81P OBSERVATIONS ON FUNCTIONAL RECEPTORS FOR CYSTEINYL-LEUKOTRIENES IN SELECTED SMOOTH MUSCLES

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The cysteinyl-containing leukotrienes (LT) C_4, D_4 and E_4 are potent inducers of airway obstruction. The $CysLT_1$ receptor in human airway smooth muscle is sensitive to all members of the first generation of antagonists, some of which currently are being introduced as new therapy in asthma. However, some effects of cysteinyl-leukotrienes are resistant to the same antagonists, and presumed to be mediated by the $CysLT_2$ receptor. A 6R-carboxyphenylthio-substitued analogue of LTE_4, BAY u9773, was reported to antagonize LTC_4 or LTD_4 at $CysLT_2$ receptors in some tissues (Tudhope et al 1994). The aim of the present study was to test if BAY u9773 caused similar inhibition of $CysLT_2$ responses in three different tissues.

Guinea-pig lung parenchyma (GPLP) or longitudinal ileum muscle (GPIL), and sheep trachea (ST) were prepared (Dahlén et al 1983; Tomioka et al 1990) and used for functional studies as described (Dahlén et al 1983). S-hexyl-glutathione (100 μM) and L-cysteine (5 mM) were included in the Tyrode's solution to inhibit catabolism of LTC_4 and LTD_4, respectively. Synthetic leukotrienes and BAY u9773 were obtained from Cascade Biochemicals (Reading UK), ICI-198,615 from Zeneca Pharmaceuticals (Alderly, UK) and other compounds and chemicals from standard commercial sources.

The concentration-response relation for LTC_4 in GPIL was unaffected even by very high concentrations of the potent $CysLT_1$ antagonist ICI-198,615 (0.1-1.0 μM, n=6 for controls and each concentration of antagonist; The pA_2 value against LTD_4 was 10.2±0.3[95% CI]). In contrast, BAY u9773 (0.3-10 μM) caused a concentration-dependent rightward displacement of the concentration-response relation for LTC_4, and Schild-plot analysis produced evidence of competitive antagonism with a pA_2 value of 6.5 ± 0.4.

In ST, ICI-198,615 failed to produce significant inhibition of the concentration-response relation for LTC_4 or LTD_4, whereas BAY u9773 (1-10 μM) caused an apparently parallell and similar rightward shift in the concentration-response relation for either leukotriene. For LTD_4, competitive antagonism was established by Schild plot analysis and the resulting pA_2 value was 6.4 ± 0.4.

In GPLP, ICI-198,615 was able to cause a small but distinct inhibition of the concentration-response curve for LTD_4. In the low range of ICI-198,615 concentrations (10-100 nM), evidence for competitive antagonism could be produced. Above this concentration, there was no further antagonism of the response to LTD_4, and the maximal shift represented only a 10 fold increase in the concentration of agonist required to cause half maximal contraction. BAY u9773 (10 μM) caused a displacement of the concentration-response relation for LTD_4 which was similar to that produced by the highest concentration of ICI-198,615. Combination of ICI-198,615 (1 μM) and BAY u9773 (10 μM) did not cause a larger antagonism than either compound alone.

Thus, the effects of BAY u9773 in GPIL and ST supported antagonist activity in both tissues at a similar $CysLT_2$ receptor. In contrast, the findings in GPLP may indicate the existence of further subclasses of receptors for cysteinyl-leukotrienes.

Dahlén et al, Acta Physiol Scand 118 (1983) 393-403.
Tomioka et al, Can J Physiol Pharmac 69 (1990) 805-811.
Tudhope et al, Eur J Pharmacol 264(1994) 317-323.

82P CHARACTERISATION OF CYCLO-OXYGENASE-2 INDUCTION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Prostanoids, produced by the enzyme cyclooxygenase (COX), are modulators of airway function. COX exists in two distinct isoforms. Constitutive COX-1 is considered to be a "housekeeping" enzyme and produces prostanoids under physiological conditions. Inducible COX-2 produces prostanoids in response to a number of inflammatory mediators. We have compared the ability of interleukin-1β (IL-1β), tumour necrosis factor α (TNFα) and interferonγ (IFNγ), alone, or in combination, to induce COX-metabolite formation in human airway smooth muscle (HASM).

HASM obtained from donor trachea was cultured in 6 or 96 well plates (Hirst *et al.*,1992).Protocols were designed to determine the relative contribution of phospholipase A_2 (PLA_2)/COX (endogenous arachidonic acid (AA)) or COX alone (exogenous AA) on the release of prostanoids in response to cytokines. Prostaglandin E_2, (PGE_2), 6 keto PGF1α and thromboxane B_2 (TXB_2) were measured by radioimmunoassay (Mitchell *et al.*, 1993) using the following protocols. Protocol I, cells were exposed to IL-1β, TNFα, and IFNγ, (10ng/ml) alone or in different combinations for 24 hours before the measurement of COX-metabolites; protocol II, in the same cells, fresh medium containing 30μM AA was added for 15 minutes and prostanoids measured. In separate experiments cells were cultured on 6 well plates and treated as described in protocol I, cells were extracted and western blot analysis performed using a specific COX-2 antibody (Cayman) as described by Mitchell *et al* (1994).

None of the individual cytokines alone produced significant levels of PGE_2 above control in either protocol I or II. The maximal increase in both protocols was seen when all the cytokines were used together (10ng/ml IL-Iβ, TNFα, IFNγ). The profile of release of prostanoids over 24 hours in response to cytmix was PGE_2 (6.04±1.8ng/ml), 6 keto PGF1α (8.14±2.1ng/ml), TXB_2 (0.40±-0.16ng/ml) control (<0.26ng/ml) n= 6-24 individual wells from 2 patients. Western blot analysis (n=3 from 2 patients) produced an inducible band at 42kD. No band was seen at the established molecular weight band of 70kD (Mitchell *et al* 1993). This discrepancy in molecular weight may be due to a degradation product, a post translational modification of COX, a novel COX isoform or a protein other than COX recognised by the polyclonal antibody.

