAMIC COMPONENTS OF MECHANICAL ALLODYNIA IN A RAT MODEL OF NEUROPATHIC PAIN

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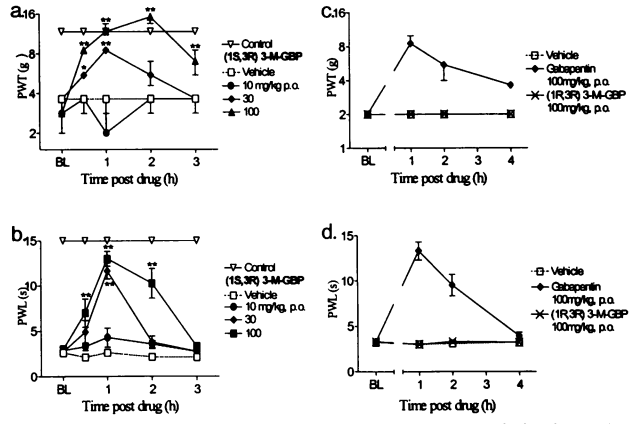
Neuropathic pain remains poorly treated, with some patients being resistant to all forms of current treatment. One of the most debilitating symptoms is allodynia where previously innocuous stimuli induce pain. Two distinct types of mechanical allodynia termed static and dynamic have been reported in the clinic. We have recently shown that both types can be detected in a diabetes model of neuropathic pain (Field *et al.*, 1999). These studies showed that gabapentin and the related compound pregabalin can block both responses. In contrast, static but not dynamic type is sensitive to morphine and amitriptyline. The mechanism of action of gabapentin is unclear. However, it has been shown that gabapentin binds selectively to the $\alpha_2\delta$ subunit of voltage dependent calcium channels (VDCC) (Gee *et al.*, 1996). Until recently, gabapentin and pregabalin were the only compounds known to bind to this site. Recent studies carried out in our laboratories have revealed that *cis*-(1*S*,3*R*)-(1-(aminomethyl)-3-methylcyclohexyl)acetic acid hydrochloride ((1S,3R) 3-Methyl-gabapentin) stereoselectively interacts with the $\alpha_2\delta$ subunit (IC_{50} = 42nM). The (1R,3R) isomer was found to possess 300 times weaker binding for this site (IC_{50} = 10,000nM) (Bryans *et al* 1998). Here, we examine and compare activities of the two isomers to block the maintenance of static and dynamic allodynia in a rat model of diabetes-induced neuropathic pain.

Male Sprague Dawley rats (200-250g), obtained from Bantin and Kingman, (Hull, U.K.) were housed in groups of 6. All animals were kept under a 12h light/dark cycle (lights on at 07h 00min) with food and water *ad libitum*. Diabetes was induced by a single i.p. injection of streptozocin (50mg/kg). Control animals received a similar administration of isotonic saline. Static and dynamic allodynia were measured as previously described (Field *et al.*, 1999).

Animals treated with streptozocin exhibited both static and dynamic allodynia with a reduced withdrawal threshold to the von Frey filaments and stroking with cotton bud respectively. No such reductions were seen in saline treated controls. The oral administration of (1S,3R) 3-methyl-gabapentin (10-100mg/kg) dose-dependently blocked the maintenance of static and dynamic allodynia with MEDs of 30mg/kg. (Figure1 a,b). Similar administration of (1R,3R) 3-methyl-gabapentin (100mg/kg) failed to have any antiallodynic action (Figure 1c,d). However, in the same experiment gabapentin (100mg/kg, p.o.) was used as a positive control which blocked both static and dynamic allodynia (Figure 1 c,d). 3-M-GBP = 3-methyl-gabapentin.

FIGURE 1.



In conclusion, these data demonstrate that (1S,3R) 3-methyl-gabapentin possesses a similar antiallodynic profile as gabapentin. These data support the potential clinical utility of gabapentin and related compounds for the treatment of neuropathic pain. The present results are also consistent with the suggestion that the $\alpha_2\delta$ subunit of VDCCs may play an important role in the mediation of the antiallodynic actions of gabapentin.

Bryans et al.,J. Med. Chem. (1998) 41: 1838.
Field, M.J., et al., Pain, 80 (1999) 391.
Gee, N.S., et al., J. Bio. Chem., 271 (1996) 5768.
Suman-Chauhan et al., Eur. J. Pharmacol-Mol. Pharmacol. Sec. (1993) 244: 293.

236P GLUTAMATE TRANSPORTERS AND MODULATION OF PRIMARY AFFERENT-MEDIATED SYNAPTIC TRANSMISSION IN THE RAT SPINAL DORSAL HORN *IN VITRO*

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Three eukaryotic glutamate transporters known as GLAST, GLT-1 and EAAC-1 have been cloned and are localized within the mammalian brain (Lehre et al., 1995). These proteins normally maintain a low basal extracellular level of glutamate at central synapses to allow for activation of receptors and to avoid neurotoxic damage. There is evidence that ionotropic and metabotropic glutamate receptors (mGluRs) are recruited in a frequency-dependent manner that reflects the concentration of glutamate within the synaptic cleft (Scanziani et al., 1997). We have examined a) the localization of these transporter proteins within the dorsal horn of the spinal cord and the spinal nucleus caudalis of the trigeminal nerve, b) the effects of L-trans-pyrrolidine-2,4-dicarboxylic acid (L-PDC), a glutamate uptake inhibitor, on primary afferent-mediated synaptic transmission. To directly activate mGluRs, we used a selective Group I agonist, (s)-3,5-dihydroxyphenylglycine (DHPG) and the Group III agonist, L-2-amino-4-phosphonobutyrate (L-AP4).

For immunohistochemistry, Wistar rats were anaesthetized (Urethane, 2 g kg^{-1}, i.p.) and the spinal cord and medulla oblongata removed and fixed in aldehydes. Spinal sections were then processed for localization of the glutamate transporters using antibodies to the glial transporters GLT-1 and GLAST, and the neuronal transporter EAAC-1 (kindly donated by Drs. N. Danbolt & D.V. Pow). Bound antibodies were visualized by the avidin-biotin complex (ABC) method. For electrophysiology, neonatal (10-12 day old) Wistar rats were anaesthetized (Urethane, 2 g kg^{-1}, i.p.) prior to removal of the spinal cord. Intracellular recordings were made from dorsal horn neurones and a lumbar dorsal root was stimulated at A- and C-fibre intensity (300 µA, 300µsec) to evoke a dorsal root-evoked synaptic potential (DR-EPSP).

Immunoreactivity for GLAST and GLT-1 was present in glial processes throughout the dorsal horn and spinal nucleus caudalis, and the labeling patterns for both transporters were similar with more intense staining in superficial laminae. EAAC-1 was localized to neuronal cell bodies and terminal varicosities throughout the dorsal horn. Inhibition of glutamate uptake by superfusion of L-PDC (1 mM, 20 min, n=6) significantly reduced (paired t-test, $P < 0.05$) the DR-EPSP amplitude (59 ± 12 %) and duration (61 ± 10 %). Superfusion of either of the mGluR agonists produced a similar DR-EPSP attenuation. L-AP4 (30 µM, 20 min, n=6) caused a significant reduction (paired t-test, $P < 0.05$) in the DR-EPSP amplitude (23 ± 6 %) and duration (35 ± 4 %). DHPG (100 µM,5 min, n=5) reduced the amplitude by 61 ± 11 % ($P < 0.05$) and the duration by 65 ± 19 % ($P < 0.05$).