Figure 1 Figure 2

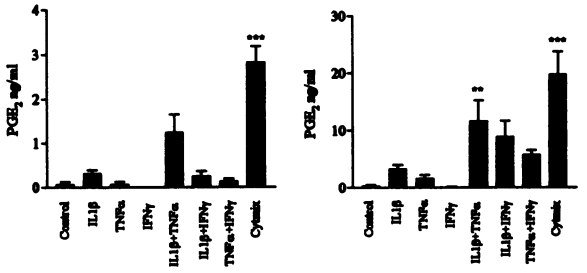

*Figure 1 and 2. The figure shows the effect of various cytokines on the release of PGE_2 from endogenous arachidonic acid (figure 1) or in the presence of exogenous arachidonic acid (figure 2). Data was analysed by ANOVA and Student-Newmans-Keuls (All treatments compared with control**$p<0.01$, ***$p<0.001$).*

In conclusion human airway smooth muscle produces prostanoids from both exogenous and endogenous arachidonic acid in response to cytokine stimulation. Maximal release of mediators requires all cytokines to be present. These results may indicate a role for smooth muscle as an inflammatory, as well as a contractile, cell.

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Hirst, S.J., Barnes, P.J., and Twort C.H. *et al.*, (1992). Am. J. Resp. Cell. Molec Biol 7, 574-582.
Mitchell, J.A., Belvisi, M.G., Akarasereenont, P. *et al.*, (1994). Br. J. Pharmacol. 113, 1008-1014.
Mitchell, J.A., Larkin, S.L., and Williams, T.J. (1995). Biochem. Pharmacol., in press.

83P DEXAMETHASONE ATTENUATES GELATINASE ACTIVITY IN STIMULATED HUMAN LUNG FIBROBLASTS AND EPITHELIAL CELLS

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Eosinophil-dependent injury of the bronchial mucosa is accompanied by an increase in the activity of the matrix metalloproteinases gelatinase A and gelatinase B (Herbert & Robinson, 1995). In cells of non-pulmonary origin the expression of these enzymes can be stimulated by cytokines in a pathway that involves protein kinase C activation and the action of transcription regulator proteins on the promoter regions of the gelatinase genes. It has been reported that gelatinase activity can be inhibited by treatment of cells with dexamethasone (Samples *et al.*, 1993; Ries *et al.*, 1994) but little information exists about whether this is an effect that is also seen in airway cells and, if so, the likely mechanisms involved. In this study we have investigated the effect of dexamethasone on the release and activation state of gelatinases released by human airway cells in culture. Confluent fibroblasts (HLF, passage 3-10) or the A549 human epithelial adenocarcinoma line (passage 125-155) were incubated for 40h in serum-free media with dexamethasone and with or without stimulation by phorbol 12-myristate 13-acetate (PMA) at 10 ng ml^{-1}. Conditions for PMA stimulation were selected from an earlier series of experiments in which a range of concentrations and exposure times were evaluated.

Analysis of conditioned media from quiescent HLF by gelatin substrate zymography indicated a single band of activity consistent with the presence of progelatinase A (apparent mass by zymography 66kDa). However, in HLF stimulated with PMA, activation products were also evident at apparent masses of 57 & 52kDa. In quiescent cells, the progelatinase A band was qualitatively unaffected by dexamethasone treatment, but in PMA-stimulated cells the activated forms of the enzyme were removed in a concentration-dependent manner. Quantitative analysis of gelatinase activity was performed by measuring the degradation of ^{14}C gelatin. In quiescent HLF, gelatinase activity was 543.0 ± 25.8 mU mg^{-1} protein (n=3), and in the presence of 1μM dexamethasone activity was 106.8 ± 2.1% of this control (n=3). PMA treatment increased the amount of gelatinase activity, with unstimulated cells having only 59.1 ± 8.0% of that in PMA-treated cells (n=12, $P<0.05$). Dexamethasone (0.01-1μM) produced a concentration-dependent inhibition of PMA-stimulated activity (IC_{75} 0.05 ± 0.02μM, n=6), returning gelatinase activity to levels found in the unstimulated cells. Even at 100μM dexamethasone gelatinase activity was not ablated (65.4 ± 1.8% of PMA control, n=3). In PMA-stimulated A549 cells bands were observed in zymography that were consistent with the activated forms of gelatinase B (89 & 80kDa). These were removed concentration-dependently by co-treatment with 0.01-0.1μM dexamethasone. Quantitative measurements supported this with dexamethasone having an IC_{50} of 0.04 ± 0.006μM (n=6). However, gelatinase activity was never reduced below levels found in unstimulated cells (at 10μM dexamethasone the activity was 32.6 ± 4.5% (n=6) of the PMA control compared to unstimulated cells which had an activity of 48.1 ± 5.9% (n=9).

This study shows that dexamethsone appears to have little effect on the activity of gelatinases A and B in unstimulated HLF or A549 cells, but in both cell types dexamethasone prevented the increase in activity caused by PMA stimulation. The failure to block basal activity contrasts with the action of dexamethsone in HL-60 cells (Ries et al., 1994) and suggests that the steroid probably acts at the level of a transcription activator rather than by binding directly to glucocorticoid responsive elements in the genes. It is also possible that dexamethasone might be affecting the expression of enzymes involved in the activation of gelatinases.

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Herbert, C.A. & Robinson, C. (1995) Br. J. Pharmacol. **115**, 13P.
Samples, J.R., Alexander, J.P. & Acott, T.S. (1993) Invest. Opthalmol Vis. Sci. **34**, 3386-3395
Ries, C., Kolb, H. and Petrides, P.E. (1994) Blood **83**, 3638-3646.

84P THE INFLUENCE OF ORGANIC PEROXIDES ON PLATELET AGGREGATION AND SENSITIVITY TO NITRIC OXIDE

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In vascular disease plasma lipid peroxides are raised and are associated with lipoproteins, particularly low density lipoproteins (LDL). Previously, hydrogen peroxide (H_2O_2) has been shown to potentiate the inhibition of platelet aggregation by nitric oxide (NO), when the two species are added simultaneously to isolated platelets (IP) before the addition of thrombin (0.02U/ml) (Naseem & Bruckdorfer 1995). Conversely, addition of H_2O_2 to IP after thrombin enhanced aggregation and antagonised the actions of NO (Naseem & Bruckdorfer 1994). The influence of 15(S)-hydroperoxyeicosatetraenoic acid (15-HpETE) and cumene hydroperoxide (cum-OOH) on aggregation of IP and their sensitivity to NO were investigated, using methods previously described (Naseem & Bruckdorfer 1995). 15-HpETE (50nmol/mg protein) was presented to IP as a complex with native LDL to form 15-HpETE-LDL. Each set of data is the mean±SD of 4 individual experiments, unless otherwise stated. Statistical analysis was performed using Students's t-test.

Cum-OOH (30μM), like H_2O_2, enhanced platelet aggregation when added after thrombin (0.005U/ml), increasing aggregation from 7.7±1.5 to 27.3±4.9% (n=3; $p< 0.01$). However 15-HpETE-LDL (0.5mg protein/ml) could only enhance aggregation by ADP, and when incubated with IP before the agonist. In contrast, both H_2O_2 and cum-OOH had a small inhibitory effect when added prior to the agonist.

Subsequently, the effects of peroxides on platelet sensitivity to NO were tested. If cum-OOH was added after thrombin (0.02U/ml), there was significant reduction in the inhibition by NO, IC_{50} increasing from 133±15 to 706±138nM ($p< 0.01$), as was the case with H_2O_2, but 15-HpETE-LDL had little effect. 15-HpETE-LDL reduced slightly the inhibitory action on ADP induced aggregation by NO (IC_{50} 63.7±8.4), increasing the IC_{50} to 76±7.7nM (n=3; $p< 0.05$): again this only occurred if the 15-HpETE-LDL was preincubated with IP. In separate experiments, the aggregatory effects of 15-HpETE-LDL and cum-OOH were tested against the synergistic actions of NO/H_2O_2. Cum-OOH had no effect on the inhibition, regardless of the time of addition. Pre-incubation of 15-HpETE-LDL with IP reduced the inhibition by the antagonists modestly from 96% to 84%. When either 15-HpETE-LDL or cum-OOH were added simultaneously with NO, they failed to alter the level of inhibition.

These results suggest that organic peroxides can modulate platelet activation and their sensitivity to NO, but to a lesser extent than H_2O_2. The effects of peroxides may be dependent in part on their permeability to the platelets: H_2O_2 and cum-OOH may enter the platelet readily, and exert direct effects on platelet signal transduction mechanisms. However, the synergism between H_2O_2 and NO seemed to be unique to H_2O_2. In contrast, the LPO associated with lipoproteins probably exert a surface mediated effect on aggregation, but may impede slightly the entry of NO into the platelet.

Naseem, K.M. & Bruckdorfer, K.R. (1994) Atherosclerosis, **109**, 187.
Naseem, K.M. & Bruckdorfer, K.R. (1995) Biochem. J., **310**, 149-153.
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85P NITRITE PRODUCTION IN THE ENDOTOXIN-TREATED ISOLATED AORTA FROM THE WATANABE HEREDITARY HYPERLIPIDAEMIC RABBIT

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Exposure of arteries to endotoxin increases the expression of the inducible isoform of nitric oxide synthase (iNOS) (Moncada et al. 1990). During the development of atherosclerosis, a variety of cytokines are released that modify the activity of the arterial wall and alter the expression of NOS (Wilcox, J.N. et al. 1994). In this study, we have investigate whether the isolated atherosclerotic aorta from the Watanabe hereditary hyperlipidaemic rabbit retains the capacity to increase nitric oxide (NO) synthesis in response to endotoxin treatment as measured by nitrite production.

The thoracic aorta was removed from Watanabe Hereditary Hyperlipidaemic (WHHL) rabbits (total cholesterol: 25 ± 1.2 mM, age: 6 months) and age-matched normocholestreolaemic New Zealand White (NZW) rabbits, dissected clean of adherent adipose tissue and washed in phosphate-buffered saline (PBS) containing penicillin and streptomycin (500 I.U. ml^{-1}) under sterile conditions. Aortic rings of 2 mm width (approximately 1-2 mg) were equilibrated in 0.5 ml of Dulbecco's modified Eagle's medium containing glutamine, penicillin and streptomycin without phenol red (DMEM) at 37 ° C in 48-well plates for 1 h under an atmosphere of 95 % O_2, 5% CO_2. Triplicate rings incubated with fresh DMEM and bacterial endotoxin (LPS: E.Coli, serotype 055:B5;10 μg ml^{-1}) or PBS for 24 h. The release of nitrite into the medium was assayed by the method of Misko et al. (1992) using 2,3-diaminonaphthalene. Data is shown as mean ± s.e. mean. Statistical analysis was performed using Student's t-test with $P<0.05$ considered significant. The influence of LPS on nitrite production in aortic rings from hyperlipdaemic and control rabbits is shown in table 1. Endotoxin induced an increase in nitrite production in the control aortic rings ($P<0.0001$) which was significantly reduced by L-NAME (100 μM) and cycloheximide (CH;10 μM) suggesting that it was due to induction of iNOS. In contrast, the release of nitrite in rings from WHHL rabbits treated with LPS showed no increase of nitrite release compared to those treated with PBS.