The glutamate transporter proteins GLT-1, GLAST and EAAC-1 are localized within the rat dorsal horn. Pharmacological inhibition of glutamate uptake attenuated primary afferent-mediated neurotransmission onto dorsal horn neurones and this effect was mimicked by selective mGluR agonists. These data suggest that use-dependent alterations in synaptic glutamate levels could differentially activate glutamate receptor populations. This may be particularly relevant under conditions of intense afferent stimulation such as may occur after peripheral tissue damage or during inflammation.

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Scanziani, M. et al., (1997), Nature 385, 630-634.
Lehre, K.P. et al., (1995), J. Neuroscience 15, 1835-1853.

237P ALTERATIONS IN NEURONAL ION CHANNEL ACTIVITY BY AMYLOID β PROTEIN THAT ARE NOT ASSOCIATED WITH NEUROTOXICITY

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Amyloid β Protein (Aβ), the major component of senile plaques, has been implicated as the mediator of neurodegeneration observed in Alzheimer's disease. Its neurotoxic actions have been suggested to involve disrupted cellular ion homeostasis possibly via a modulatory action on ion channel activity.

Here we use the whole-cell configuration of the patch-clamp technique to study the effect of $A\beta_{1-40}$ on K^+ and Ca^{2+} channel currents in primary cultures of rat cerebellar granule and cortical neurones (Price *et al*, 1998). Electrophysiological changes were correlated to cell death using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay (Mosmann *et al*, 1983). For incubation of cells 1μM $A\beta_{1-40}$ or $A\beta_{40-1}$ (control) was added to the culture medium 24 hours before recording.

Incubating cortical neurones with non-aggregated 1μM $A\beta_{1-40}$ for 24 hours increased the Ca^{2+} channel current by almost 50% when compared to controls (control –94.5±10.0 pA/pF, n=39; $A\beta_{1-40}$ –141.1±11.6 pA/pF, n=47, P<0.01). Previous studies found 24 hour pretreament with $A\beta_{1-40}$ to increase N-type Ca^{2-} current in granule neurones (Price *et al*, 1998). In the presence of 1μM ω-conotoxin GVIA, $A\beta_{1-40}$ still increased cortical Ca^{2+} channel current by approximately 30% at the +10mV potential (control –37.0±3.0 pA/pF, n=42; $A\beta_{1-40}$ –48.3±4.8 pA/pF, n=39, P<0.05, *t* test). The addition of P-type antagonist ω-agatoxin IVA (30nM) prevented the increase in Ca^{2+} channel current induced by $A\beta_{1-40}$ (control –66.5±7.0 pApF, n=22; $A\beta_{1-40}$ – 79.5±9.7 pA/pF, n=20) suggesting the enhanced Ca^{2+} current in cortical neurones is comprised of an N and P-type component.

Incubation with 1μM $A\beta_{1-40}$ increased the K^+ channel current in cerebellar granule neurones by 27% at the +50mV potential (control 1.8±0.1 nA/pF, n=24; $A\beta_{1-40}$ 2.29±0.16 nA/pF, n=29, P<0.01, *t* test), an effect confined to the inactivating component of current (I_A) which was augmented by over 50%. Identical treatment protocols have no effect on I_A or I_K in cortical pyramidal neurones (control 0.45±0.05 nA/pF, n=23; $A\beta_{1-40}$ 0.5±0.05 nA/pF, n=20). The increase in granule neurone I_A was not Ca^{2+} activated as it was also observed when the experiment was repeated in the presence of 10μM $CdCl_2$ (control 0.63±0.04 nA/pF, n=13; $A\beta_{1-40}$ 0.98±0.07 nA/pF, n=16, P<0.001, *t* test).

The MTT assay for cell viability showed that 1μM $A\beta_{1-40}$ was only neurotoxic to these cells when allowed to aggregate.

These data suggest that unaggregated $A\beta_{1-40}$ has modulatory effects on both K^+ and Ca^{2+} channel activity which are not associated with cell death. This suggests the possibility of a physiological role for non-aggregated Aβ in the function of central neurones.

Price S.A. *et al* (1998), *NeuroReport.* 9, 539-545.
Mosmann T. *et al* (1983), *J.Immunol.Methods* 65, 55-63.

238P CLOTRIMAZOLE INHIBITION OF HUMAN RECOMBINANT CARDIAC L-TYPE Ca^{2+} CHANNEL α_{1C} SUBUNITS STABLY EXPRESSED

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Clotrimazole is an antifungal antibiotic which has received attention as a potentially useful agent in the treatment of cancer, because of its antiproliferative properties (Benzaquen *et al.*, 1995). Its usefulness may be limited, however, since a recent report has described a potent inhibitory action on L-type Ca^{2+} currents recorded in isolated guinea pig ventricular myocytes (Thomas *et al.*, 1999). The pharmacological properties of cardiac Ca^{2+} currents show marked species variation (e.g. Lacampagne *et al.*, 1995; Campbell *et al.*, 1996), and the actions of clotrimazole have not yet been studied on human cardiac Ca^{2+} currents.

Here, we describe the effects of clotrimazole on Ca^{2+} currents (recorded using the whole-cell configuration of the patch clamp technique with 20mM Ba^{2+} as charge carrier) in HEK 293 cells which have been stably transfected with the α_{1C} subunit of the human L-type cardiac Ca^{2+} channel, in the absence of auxiliary subunits (see Fearon *et al.*, 1997, for solution compositions and construction of cell line).

Using 100ms step depolarizations from -80mV to 0mV (0.2Hz), bath application of clotrimazole (0.25-25μM) caused reversible, concentration-dependent inhibitions of Ca^{2+} current amplitudes. For example, currents (measured at the end of step depolarizations) were inhibited 37.5 ± 3.8% (n=4 cells, P<0.02, paired Student's t-test) at 250nM clotrimazole. At 25μM, clotrimazole almost completely inhibited currents (95.7 ± 2.4% inhibition, n=3). Analysis of current-voltage relationships (n=11) revealed that clotrimazole inhibited current amplitudes at all activating test potentials examined (-30mV to +30mV). Clotrimazole also caused marked inactivation of Ca^{2+} currents: the time constant of decay of control currents, recorded at 0mV, was 278 ± 49ms, and this was significantly reduced (P<0.01) to 160 ± 35ms (n=11) in the presence of 1μM clotrimazole.

Our findings indicate that clotrimazole inhibits the recombinant human L-type Ca^{2+} channel α_{1C} subunit expressed in HEK 293 cells. The inhibitory effects we find are more potent than those reported in native guinea pig cardiac myocyte Ca^{2+} currents (Thomas *et al.*, 1999). Whether this is due to species variation, lack of auxiliary subunits in our HEK 293 cells or other reasons remains to be determined. However, if such effects of clotrimazole are also reproduced on native human Ca^{2+} channels, this would have potentially important implications for the clinical usefulness of this compound.

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Benzaquen, L.R. *et al.* (1995) *Nature Med.* 1, 534-540.
Campbell, D.L. *et al.* (1996) *J. Gen. Physiol.* 108, 277-293.
Fearon, I.M. *et al.* (1997) *J. Physiol.* 500, 551-556.
Lacampagne, A. *et al.* (1995) *Cardiovasc. Res.* 30, 799-806.
Thomas, G.P. *et al.* (1999) *Br. J. Pharmacol.* 126, 1531-1533.