Table 1. Nitrite release from rabbit aortic rings

Treatment	Nitrite release (pmol/mg tissue)	
	NZW n=6	WHHL n=6
PBS	90.8 ± 3.9	81.1 ± 4.6
PBS + L-NAME	5.2 ± 1.3	7.6 ± 2.1
PBS + CH	80.6 ± 6.1	52.3 ± 3.4
LPS	150.6 ± 5.0	78.0 ± 2.5
LPS + CH	99.1 ± 1.1	40.5 ± 1.7
LPS + L-NAME	93.0 ± 5.4	41.9 ± 2.0

This study suggests that as a result of hypercholesterolaemia, the ability of LPS to induce iNOS has been lost in the aorta of the WHHL rabbit or the metabolism of NO has been affected.

Misko, T.P., Schilling, R.J., Salvemeni, D. *et al.* (1993) *Anal. Biochem.* 214, 11-16.
Moncada, S., Palmer, R.M.J. & Higgs, E.A. (1991) *Pharmacol. Reviews* 43, 109-135.
Wilcox, J.N., Romesh, R., Subramanian, C.E. *et al.* (1994) *Circulation* 90, I-298

86P EXPRESSION OF FUNCTIONAL BRAIN AND MACROPHAGIC NO-SYNTHASES IN THE ATHEROSCLEROTIC RABBIT AORTA

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Atherosclerotic lesions profoundly alter contractile and relaxatory properties of the arterial wall (Verbeuren et al., 1986). Evidence for the presence of non endothelial NO-synthases (NOS) in aortas of cholesterol-fed rabbits has been reported (Verbeuren et al., 1993). The present investigation was designed to evaluate the functionality of these NOS on guanylate cyclase and to characterize *in situ* the isoform(s) responsible for this activity.

The studies were performed on aortas of White New Zealand rabbits fed either a control or a cholesterol rich (0.3%) diet for 45 weeks. Some rabbits were treated with the non selective NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) for 4 weeks prior to experimentation. The aortic cGMP was quantified on saline extracts using a competitive immunoenzymatic assay in which rabbit anti-cGMP immunoglobulins G were covalently linked to the solid phase to avoid false positive results due to high rabbit immunoglobulin G concentrations in the atherosclerotic saline extracts. Protein concentrations of homogenized aortas were determined by the Lowry procedure. Serial frozen sections were prepared from intact aortas and treated with monoclonal antibodies against the macrophagic and brain NOS isoforms (macNOS and bNOS). The immunologic reactions were revealed by immunofluorescence using an amplification procedure with the streptavidin-biotin system.

Using the saline extraction procedure, and the adapted cGMP protocol described above, it was found that the atherosclerotic aortas contained at least 5 fold more cGMP than control aortas (5.71 ± 0.65 pmol/mg of protein versus 1.05 ± 0.38 pmol/mg of protein, n=5, $P<0.001$ unpaired t-test). However, the chronic administration of L-NAME, at 12 mg/kg/day for one month did not reduce the cGMP concentration of atherosclerotic aortas (n=5) while the cGMP concentration of aortas from treated control rabbits (n=5) decreased by 63.4 ± 7.6% ($P<0.05$, unpaired t-test). Whatever the anti-NOS antibody used, no fluorescence was detected in sections of control aortas. In contrast, in all the atherosclerotic aortas studied (n=4), macNOS and bNOS were revealed as numerous intense spots in the neointima. Moreover a diffuse staining was also revealed just below the endothelial layer with both anti-NOS antibodies in 3 out of 4 aortas and anti-macNOS antibodies strongly labelled the atherosclerotic endothelium in 3 out of 4 aortas.

Our data demonstrate that macNOS and bNOS are present in the neointima of advanced atherosclerotic lesions in aortas of hypercholesterolemic rabbits which confirms our previous findings on blood vessel reactivity (Verbeuren et al., 1993). The high cGMP levels found in these atherosclerotic arteries suggest that these NOS isoforms are functional and that the NO produced can diffuse to reach the aortic guanylate cyclases. Further studies are necessary to elucidate the reasons why the atherosclerotic NOS are insensitive to an in vivo L-NAME treatment.

Verbeuren T.J., Jordaens F., Zonnekeyn L., et al. (1986), Circ. Res., 58, 552-564.
Verbeuren T.J. Bonhome E., Laubie M. & Simonet S. (1993), J. Cardiovasc. Pharmacol., 21, 841-845.

87P MODULATION OF INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) ACTIVITY IN THE HUMAN COLON EPITHELIAL CELL LINE HT-29: DUAL EFFECT OF INTERLEUKIN-13 (IL-13)

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Nitric oxide (NO) synthesis is increased in rectal biopsies from patients with ulcerative colitis (Middleton *et al.*, 1993). The combination of IL-1α/IFN-γ induces iNOS mRNA expression and activity in the human colon epithelial cell line HT-29, while the addition of TNF-α up-regulates this effect at the post-transcriptional level (Kolios *et al.*, 1995). We have examined the effect of IL-13 on the iNOS expression and activity in the human colon epithelial cell line HT-29. We have measured nitrite levels by a fluorescent substrate (Misko *et al.*, 1993), iNOS mRNA expression by Northern analysis, and iNOS protein expression by Western analysis. HT-29 cells were treated in McCoys 5A medium plus 10% FCS in six well plates until confluent, then in the absence of FCS for 24h prior to and up to 48h following the addition of human recombinant cytokines.

Confluent monolayers after 1h pre-treatment with different concentrations of IL-13 (0.1-30ng/ml) were stimulated with either IL-1α(10ng/ml)/IFN-γ(300U/ml) or IL-1α(10ng/ml)/IFN-γ(300U/ml)/TNF-α(100ng/ml). iNOS mRNA expression, iNOS protein expression and nitrite levels were determined at 24h, 30h and 48h respectively. Following stimulation with IL-1α/IFN-γ/TNF-α, IL-13 produced a concentration dependent highly significant ($p<0.001$) suppression of nitrite generation by HT-29 cells, from 891 ± 52 to 307 ± 37 nM/10^6 cells, at 30ng/ml of IL-13 (n=3). Low concentrations of IL-13 (0.1-3ng/ml) produced a significant enhancement of IL-1α/IFN-γ-induced nitrite production from 301 ± 20 to 401 ± 31 nM/10^6 cells (n=3) ($p<0.01$), while higher concentrations of IL-13 reduced the nitrite generation by HT-29 cells (215 ± 15 nM/10^6 cells (n=3) $p<0.01$ at 30ng/ml of IL-13). Low concentrations of IL-13 (0.1-3ng/ml) were found to increase, while high concentrations of IL-13 (10-30ng/ml) were found to suppress significantly both the IL-1α/IFN-γ and IL-1α/IFN-γ/TNF-α-induced iNOS mRNA expression by HT-29 cells. Western analysis revealed that IL-13 suppresses in a concentration dependent manner the IL-1α/IFN-γ/TNF-α-induced iNOS protein expression by HT-29 cells, while low concentrations of IL-13 (0.1-3ng/ml) increased and high concentrations (10-30ng/ml) suppressed the IL-1α/IFN-γ-induced iNOS protein expression. These data suggest that low concentrations of IL-13 potently inhibit nitrite generation and iNOS protein expression by HT-29 cells, while higher concentrations in addition block iNOS mRNA induced by the optimum combination of IL-1α/IFN-γ/TNF-α. Therefore, IL-13 may have an anti-inflammatory role in intestinal inflammation.

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Kolios, G., Brown, Z., Robson, R.L., *et al.* (1995) *Br. J. Pharmacol.*, (in press).
Middleton, S.J., Shorthouse, M. & Hunter, J.O. (1993) *Lancet*, **341**, 465-466.
Misko, P., Schilling, R.J., Salvemini, D. *et al.* (1993) *Anal. Biochem.*, **214**, 11-16.

88P TACHYKININS RELAX THE GUINEA-PIG TRACHEA BY STIMULATING AN EPITHELIAL 'SEPTIDE-INSENSITIVE' NK_1 RECEPTOR WHICH RELEASES NO: EVIDENCE FOR NK_1 RECEPTOR SUBTYPES?

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The existence of a 'septide-selective' tachykinin NK_1 receptor subtype has been proposed in various tissues (Petitet et al., 1992) including the guinea-pig airways (Zeng & Burcher, 1994). The possibility that epithelium-derived nitric oxide (NO) mediates the relaxation of the guinea-pig airway smooth muscle induced by tachykinins, and that different potencies of selective agonists for tachykinin NK_1 receptor in contracting the guinea-pig airways may depend on their ability to relax the smooth muscle *via* NO release was studied.

Isometric tension was recorded in isolated tracheal tubes in which compounds were administered intraluminally (Nijkamp et al, 1993) in the presence of phosphoramidon, indomethacin (both 1 μM) and the tachykinin NK_2 receptor antagonist, SR 48968 (0.1 μM).

In the presence of D-NMMA (100 μM) SP, NKA, NKB, [Sar^9, $Met(O_2)^{11}$]-SP and [Pro^9] SP (0.1-10 nM) relaxed the trachea precontracted with acetylcholine (Ach, 10 μM). This relaxation was changed (in a L-Arg-dependent manner) into a contraction by pretreatment with L-NMMA (Table 1). After removal of the epithelium SP, NKA, NKB, [Sar^9,$Met(O_2)^{11}$]-SP and [Pro^9] SP produced a contraction in the presence of either L-NMMA or D-NMMA. The contraction and the relaxation caused by natural tachykinins, [Pro^9] SP and by [Sar^9, $Met(O_2)^{11}$]-SP were abolished by pretreatment with the selective tachykinin NK_1 receptor antagonist, CP-99,994 (1 μM), but not by its inactive enantiomer CP-100,263 (1 μM). In the presence of either D-NMMA or L-NMMA septide, [$pGlu^6$]-SP (6-11) and SP-O-CH_3 (0.1-10 nM) caused a contraction (Table 1) of tracheal tubes that was abolished by CP-99,994 (1 μM). Concentration-response curves to septide, [$pGlu^6$]SP (6-11) and SP-O-CH_3 after L-NMMA were not different from those after D-NMMA.

Evidence is presented that tachykinins relax the guinea-pig trachea by activation of an epithelial NK_1 receptor that releases NO. This receptor, being sensitive to SP, NKA, NKB, [Sar^9, $Met(O_2)^{11}$]-SP, but not to septide, [$pGlu^6$]-SP (6-11) and SP-O-CH_3 is pharmacologically distinct from the NK_1 receptor that mediates contractions, that is stimulated markedly by all these agonists, including septide, [$pGlu^6$]-SP (6-11) and SP-O-CH_3. The present data do not support the hypothesis of the existence of a 'septide-selective' NK_1 receptor in the guinea-pig trachea.