239P INHIBITION OF CONTRACTILE RESPONSES BY THAPSIGARGIN IN PRECAPILLARY ARTERIOLAR SMOOTH MUSCLE CELLS

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It has been suggested there may be non-contractile Ca^{2+} compartments in smooth muscle. Furthermore, inhibition of sarcoplasmic reticulum (SR) Ca-ATPase, by thapsigargin (TG) or cyclopiazonic acid, has been shown to potentiate contractile responses to high concentrations of K^+, giving rise to the concept of the SR acting as a superficial buffer barrier (van Breemen *et al.*, 1995). The aim of this study was to investigate this hypothesis in arteriolar smooth muscle cells.

Male rabbits (1-1.5 kg) were killed by an intravenous overdose of 70 mg kg^{-1} sodium pentobarbitone and the pial membrane was removed from the brain to enzymatically and mechanically isolate small arteriolar fragments (20-40 μm external diameter). As described previously (Guibert & Beech, 1999), $[Ca^{2+}]_i$ was recorded by loading arterioles with fura-PE3AM (1 μM). $[Ca^{2+}]_i$ signals from smooth muscle cells are given as ratios of emission intensities for 355/380 nm excitation. Contraction was recorded with a video edge-detection system. Values are given as mean±s.e.mean and statistical significance was assessed by Student's *t*-test.

Arteriolar constriction in response to 60 mM K^+ was strongly inhibited by 1 μM TG pretreatment for 1 hr. Under control conditions, arterioles constricted to 69.3±5.6 % (n=9) of the initial diameter, whereas after TG-treatment constriction was to 98.4±0.8 % of initial diameter (n=9). The vehicle for TG was dimethylsulphoxide (DMSO); this had no effect on the constrictor response to 60 mM K^+ (n=6, $P>0.05$). By contrast, the Ca^{2+} response to 60 mM K^+ solution was not significantly modified by TG (fura-PE3 ratio of 0.032±0.006 versus 0.017±0.006 for control conditions; n=8 and 9 respectively; $P>0.05$).

Similarly, TG strongly inhibited the constrictor response to endothelin-1 (ET1) acting at ET_A receptors. Time-matched diameter recordings with 1 nM ET1 were performed in control conditions (giving constriction to 67±6.4 % of the initial diameter, n=4), in the presence of DMSO (constriction to 56.2±5.3 %, n=5; $P>0.05$ compared with control) and after pretreament with TG (constriction to 99.7±0.2 %, n=5). By contrast, TG did not significantly change the sustained Ca^{2+} response to 10 nM ET1, although it abolished the initial transient Ca^{2+} response (Guibert & Beech, 1999).

Thus, inhibition of SR Ca^{2+}-ATPase reduces the contractile effects of high K^+ solution and endothelin-1 in arteriolar smooth muscle cells without altering the sustained $[Ca^{2+}]_i$ response. The data may be explained by the existence of a non-contractile Ca^{2+} compartment that controls access of Ca^{2+}-influx to the contractile apparatus via SR Ca^{2+}-ATPase.

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Guibert C. & Beech D.J. (1999). *Journal of Physiology* 514, 843-856.
Van Breemen, C., Chen, Q. & Laher, I. (1995). *Trends in Pharmacological Sciences* 16, 98-105.

240P IONIC CURRENTS ACTIVATED BY DEPOLARISATION IN SMOOTH MUSCLE CELLS OF CEREBRAL PRECAPILLARY ARTERIOLES

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The purpose of this study was to determine the identity and relative amplitudes of K^+ and Cl^- channel currents activated by depolarisation in precapillary arteriolar smooth muscle cells.

Pial membrane was obtained from male 1.5 kg rabbits given a lethal dose of sodium pentobarbitone. Whole-cell patch-clamp recordings were made from freshly isolated arteriolar segments with an external diameter of 20-40 μm. The KCl-based patch pipette solution included 0.2 mM EGTA, and the standard 130-mM NaCl bath solution included 5 mM K^+ and 1.5 mM Ca^{2+}. Data are given as mean±s.e.mean.

Application of 1-s depolarising ramp changes in voltage from the holding potential of –80 mV to +40 mV elicited a voltage-dependent outward current that was often preceded by an inward current. The inward current was abolished if Ca^{2+} in the bath solution was replaced by Mg^{2+} (n=9).

Bath application of 100 nM penitrem A to block BK_{Ca} channels inhibited the outward current in some arterioles, giving a mean reduction of 39.7±43.3 pA at +20 mV and 18.0±13.8 pA at –10 mV, and no effect at –40 mV (n=8 for each). The mean reduction of current at +20 mV caused by 100 nM iberiotoxin was 7,5±5.9 pA (n=5).

3,4-diaminopyridine (3,4-DAP) was used to block K_V channels in zero-Ca^{2+} bath solution containing 100 nM penitrem A, 1 μM glibenclamide and 50 μM niflumic acid. 3,4-DAP (1 mM) inhibited the outward current by 164.9±32.9 pA at +20 mV, by 72.9±9.9 pA at –10 mV and 15.8±16.1 pA at –40 mV (n=10 for each). Measurement of current at the end of 1-s square voltage steps from –80 mV indicated the threshold for activation of K_V channels was –56.4±3.0 mV (n=10). In one experiment in which the smooth muscle cell recorded from was not coupled to other cells in the arteriole the threshold for activation was -55mV.

In Ca^{2+}-containing solution there was a residual current that was resistant to block by 0.1 mM Ba^+, 4 mM TEA^+, 100 nM penitrem A, 1 μM glibenclamide, 1 mM 3,4-DAP and 100 nM apamin. This current was evident during 1-s ramp changes in voltages from –80 to +40 mV and as a slow tail current upon repolarisation to –80 mV. It was inhibited by niflumic acid (EC_{50} 59.9 μM at +35 mV for 8 experiments) or substitution of bath Cl^- by 130 mM aspartate (n=5). Niflumic acid-sensitive current reversed at –19.1±5.1 mV (n=21).

The data suggest that BK_{Ca}, K_V and Ca^{2+}-activated Cl^- channels are expressed and functional in cerebral arteriolar smooth muscle cells. K_V channel current appears to be dominant and activate at the most negative voltage.

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241P A ROLE FOR NF-κB IN THE STIMULATION BY CYTOKINES OF ENDOTHELIN-1 RELEASE IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

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Endothelin-1 (ET-1) mRNA expression and peptide production in human vascular smooth muscle cells (HVSMCs) are markedly increased by exposure to cytokines (Woods *et al.*, 1999). Transcription factor NF-κB often mediates the effects of cytokines in target cells (Siebenlist *et al.*, 1994; Thanos and Maniatis, 1995). In this study we have attempted to determine whether exposure of HVSMCs to cytokines results in activation of NF-κB.

Saphenous vein (SV) was obtained from patients undergoing coronary artery bypass graft surgery. Explants of HVSMCs were grown in DMEM supplemented with 2mM glutamine, streptomycin ($100\mu g.ml^{-1}$), penicillin ($100U.ml^{-1}$) and 15% foetal calf serum (37°C; 5% CO_2; 95% air). HVSMCs were identified by α-actin staining. HVSMCs were incubated with TNF-α ($10ng.ml^{-1}$) and IFN-γ ($1000U.ml^{-1}$) for 48h in the presence or absence of BAY 11-7082 (an inhibitor of cytokine induced IκB-α phosphorylation). ET-1 levels were measured by specific sandwich ELISA (R&D Systems). Total RNA was isolated by a guanidinium thiocyanate/isopropanol method with minor modifications (Chomczynski and Sacchi, 1987). Reverse transcription coupled with polymerase chain reaction was performed using standard methods. or Western blotting, nuclear extracts were prepared (Dignam *et al.*, 1983) and NF-κB was detected by immunoblotting using anti-NFκB p65 antibody (Biomol Research Laboratories).