Table 1
Effect (tension, mg) of various agonists (10 nM) on isolated and perfused tracheal tubes (+ = contraction, - = relaxation)

	D-NMMA	L-NMMA
SP	-12 ± 3 (n = 6)	$+24\pm6$ (n = 6)
[Sar^9,$Met(O_2)^{11}$]-SP	-14 ± 3 (n = 5)	$+26\pm5$ (n = 5)
Septide	$+34\pm7$ (n = 5)	$+28\pm5$ (n = 5)
SP-O-CH_3	$+26\pm4$ (n = 5)	$+31\pm5$ (n = 5)

Nijkamp, F.P., Van Der Linde, H.J. & Folkerts, G. (1993). *Am. Rev. Respir. Dis.*, 148, 727-734.
Petitet F., Saffroy M., Torrens Y., et al. (1992). *Peptides*, 13, 383-388.
Zeng X.-P. & Burcher E. (1994) *J. Pharmacol. Exp. Ther.* 270, 1295-1300

89P CONTRIBUTION OF 5-HYDROXYTRYPTAMINE RELEASE FROM MAST CELLS TO RAT PAW OEDEMA INDUCED BY WASP VENOM AND SYNTHETIC KININS

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We have reported previously (Griesbacher *et al.*, 1994) that the nociceptive actions of the venom of the wasp *Vespula vulgaris* are entirely due to the kinins from the venom, whereas the increase in vascular permeability is only partially inhibited by the bradykinin B_2 receptor antagonist, icatibant (Hoe 140, Lembeck *et al.*, 1991). The residual part of the oedema was not inhibited by the histamine H_1 antagonist, mepyramine, and also is not affected by the H_2 blocker, cimetidine.

The increase in paw volume (measured by volume displacement with a Ugo Basile plethysmometer) 30 min after subplantar injection of 10 µg of the venom (minus volume increase of the contralateral paw injected with 50 µl phosphate-buffered saline) in icatibant-pretreated rats (18±3%) was almost abolished (3±1%, $P<0.05$, multiple nonparametric comparisons) by the 5-hydroxytryptamine antagonist, ketanserin (20 µmol kg^{-1}, i.p.). However, ketanserin also has unspecific actions and reduced systemic arterial pressure by 59±6 mmHg.

Therefore, the experiments were repeated with methysergide (20 µmol kg^{-1}, i.p.). This treatment reduced the venom-induced paw oedema from 46±4% to 23±3% ($P<0.05$). The paw oedema in rats pretreated with icatibant (300 nmol kg^{-1}, s.c.) was reduced from 30±3% to 3±2% ($P<0.05$).

Pretreatment with methysergide also reduced systemic arterial blood pressure by 35±1 mmHg. In order to investigate whether a reduction of systemic blood pressure by this amount could inhibit the paw oedema by itself, the effects of phentolamine on the paw oedema induced by bradykinin (30 nmol) were investigated. Phentolamine at a dose of 7.5 µmol kg^{-1}, i.p. reduced blood pressure by a similar extent (30±4 mmHg). However, the icatibant-resistant part of the bradykinin-induced paw oedema (18±3%) remained unchanged (16±6%).

In order to determine the source of the 5-hydroxytryptamine released by the venom, rats were pretreated with compound 48/80 to deplete mast cells. On 3 consecutive days, compound 48/80 (5 mg kg^{-1}, i.p.) was injected 40 min after protective i.p. treatment with mepyramine, cimetidine and methysergide (20 µmol kg^{-1} each). On the fourth day, this procedure was carried out twice. Compound 48/80 reduced the venom-induced paw oedema from 35±2% to 15±3% ($P<0.05$). In rats pretreated with icatibant 30 min before the subplantar injections, compound 48/80 reduced the oedema from 22±2% to 2±2% ($P<0.05$).

The experiments were repeated using subplantar injections of bradykinin (30 nmol) and Thr^6-bradykinin (30 nmol). The pattern of inhibition by ketanserin, methysergide and compound 48/80 were identical to those described above.

It is concluded that in the rat the inflammatory actions of kinins from wasp venom and of synthetic kinins rely in part on the release of 5-hydroxytryptamine, but not histamine, from mast cells. This part of the kinin action does not involve bradykinin (B_2) receptors.

Griesbacher, T., Zenz, M., Althuber, P. & Lembeck, F. (1994). *Br. J. Pharmacol.* 112: 126P.

Lembeck, F., Griesbacher, T., Eckhardt, M.,Henke, S., Breipohl, G. & Knolle, J. (1991). *Br. J. Pharmacol.* 102: 297–304.

90P EFFECTS OF BRADYKININ AND DESARG9-BRADYKININ ON AFFERENT NEURAL DISCHARGE IN INTERLEUKIN-1β-TREATED RAT KNEE JOINTS

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The inflammatory cytokine Interleukin-1β (IL-1β), injected intra-articularly (i.art.) into the rat knee joint, has been shown to induce mechanical hyperalgesia and also bradykinin B_1 receptor mediated hyperalgesia (Davis & Perkins, 1994). We investigated the effects of i.art. injection of IL-1β on the background neural discharge and on the changes in the activity evoked by intra-arterial (i.a.) injection of bradykinin (Bk) and $desArg^9$-Bk in rat knee joint mechanonociceptors in-vivo.

Male wistar rats were anaesthetised with urethane (25%w/v, 0.6ml $100g^{-1}$ weight i.p.). The trachea, carotid artery (for monitoring blood pressure) and right femoral artery (i.a. drug administration) were cannulated. Recording of afferent neural activity from high threshold mechanonociceptors was similar to that described elsewhere (Birrell et al., 1993) except the medial articular nerve innerveting the knee joint was dissected from the saphenous nerve. During experiments either IL-1β, boiled (inactivated) IL-1β or a combination of IL-1β and IL-1 receptor antagonist (IL-1ra) was injected i.art. into the rat knee joint.