BAY 11-7082 inhibited cytokine-stimulated ET-1 release from SV with an IC_{50} value of 43.6μM (33.9-56.1μM; n=4). Densitometry indicated that BAY 11-7082 (100μM; n=3) also inhibited by 54.2±1.3% the cytokine-stimulated elevation in expression of mRNA for prepro-ET-1 seen over 48h. Exposure of SV HVSMCs to a combination of TNF-α and IFN-γ resulted in a translocation of NF-κB from the cytosol to the nucleus as determined by Western blotting. This nuclear translocation of NF-κB was detectable after 15 min and was still visible up to 48h following treatment with cytokines.

In conclusion, NF-κB activation is involved in the stimulation by cytokines of ET-1 release from HVSMCs. As up-regulated production of ET-1 within VSMCs may underlie the causative role of ET-1 in a number of disease states this finding indicates that NF-κB within HVSMCs could be central to a number of vascular pathologies.

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Woods, M., Mitchell, J.A., Wood, E.G. *et al.* (1999). *Mol. Pharmacol.*, **55**, 902-909.
Thanos, D. and Maniatis, T. *(1995). Cell*, **80**, 529-532.
Siebenlist, U., Franzoso, G., and Brown, K. (1994*). Annu. Rev. Cell Biol.*, **10**, *405-455.*
Chomczynski, P. and Sacchi, N. (1987). *Anal. Biochem.*, **162**, 156-159.
Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983). *Nucleic Acids Res.*, **11**, 1475-1489.

242P COX-2 DIFFERENTIALLY REGULATES GM-CSF AND G-CSF PRODUCTION BY HUMAN VENOUS SMOOTH MUSCLE CELLS

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Neutrophil recruitment and activation are primary events in the development of a number of vascular diseases. Once present in the vessel wall neutrophils do not differentiate and rapidly die. Neutrophil survival can be promoted by the cytokines granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF). We have recently shown that human venous smooth muscle cells can be induced to release GM-CSF and to express cyclo-oxygenase-2 (COX-2) when stimulated with inflammatory cytokines such as IL-1β and TNFα (Mitchell *et al.*, 1998). Furthermore, we have shown that GM-CSF release from these cells is further increased when COX-2 activity is blocked by indomethacin and a range of other NSAIDs including the COX-2 selective compound, L-745,337 (Stanford *et al.*, 1998). Here we show that venous cells are also capable of releasing G-CSF after stimulation with inflammatory cytokines. Furthermore we have investigated the effects of COX-2 on G-CSF release from human venous smooth muscle cells.

Samples of saphenous vein (SV) were dissected clean, cut into small pieces and placed in supplemented culture medium as described previously (Bishop-Bailey *et al.*, 1997). Following explantation, cultured human venous smooth muscle cells were plated onto 96 well plates. When cells reached confluence culture medium was replaced with new medium. Cells were stimulated with increasing concentrations of IL-1β (0.01-10ng/ml), TNFα (0.01-10ng/ml) or a combination of the two cytokines (10ng/ml). In some experiments cells were pretreated with indomethacin (10μM). After 24 hours the medium was removed. GM-CSF and G-CSF release were measured by ELISA.

As observed previously IL-1β and TNFα induced a concentration-dependent stimulation of GM-CSF release producing an Emax at 1.0ng/ml and 10ng/ml respectively (n=6). Similarly G-CSF release was stimulated in a concentration-dependent manner by IL-1β reaching an Emax at 1.0ng/ml (n=6). However only the highest concentration of TNFα tested (10ng/ml) stimulated G-CSF release (Figure 1b). Indomethacin significantly potentiated the release of GM-CSF from stimulated cells. In contrast, G-CSF levels were significantly reduced in the presence of the COX inhibitor.

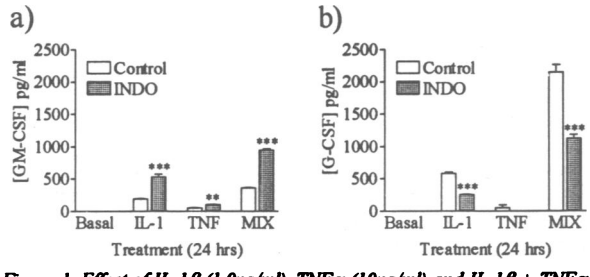

*Figure 1. Effect of IL-1β (1.0ng/ml), TNFα (10ng/ml) and IL-1β + TNFα (MIX: 10ng/ml) on the release of a) GM-CSF and b) G-CSF from human vascular smooth muscle cells in the presence and absence of indomethacin (INDO: 10μM). Data is shown as mean±s.e.m: n=6: **P<0.01, ***P<0.001 (Unpaired t test).*

Here we show that human venous smooth muscle cells release both G-CSF and GM-CSF following stimulation with inflammatory cytokines. Furthermore we show that, in contrast to its suppression of GM-CSF release, COX-2 activity potentiates G-CSF release. These observations add further support to the hypothesis that COX-2 mediates inflammatory responses at the level of cytokine release.

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Bishop-Bailey, D., *et al.*, (1997). *Br. J. Pharmacol.* 120, 73P.
Mitchell, J.A., *et al.*, (1998). *Br. J. Pharmacol.* 123, 74P.
Stanford, S.J., Pepper J.R. & Mitchell, J.A. (1998). *Br. J. Pharmacol.* 125, 20P.

243P ENDOTOXAEMIA ALTERS INOTROPIC RESPONSE TO ISOPRENALINE IN PAPILLARY MUSCLES: AN EFFECT THAT IS MODULATED BY L-NAME

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Nitric oxide (NO) has been implicated in the control of myocardial function under physiological conditions (Brutsaert *et al*, 1988). In sepsis, large amounts of NO are produced as a result of induction of NO synthase (iNOS), however, controversy exists as to whether this results in the depression of myocardial function seen (Brady *et al*, 1992, Herbertson *et al*, 1996, Keller *et al*, 1995). We have previously shown that in endotoxaemia, L-arginine becomes rate-limiting for the production of NO in atria, and that this tissue has a decreased inotropic response to the beta agonist isoprenaline (Price *et al* 1999). Moreover, when L-arginine is added, the increased NO produced restores the diminished response to isoprenaline, and is therefore positively inotropic. Here we have furthered these studies by assessing the effects of endotoxaemia and NOS inhibition on left ventricular papillary muscle contractility in response to isoprenaline.

Control and endotoxaemic (LPS 20mg^{-1}kg^{-1} i.p 4h) male Wistar rats (250-300g) were anaesthetised with sodium pentabarbitone (100mg^{-1}kg i.p) and killed by cervical dislocation. Hearts were removed and left ventricular papillary muscles were dissected out, and attached to a force-transducer in gassed (95%O_2:5%CO_2) Krebs' buffer. Preparations were maintained at 37°C and paced at 0.5Hz (100% above the threshold voltage). Peak-generated tension was measured in the presence and absence of L-NAME (10^{-3}M) and L-arginine (10^{-3}M), and concentration-response curves in response to isoprenaline (10^{-10} to $3x10^{-6}$M) were performed.

All data are shown as mean ± S.E.M unless otherwise stated. There was no difference in baseline contractility (dP/dT, milligrammes/second) between papillaries from control or endotoxaemic rats (control; 6.23±0.54, n=34: LPS; 5.35± 0.59, n=27). Neither L-arginine nor L-NAME altered baseline papillary contractility in either group (control+L-arginine;6.2±1.6:n=6,control+L-NAME;5.76±0.7:n=5, LPS+Larginine;5.30±1.15:n=4,LPS+LNAME;5.50±0.2:n=4). Endotoxaemia increased the inotropic response to isoprenaline, shown as percentage increase rate of developed tension *(p<0.0001 Two-way ANOVA)*(Figure 1). L-NAME did not alter the inotropic response to isoprenaline in papillaries from control rats (Emax (% control; 50.56±11.46 (n=9): control+L-NAME; 51.91±15.6:n=4), however, in papillaries from endotoxaemic rats, L-NAME reduced this response*(p<0.011 Two-way ANOVA)*(Figure 2).

Figure 1

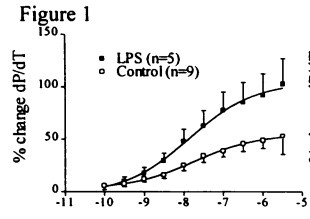
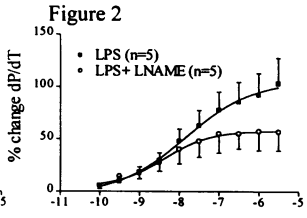


Figure 2



This data shows that left ventricular papillary muscles display hyperreactivity to isoprenaline *ex vivo* after *in vivo* administration of endotoxin. The increased response to isoprenaline seen in papillary contractility was reversed by L-NAME, suggesting that as in atrial tissue, NO from iNOS is positively inotropic and may be cardioprotective in this model. These observations suggest that inhibitors of iNOS may have detrimental effects on cardiac function in some patients with septic shock.

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Brady A.J. *et al.* (1992) *Am. J. Physiol.* 263, H1963-6
Brutsaert D.L.M. *et al.* (1988) *Circ. Res.* 62, 358-66
Herbertson M.J. *et al.* (1996) *Am. J. Physiol.* 270, H1979-84
Keller R.S. *et al.* (1995) *Shock.* 4, 338-44
Price S. *et al* (1999) *Br. J. Pharmacol.* 126, 77P

244P ODQ, AN INHIBITOR OF SOLUBLE GUANYLYL CYCLASE, REDUCES THE RENAL/LIVER DYSFUNCTION/INJURY IN *IN VIVO* MODELS OF GRAM-POSITIVE AND GRAM-NEGATIVE SHOCK IN THE RAT

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Endotoxaemia, sepsis and Gram-positive shock lead to circulatory failure and multiple organ dysfunction syndrome (MODS) (Natanson *et al.*, 1989). Here we investigate the effects of ODQ ([1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxaline-1-one]), an agent which inhibits soluble guanylyl cyclase (Garthwaite *et al.*, 1995), on the renal/liver dysfunction/injury associated with Gram-positive and Gram-negative shock in the anaesthetised rat.

Male Wistar rats (240-300 g, n=57) were anaesthetised with sodium thiopentone (120 mg·kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration. Catheters were inserted in the right carotid artery for the measurement of mean arterial blood pressure (MAP) and heart rate (bpm), and the jugular vein for drug administration. At completion of the surgical procedure, animals were allowed to equilibrate for 15 min after which they received a bolus of ODQ (2 mg·kg^{-1} i.p.) or its vehicle (30% v/v DMSO in saline, 2 ml·kg^{-1} i.p., *in vivo* concentration < 1%). After 2 h, either (i) Gram-positive or (ii) Gram-negative septic shock was induced by administration of (i) two cell wall fragments [lipoteichoic acid (LTA, 3 mg·kg^{-1} i.v.) + peptidoglycan (PepG, 10 mg·kg^{-1} i.v.)] of the pathogenic Gram-positive bacterium *Staphylococcus aureus* (Kengatharan *et al.*, 1998) or (ii) lipopolysaccharide (LPS, 6 mg·kg^{-1} i.v.) of the pathogenic Gram-negative bacterium *Escherichia coli* (Leach *et al.*, 1998). After this, an infusion of saline was started (1.5 ml·kg^{-1}·h^{-1} i.v.) and maintained for the duration of the experiments. All values are expressed as mean ± s.e.mean.

Gram-positive and Gram-negative shock for 6 h resulted in significant rises in the serum levels of urea and creatinine (creat) (indicators of renal dysfunction/failure), aspartate aminotransferase (AST, a non-specific marker for hepatic injury) and alanine aminotransferase (ALT, an indicator of liver injury). ODQ significantly attenuated the renal dysfunction as well as the liver injury/dysfunction caused by LTA/PepG or LPS (Table 1). In rats not receiving LTA/PepG or LPS (sham + ODQ, n=6), ODQ did not affect any of the parameters measured (data not shown). In rats receiving LTA/PepG or LPS (shock + vehicle of ODQ, each n=6), the vehicle of ODQ did not affect any of the parameters measured (data not shown).

Table 1: Effects of ODQ after 6 h on organ injury/dysfunction (urea: mmol·L^{-1}, creat: μmol·L^{-1}, AST, ALT: i.u.·L^{-1}) caused by LTA/PepG or LPS (* p<0.05 vs. sham + saline, # p<0.05 vs. shock + saline. ANOVA followed by Bonferroni's test).

	urea	creat	AST	ALT
Sham + saline (n=6)	5±0.4	35±3	176±20	100±10
LTA/PepG + saline (n=8)	16±2 *	58±6 *	464±63 *	251±70
LTA/PepG + ODQ (n=9)	9±0.5 #	39±1 #	200±15 #	77±8 #
LPS + saline (n=8)	24±1 *	61±5	553±60 *	378±72 *
LPS + ODQ (n=8)	18±1 *#	53±12	374±41 *#	186±28 #

Thus, pretreatment of rats with ODQ significantly reduced the renal and liver dysfunction and/or injury caused by Gram-positive or Gram-negative septic shock. The mechanism of action of ODQ warrants further investigation.

Garthwaite, J., Southam, E., Boulton, C.L., *et al.*, (1995) *Mol. Pharmacol.*, 48, 184-188.
Kengatharan, K.M., De Kimpe, S., Robson, C., *et al.*, (1998) *J. Exp. Med.*, 188, 1-11.
Leach, M., Frank, S., Olbrich, A. *et al.*, (1998) *Br. J. Pharmacol.*, 125, 817-825.
Natanson, C., Danner, R.L., Elin, R.J., *et al.*, (1989) *Clin. Invest.*, 83, 243-251.

245P EFFECT OF THE NITRIC OXIDE-ASPIRIN ADDUCT, NCX 4016, ON RELAXATRION OF THE CORPUS CAVERNOSUM AND PLATELET FUNCTION *IN VITRO*: POTENTIAL ROLE IN THE TREATMENT OF ERECTILE DYSFUNCTION

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Platelets may be involved in the aetiology of erectile dysfunction (ED) since they release vasoconstrictors (Khan et al., 1999a). The nitric oxide (NO)-aspirin (ASA) adduct, NCX 4016 possesses the capacity not only to inhibit platelet activity but also to relax vascular smooth muscle tissue. It is reasonable to suggest, therefore, that NCX4016 may be useful in the treatment of ED through a direct NO-mediated relaxant effect on the corpus cavernosum (CC) and inhibition of platelet activity by both its ASA-and NO-moieties. In order to test this possibility, the effect of NCX4016 on relaxation of the rabbit corpus cavernosum and on the expression of adhesion molecules and the formation of cGMP and thromboxane A_2 by human platelets in vitro was assessed.