Mechanonociceptor units studied had a basal discharge of 0.4 ± 0.2 impulses per second (i.p.s.), were activated by capsaicin (1-3µg, i.a.) and had conduction velocities within the C-fibre range (0.46±0.1ms^{-1}, range 0.1 - 1.2ms^{-1}). IL-1β [10-100units (100µl), i.art.] but not boiled IL- 1β 100µl (i.art.) increased background neural discharge. The increase had two phases, an initial peak increase above basal 10 minutes after injection (10units: 1.3 ± 0.2 i.p.s., 100units: 1.5 ± 0.2 i.p.s.) and a delayed peak increase above basal 40 minutes after injection (10 units: 1.4 ± 0.6 i.p.s., 100units: 1.7 ± 0.5 i.p.s.). These were blocked by co-injection of IL-1ra (i.art.). One hour after IL-1β, but not boiled IL-1β or IL-1β (IL-1ra) bradykinin-induced activation of mechanonociceptors was increased and responses to $desArg^9$-Bk were evident (Table 1). In all cases the Bk-induced increase in neural discharge was blocked by the B_2 antagonist (Hoe 140 10µg kg^{-1}, i.a.) and in IL-1β-treated knee joints the response to $desArg^9$-Bk was blocked by the B_1 antagonist ($desArg^9Leu^8$-Bk, 1mg kg^{-1}, i.a.)[Table 1].

These data suggest that the inflammatory cytokine IL-1β, injected into the knee joint, not only increases background neural discharge from joint mechanonociceptors, but also sensitises these afferents to Bk and induces a B_1 receptor mediated activation.

Birrell, G.J., McQueen, D.S., Iggo, A. et al. (1993). Neuroscience. 54, 537-544.

Davis, A.J. & Perkins, M.N. (1994). Br. J. Pharmacol. 113, 63-68.

Table 1. Effects of i.art. IL 1-β on Bk and $desArg^9$-Bk activity in rat knee joint mechanonociceptors.

	UNTREATED (n=11) *peak increase (i.p.s.)*	boiled IL-1β (n=3) *peak increase (i.p.s.)*	IL-1β (n=4) *peak increase (i.p.s.)*	IL-1β (IL-1ra 0.1µg)(n=4) *peak increase (i.p.s.)*
Bk 3µg	2.0 ± 0.5	1.4 ± 0.3	11.8 ± 5.7 *	2.4 ± 0.7
Bk 10µg	2.5 ± 0.6	3.6 ± 0.8	12.8 ± 4.0 *	3.1 ± 0.9
Bk 30µg	4.3 ± 1.1	5.3 ± 0.8	12.2 ± 2.7 *	5.6 ± 1.2
Bk 30µg (Hoe 140)	0.3 ± 0.1 †	0.5 ± 0.3 †	0.8 ± 0.2 †	0.6 ± 0.2 †
$desArg^9$-Bk 100µg	0.3 ±0.1	0.4 ± 0.1	1.6 ± 0.1 *	0.3 ± 0.1
$desArg^9$-Bk 100µg ($desArg^9Leu^8$-Bk)		-	0.8 ± 0.3 ‡	-

*p<0.05 Mann Whitney U-test, compared to untreated or boiled IL-1β. † p<0.05, paired t-test, compared to Bk 30µg. ‡ p<0.05, paired t-test, compared to $desArg^9$-Bk 100µg

91P SPECIFIC LOCALISATION OF THE 5-HT TRANSPORTER IN THE RAT ADRENAL MEDULLA

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The serotonin (5-HT) transport protein occurs not only in the CNS, but also in several peripheral tissues, including lung, placenta (Balkovetz *et al.*, 1989) and platelets (Rudnick, 1977). Blakely *et al.* (1991) demonstrated a high level of 5-HT transporter mRNA expression in the rat adrenal gland, a tissue not thought to synthesize 5-HT. This finding was confirmed by *in situ* hybridisation, detecting high level gene expression by medullary chromaffin cells (Blakely *et al.*, 1994). Blakely *et al.*, 1994, hypothesised that the 5-HT transporter loads chromaffin granules with 5-HT for later release, analogous to the process of the uptake, storage and release of 5-HT by platelets (Rudnick, 1977). The steroidogenic actions of 5-HT are confined to the zona glomerulosa, where it is a potent stimulus to aldosterone (Haning *et al.*, 1970). The aim of this study was to analyse sections of rat adrenal gland, initially using [^{3}H]paroxetine binding, and secondly using immunohistochemistry, to determine whether the 5-HT transporter was confined to the medulla or if the transporter was also present in adrenal cortex.

Sections of adrenal gland were cut in a cryostat (-20°C), thaw-mounted onto gelatin covered glass slides and stored at -70°C for subsequent [^{3}H]paroxetine autoradiographic binding analysis of 5-HT uptake sites. Slide-mounted sections were processed for [^{3}H]paroxetine autoradiography according to the protocol described by Battaglia *et al.*, 1987. Briefly, sections were incubated in buffer containing a saturating concentration (250 pM) of [^{3}H]paroxetine. Non-specific binding was defined in adjacent sections by [^{3}H]paroxetine binding in the presence of 4μM citalopram.

Analysis of [^{3}H]paroxetine autoradiographs was performed using a computer-based image analysis system (Cambridge Instruments Quantimet 970). Tissue isotope concentrations were measured from autoradiographic images of rat adrenal gland sections, relative to appropriate [^{3}H] standards (Amersham International). Specific [^{3}H]paroxetine binding was determined by subtraction of image densities from total and non-specific binding images. Data are presented as mean binding density±standard deviation. [^{3}H]paroxetine binding (fmol/mg of tissue) was evident in the medulla of the rat adrenal gland (220±20), but not in the rat adrenal cortex (7±6).