New Zealand White rabbits were killed by cervical dislocation, cavernosal tissue excised and mounted in organ baths for recording of isometric tension (Khan et al., 1999b). Tissues were pre-contracted with phenylephrine (10 μM) and relaxation responses to carbachol assessed in the presence of NCX4016. Blood was taken from normal subjects, stimulated with 100 μM ADP and P-selectin and GPIIbIIIa expression measured using flow cytometry. Platelet rich plasma (PRP) was also prepared and the effect of NCX4016 on Ca^{2+} ionophore A23187 (10 μM)-stimulated thromboxane A_2 (TXA_2) and nitroprusside-stimulated cyclic GMP (cGMP) assessed by radioimmunoassay (Jeremy et al., 1993).

NC4016 relaxed rabbit corpus cavernosum and inhibited ADP-stimulated expression of GPIIbIIIa and P-selectin and A23187-stimulated TXA_2 but promoted cGMP formation by human platelets in dose dependent manners (tables 1and 2)

Table 1. Effect of NCX4016 on % maximal inhibition of ADP-stimulated expression of GPIIbIIIa and P-selectin and A23187-stimulated TXA_2 (C) by human platelets and % relaxation of rabbit corpus cavernosum (± s.e.m., n = 10) * $p < 0.05$, (ANOVA) compared to control values.

	[NCX] (μmol.l-1)		
	1	10	100
A) GPIIbIIIa	1±0.2	45±8.4*	96±12*
B) P-selectin	3±0.5	48±9.3*	98±20*
C) TXA_2	11±1.4	53±8.6*	92±24*
D) CC relaxation	3±0.2	15±3.2*	48±7.2*

Table 2 Effect of NCX 4016 on cyclic GMP formation (fmoles / 10^8 platelets / min) by human platelets (mean ± S.E.M., n = 10)
* $p < 0.05$, (ANOVA) compared to control values.

	[NCX] (μmol.l-1)			
	0	1	10	100
[cGMP]	6.4±1	15±1.4*	130±30*	370±45*

These data indicate that NCX4016 may prove useful in the treatment of ED through a direct relaxatory effect of its NO moiety on the corpus cavernosum. Since ED is associated with endothelial dysfunction it is reasonable to suggest that platelets may adhere to cavernosal walls during erection due to increased shear stress. In turn, the local release of TXA_2 by adherant platelets would promote contraction of the cavernosal tissue and therefore impair erection. Thus, the suppression of platelet adhesion molecules and TXA_2 formation by NCX 4016 may also be a beneficial property in reducing acute ED as well as having potentially long term beneficial effects.

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Jeremy, J.Y., *et al.*, (1993)*Eur. J. Pharmacol.* **245**, 67-73.
Khan, M., *et al.*, (1999a) *Prostagl. Leukotr. Essential.Fatty Acids.* in press.
Khan, M., *et al.*, (1999b) *Br.J.Urol.* in press

246P ANTISERUM TO TUMOR NECROSIS FACTOR OR INTERLEUKIN 1 DOES NOT ABOLISH THE SECOND WINDOW OF PROTECTION INDUCED BY LIPOTEICHOIC ACID IN THE RAT HEART

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Pretreatment of rats with a low dose of endotoxin (lipopolysaccharide, LPS) for 16 h protects the heart against ischaemia-reperfusion injury (Zacharowski *et al.*, 1999). Similarly, pretreatment of rabbits with monophosphoryl lipid A, a non-pyrogenic derivate of *Salmonella* lipopolysaccharide, also reduces myocardial infarct size (Baxter *et al.*, 1996). This study was designed to investigate the effects of pretreatment with lipoteichoic acid (LTA), a cell wall fragment of *Staphyloccocus aureus*, on (i) myocardial infarct size in the rat, and (ii) mRNA levels of TNFα and IL-1β in the heart. Finally, we have investigated whether antibodies against TNFα or IL-1β reduce the cardioprotective effects of LTA.

Twenty-eight male Wistar rats (240-300 g) were pretreated with i.p. boluses of (1) an antibody against TNFα (TNFα-Ab, 2 h before LTA treatment, 100 $mg \cdot kg^{-1}$; n=6), (2) an antibody against IL-1β (IL-1β-Ab, 2 h before LTA treatment, 100 $mg \cdot kg^{-1}$; n=6), (3) LTA (16 h pretreatment, 1 $mg \cdot kg^{-1}$; n=8) or saline (1 $ml \cdot kg^{-1}$; n=8). 16 h after LTA or saline pretreatment, rats were anaesthetised (thiopentone sodium, 120 $mg \cdot kg^{-1}$ i.p.), tracheotomised and ventilated (tidal volume: 10 $ml \cdot kg^{-1}$, 70 $strokes \cdot min^{-1}$, inspiratory oxygen-concentration: 30%, positive end-exspiratory pressure: 1-2 mmHg). The carotid artery was cannulated to measure mean arterial blood pressure. Following a left-sided thoracotomy, a needle was placed around the left anterior descending coronary artery (LAD). The animals were allowed to recover for 30 min and subsequently the LAD was occluded for 25 min and then reperfused for 2 h. At the end of the experiment, the LAD was re-occluded and 1 ml of Evans Blue dye (2% w/v) was injected to determine the perfused and the non-perfused (area at risk, AR) myocardium. Infarct size (IS) was determined by incubation of the slices of the heart with p-nitro-blue tetrazolium (NBT, 0.5 $mg \cdot ml^{-1}$). The mRNA levels of TNFα/IL-1β (n=3) were determined as described previously (Frank *et al.*, 1999).

The AR was similar in all groups studied (Table 1). When compared to vehicle, pretreatment of rats with LTA for 16 h caused a significant reduction in IS of approximately 60 %. Antibodies against TNFα or IL-1β did not abolish the LTA-induced cardioprotection. There was a small increase in the cardiac levels of TNFα/IL-1β mRNA after LTA pretreatment. There were no haemodynamic differences between any of the groups studied (data not shown).

Table 1: Infarct size and mRNA data (expressed as x-fold induction of control). ND = not determined. (* $p<0.05$ vs. saline, ANOVA followed by Bonferroni's test).

	IS (%)	AR (%)	TNFα	IL-1β
(1) TNFα-Ab + LTA	20±7 *	51±2	ND	ND
(2) IL-1β-Ab + LTA	20±6 *	52±1	ND	ND
(3) LTA	18±6 *	53±2	5 *	4 *
(4) saline	60±3	48±4	1	1

Pretreatment of rats with LTA causes a substantial protection against a subsequent period of myocardial ischaemia and reperfusion. The cardioprotective effects of LTA were not abolished by antibodies against TNFα or IL-1β, suggesting that these cytokines are not involved in the mechanism of cardioprotection of LTA.

Baxter, G.F., Goodwin, R.W., Wright, M.J., *et al.*, (1996). *Br. J. Pharmacol.*, 117, 1685-1692.
Frank, S., Zacharowski, K., Wray, G.M., *et al.*, (1999). *FASEB J*, 13, 869-882.
Zacharowski, K, Thiemermann, C. (1999). *Br. J. Pharmacol.*; in press

247P OXIDATIVE STRESS ACTS SYNERGISTICALLY WITH IL-1β, BUT NOT TNFα, TO ENHANCE IL-8 RELEASE BY HUMAN PULMONARY ARTERY SMOOTH MUSCLE CELLS

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Neutrophil recruitment and activation play crucial roles in many disease processes affecting the lung and are mediated the chemokines. Interleukin (IL)-8 is a powerful chemotactic agent for neutrophils and stimulates vascular smooth muscle cell migration (Yue et al. 1994). Recently we have suggested that vascular smooth muscle cells are an important source of IL-8 formation in the lung (Jordan et al. 1999). In other cell types, IL-8 release is stimulated by cytokines as well as oxidative stress (Lakshminarayananet et al. 1997). Thus, the purpose of this study was to investigate the effects of these stimuli on IL-8 release by human pulmonary artery smooth muscle cells.

Human Pulmonary artery (HPA) was obtained from surgically resected lung, and smooth muscle cells cultured in supplemented Dulbecco's Modified Eagle Medium, with 10% foetal calf serum (FCS), as described previously (Jourdan. 1999). Cultured myocytes were seeded into 96 well plates, growth arrested with serum free medium, and treated the following day for 24 hours with drugs and cytokines dissolved in DMEM with 10% FCS. IL-8 release was measured by ELISA (R&D Systems). Cellular respiration was measured by the ability of cells to convert 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan, quantified by the absorbance of treated cells at 550nm, as described previously (Jourdan. et al 1999) All data are n=9 from 3 experiments.

When stimulated with IL-1β or TNF-α (0.001 to 10 ng ml^{-1}) IL-8 was released by myocytes in a concentration dependant manner (EC_{50} = 0.25 ng ml^{-1} and 1.31 ng ml^{-1} respectively). Xanthine oxidase (XO; 10 iu ml^{-1}), which catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid with the concomitant production of superoxide anions, significantly inhibited the conversion of MTT to formazan by these cells (O.D. at 550nm. control, 0.554±0.039; plus XO, 0.399±0.04. $p<0.05$), indicative of an oxidant stress and stimulated the release of low, but measurable levels of IL-8 (figure 1). When stimulated with a sub-maximal concentration of IL-1β (0.1 ng ml^{-1}), but not TNF-α (1 ng ml^{-1}), IL-8 release was increased in a synergistic fashion by co-stimulation with XO (figure 1).

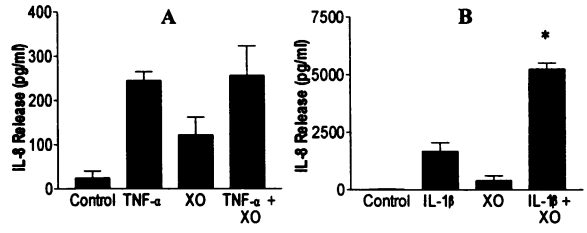

Figure 1: The effect of xanthine oxidase ± TNFα (A) and IL-1β (B) on IL-8 release by HPA myocytes.*$P<0.05$ vs. IL-1β alone.

The findings here that XO synergises with IL-1β, but not TNFα, to release IL-8 suggests a complex interaction between oxidant stress and cytokines in the induction of chemokines. Moreover, this data implicates IL-8 release as a mechanism by which oxygen derived free radicals mediate inflammation in the lung in diseases such as ARDS.

This work was funded by a grant from the Wellcome Trust.

Jordan, S. et al, (1999). Am. J. Resp. Crit. Care Med. 159(3). A511.
Jourdan, K. et al., (1999). FASEB, 13, 1025-1030
Lakshminarayanan, V. et al., (1997). J. Biol. Chem. 272(52): 32910-8.
Yue, T.L. et al., (1994). Circ. Res. 75(1). 1-7.

248P EFFECTS OF DISEASE MODIFYING ANTI-RHEUMATIC DRUGS AND CORTICOSTEROIDS ON PROSTAGLANDIN E_2 AND GMCSF PRODUCTION BY CYTOKINE-STIMULATED HUMAN SYNOVIOCYTES

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Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used treatment for osteo- and rheumatoid arthritis (RA). Despite their popularity NSAIDs are not disease modifying and produce well-characterised side effects, such as gastric ulceration, generally attributed to their ability to inhibit cyclo-oxygenase-1, (COX-1), (Vane *et al*, 1998). Prostaglandin $(PG)E_2$, a product of COX activity has been shown to inhibit the production of granulocyte macrophage colony stimulating factor (GMCSF), (Agro *et al*, 1996). GMCSF, which is elevated in rheumatoid arthritis (al-Janadi *et al* 1996), increases neutrophil activity and longevity. Neutrophils are strongly associated with joint damage in RA and NSAID induced gastric damage is clearly associated with neutrophil activity (Wallace *et al*, 1990). We have previously shown that NSAIDs reduce PGE_2 production by stimulated human synoviocytes and that this causes a reciprocal increase in GMCSF production (Breese *et al*, 1999). Here we have examined the effect of disease modifying anti-inflammatory drugs (DMARDs) and corticosteroids on PGE_2 production and looked for influences on the production of GMCSF.

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

Dexamethasone and prednisolone reduced both PGE_2 and GMCSF production. Interestingly chloroquine and penicillamine also reduced PGE_2 production without effect on GMCSF production. In addition, 10^{-6}M auranofin reduced PGE_2 production from stimulated synoviocytes to less than 43 ± 12% of control (n=4) without effect on GMCSF production.

Drug	PGE_2 IC_{50} nM	GMCSF IC_{50} nM
Dexamethasone (n=9)	0.39	0.003
Prednisolone (n=7)	3.3	6.5
Penicillamine (n=4)	17,000	-
Chloroquine (n=6)	589	-

Table 1: Effect of inhibitors on PGE_2 and GMCSF release from cytokine stimulated human synoviocytes.

Our results suggest steroids may be a successful treatment of arthritis because they inhibit both COX activity and GMCSF production. This later effect would reduce GMCSF-dependent neutrophil recruitment in inflammation. Moreover, penicillamine and chloroquine also reduce PGE_2 without increasing GMCSF production. This may partly explain why these DMARDs are disease modifying and gastric sparing.

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Agro, A. *et al.* (1996) *J. Rheumatol.*, **23**,862-8.
al-Janadi, N *et al.* (1996) *J. Rheumatol.*,**23**,809-14.
Breese, E.J. et al (1999) *British J Pharmacol.*, **126**, suppl 155P.
Vane, J.R. *et al.* (1998) *Annu.Rev.Pharmacol.Toxicol.*, **38**,97-120.
Wallace, et al (1990) Am. J .Physiol., **259**,462.

249P EFFECTS OF HEPARIN UPON ADHESION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS TO HUMAN STIMULATED UMBILICAL VEIN CELLS AND ADHESION MOLECULE EXPRESSION *IN VITRO*

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Heparin and other glycosaminoglycans are known to possess anti-inflammatory properties. We have demonstrated previously that heparin inhibits adhesion of polymorphonuclear leucocytes to stimulated human umbilical vein endothelial cells (HUVECs) in a manner which is not dependent on the expression of endothelial cell adhesion molecules (Lever & Page, 1998a) and is *via* a mechanism unrelated to anticoagulant activity (Lever & Page, 1998b). In the present study, we have investigated the effects of unfractionated heparin upon the adhesion of human peripheral blood mononuclear cells (HPBMNC) to HUVECs. Mononuclear cells were isolated from the venous blood of healthy volunteers (n=6) by density-dependent centrifugation and were radiolabelled with ^{51}Cr. Monolayers of HUVECs were grown to confluency in flat-bottomed 96 well plates and were stimulated for six hours with interleukin-1β (IL-1β; 100 Uml^{-1}), tumour necrosis factor-α (TNF-α; 1000 Uml^{-1}) or bacterial lipopolysaccharide (LPS; 100 μgml^{-1}) in the absence and presence of heparin (50 - 1000 Uml^{-1}). Following stimulation, endothelial cells were washed and 2 x 10^5 radiolabelled HPBMNC added to each well. Cells were allowed to adhere for 30 minutes at 37°C, following which, non-adherent cells were removed and adherent cells lysed with 1% Nonidet-P40. Lysates were γ-counted, and adhesion quantified. Data were analysed using ANOVA followed by Dunnett's test. Heparin was found to inhibit the adhesion of HPBMNC to stimulated HUVECs at most concentrations tested {$P<0.05$; maximum inhibition 98 ± 7% using 1000Uml^{-1} heparin (IL-1β-stimulated cells)}.

Enzyme linked immunosorbant assays (ELISA) were performed to assess the effects of heparin upon expression of the endothelial adhesion molecules intercellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both of which are involved in mononuclear cell adhesion. HUVECs were treated with stimuli ± heparin as before, were washed and a primary anti-human-ICAM-1, -VCAM-1 or control mouse IgG_1 added. Following a one hour incubation period at room temperature, cells were washed and the secondary, goat-anti-mouse-IgG_1 peroxidase-linked antibody was added for a further hour. Finally, cells were washed, substrate added and allowed to develop at room temperature. Plates were analysed colorimetrically at 450nm and data treated as for adhesion experiments. Heparin inhibited significantly ($P<0.05$) the expression of both ICAM-1 and VCAM-1 on stimulated HUVECs, although only at the higher concentrations used {maximum inhibition of ICAM-1 expression 78 ± 8%; maximum inhibition of VCAM-1 expression 78 ± 13%; in both cases using 1000Uml^{-1} heparin (IL-1β-stimulated cells)}. Nonetheless, these results suggest that heparin is able to interfere with the upregulation of these immunoglobulin-like adhesion molecules on activated vascular endothelium - an effect which leads to a reduction in cellular adhesion. However, given that adhesion was inhibited by heparin at concentrations lower than those required to modulate adhesion molecule expression, we would conclude that additional mechanisms are also involved in the inhibition of mononuclear cell adhesion to vascular endothelium by heparin.

Lever, R. & Page, C.P. (1998a). *Br. J. Pharmacol.* 125 33P.
Lever, R. & Page, C.P. (1998b). *Am. J. Respir. Crit. Care Med.* 157 A190.

250P TIME COURSE OF CELLULAR RECRUITMENT AND EXPRESSION OF VCAM-1 FOLLOWING INHALED ANTIGEN CHALLENGE IN THE NEONATALLY-SENSITISED RABBIT

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Previous studies have shown that antibodies against the integrin subunit α_4 can inhibit the recruitment and activation of airway eosinophils following antigen challenge in the neonatally immunised rabbit (Metzger 1995; Gascoigne *et al*, 1999), suggesting a role for very late antigen-4 (VLA-4; $\alpha_4\beta_1$) in this model. To extend these studies, we have investigated the time course of antigen-induced cellular infiltration into the airways. Additionally, the number of α_4 positive cells and the expression of the VLA-4 counter ligand vascular cell adhesion molecule-1 (VCAM-1) was determined.

Four groups (n≥6) of NZW rabbits (2.4-3.4Kg) were immunised within 24h of birth and until 12 weeks of age with *Alternaria tenuis* antigen (Ag) in $Al(OH)_3$ gel (*i.p.*) (Herd & Page, 1996). Rabbits were then sedated with diazepam (2.5 $mg.kg^{-1}$ i.v.) and Hypnorm™ (0.4 $ml.kg^{-1}$ i.m.) and challenged with aerosolised Ag (20,000 PNU ml^{-1} in saline for 20 min). A bronchoalveolar lavage (BAL) was performed pre-Ag then +30 mins, +8h and +24h post Ag challenge. The lungs were then removed for histological assessment. All data were compared to pre-Ag values by Kruskal-Wallis; Dunns post test (*$p<0.05$, **$p<0.01$). Eosinophils were identified using a cyanide resistant peroxidase method (Li *et al.*, 1998). The primary antibodies used to identify inflammatory cells were MCA 800 (T cells, Serotec, UK), RAM II (monocytes, Dako, UK) and MCA 802 (neutrophils and monocytes, Serotec, UK). VCAM and α_4 were revealed using the monoclonal antibodies 8.1F8 and Max68-P (Celltech, UK) respectively.

The number of leucocytes recovered in the BAL fluid were increased at 8h and 24h post Ag challenge. Eosinophil and lymphocytes numbers were elevated by 8h, with a maximal increase at 24h. Neutrophils were significantly elevated at 8h and 24h, with maximal increase at 8h (Table 1).

Table 1. Total and differential cell counts in BAL fluid (10^4 cells/ml BAL fluid)

	totals	eosinophils	neutrophils	Lymphocytes
Pre- Ag	34.1±3.6	0.04±0.2	0.6±0.2	0.06±0.1
+30min	36.5±5.0	0.02±0.02	0.08±0.04	0.16±0.1
+8 h	200±16.9**	0.58±0.3	145±24**	0.56±0.3
+24 h	154±55.3*	9.42±2.8*	67.8±34.1*	4.1±1.6*

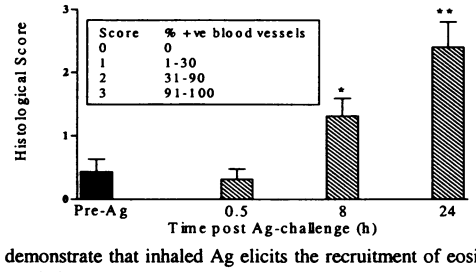
There was no change in mononuclear/macrophage numbers. Histological examination of the lung bronchial tissue revealed a similar time-course of eosinophil, neutrophil and lymphocyte recruitment as that detected in BAL fluid (Table 2).

Table 2. Histological evaluation of bronchial lung tissue (cells/5 fields x40).

	eosinophils	neutrophils	Lymphocytes
Pre- Ag	28.4±6.9	67±9.4	70±10.3
+30min	28.4±6.2	58±6.8	65±6.8
+8 h	42.7±11	102±12*	65±19
+24 h	118.5±29**	131±26*	108±22.8

There was a pronounced increase in the expression of VCAM-1 on endothelial cells at both 8h and 24h following Ag, with peak expression at 24h (figure 1). This corresponded with a significant increase in α_4-positive granulocytes cuffing blood vessels in lung tissue (pre-Ag 68.3±8.4 vs +24h 284±32($p<0.01$) cells/5 fields x40).

Figure 1. VCAM-1 expression on pulmonary endothelial cells following Ag-challenge.



These data demonstrate that inhaled Ag elicits the recruitment of eosinophils, neutrophils and lymphocytes in the rabbit lung. Futhermore, VCAM-1 expression parallels the recruitment of α_4-positive cells into the airways. These data extend our previous observations (Gascoigne *et al.*, 1999) that inflammatory cell recruitment is α^4-dependent in the neonatally sensitised rabbit model.

Gascoigne *et al.*, 1999 *Am. J. Resp. Crit. Care. Med.* 159 (3), A326.
Herd & Page, 1996 *In modelling the asthmatic response in-vivo. Ed: Reaburn D & Giembycz M.A. Birkhauser Verlag.*
Li *et al.*, 1998 *J. Immunol.* 1998 161, 3128-3135.
Metzger, 1995 *Springer Sem Immunopath* 16, 467-478.