For immunohistochemistry studies two different antibodies to the 5-HT transporter were used. The first antibody S-240-KLH was raised in rabbits against a 14 amino acid sequence present in the second extracellular loop of the 5-HT transporter. The second antibody S-387-KLH was raised against a 14 amino acid sequence present in the fourth extracellular loop of the transporter. The adrenal glands were fixed in 10% neutral buffered formalin for 24 hours, then paraffin processed and 4μm sections cut onto adhesive slides. Immunostaining was by an indirect peroxidase method of antigen retrieval using microwave according to the method of Gerdes *et al.*,1992. Negative controls consisted of replacement of primary antibody with an equivalent concentration of normal rabbit serum. Endogenous peroxidase was blocked using 3% aqueous hydrogen peroxide, and before primary antibody incubation, sections were incubated with 20% normal sheep serum to reduce background staining. This was drained off and primary antibody added. Incubations in primary antibody were 60 minutes at room temperature and dilutions established by a titration series resulting in a dilution of 1:200 for both S-387-KLH and S-240-KLH. Following a 10 minute TBS wash, sections were incubated in peroxidase conjugated anti-rabbit antibody (diluted 1:50) for 60 minutes. A further 10 minute wash in TBS preceded visualisation in a standard hydrogen peroxide/diaminobenzidine substrate/chromagen solution. Finally sections were counterstained in haematoxylin, dehydrated in ethanol, cleared in xylene and mounted in DPX.

S-240-KLH demonstrated background staining throughout the adrenal gland, with darker staining in the adrenal medulla . In contrast S-387-KLH located specific immunoreactivity in the medulla, with no significant staining in the adrenal cortex.

We conclude that the 5-HT transporter is confined to the medullary tissue of the rat adrenal gland. In addition, antibody S-387-KLH may serve as a useful reagent for the localisation of the 5-HT transporter in immunohistochemical studies.

Balkovetz, D.F., Tiruppathi, C. *et al.* (1989) *J.Biol.Chem.* 105, 2195-2198.
Battaglia, G., Yeh, S.Y.*et al.*,(1987) *J.Pharmacol.Exp.Ther.* 242, 911-916.
Blakely, R.D., Berson, H.E.*et al.* (1991) *Nature* 354, 66-70.
Blakely, R.D., Felice, L.J.D.*et al.* (1994) *J.Exp.Biol.* 196, 263-281.
Gerdes, J., Becker, M.H.G. *et al.* (1992) *J.Pathol.* 168, 85-87.
Haning, R., Tait, S.A.S. & Tait, J.F. (1970) *Endocrinology* 87, 1147-1167.
Rudnick, G. (1977) *J.Biol.Chem.* 252, 2170-2174.

92P 8-OH-DPAT MAY HAVE 5-HT UPTAKE BLOCKING PROPERTIES IN RAT HIPPOCAMPUS

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The 5-HT_{1A} ligand, 8-OH-DPAT also labels 5-HT uptake sites (e.g. Sprouse *et al.*, 1993). To study the functional consequences of this property, the effects of 8-OH-DPAT were compared to those of the 5-HT uptake inhibitors paroxetine and clomipramine, and of the 5-HT_{1A} agonist flesinoxan, on 5-HT uptake *in vitro*, and on 5-HT levels *in vivo* using microdialysis. As 5-HT uptake inhibitors prevent dexfenfluramine-induced increase of 5-HT levels (Kreiss *et al.*, 1993), the effects of paroxetine and 8-OH-DPAT on this increase were examined.

The uptake of [^{3}H]5-HT *in vitro* was measured in hippocampus of Sprague Dawley rats. Aliquots of crude homogenate (2.5 mg/50 μl) were incubated with 10 nM [^{3}H]5-HT for 5 min at 37°C with or without the compounds (5 concentrations) in a final volume of 500 μl. Microdialysis studies in the left ventral hippocampus of chloral hydrate (500 mg/kg i.p.) anaesthetised rats (5 per group) were performed as described by Sharp *et al.* (1989). Perfusates were collected every 20 min and analysed for 5-HT content. After 4 samples, collected to check the stability of the baseline, the compounds were added to the perfusion medium, saline or dexfenfluramine 10 mg/kg, i.p. were injected 40 min later and samples were collected for a further 140 min.

8-OH-DPAT, paroxetine and clomipramine, significantly increased hippocampal 5-HT levels, in a manner consistent with their potencies to inhibit 5-HT uptake (Table 1). Flesinoxan did not block 5-HT uptake *in vitro*, and did not significantly alter 5-HT levels *in vivo*. Dexfenfluramine increased 5-HT levels to a maximum of 133±15 fmol/20μl and these levels were reduced to 41±6 and 35±7 fmol/20μl ($P<0.01$, Newman-Keuls test) by 0.1 μM paroxetine and by 100 μM 8-OH-DPAT, respectively. It is suggested that the 8-OH-DPAT-induced increase of 5-HT levels does not involve 5-HT_{1A} receptors, but results, at least in part, from its 5-HT uptake blocking properties.

Table1. Effects of various compounds on [^{3}H]5-HT uptake *in vitro* and on 5-HT levels *in vivo*. pIC_{50} values ± s.e. (3 experiments) were calculated with the program Allfit. Microdialysis results are mean AUC ± s.e.m. (fmol/20 μl) calculated for the 3 h period during which the compounds were present in the perfusion medium. *P<0.05, **P<0.01 significantly different from control, Dunnett's test.

Compound (μM)	mean AUC	pIC_{50}
control	3.67 ± 0.39	
8-OH-DPAT (10)	12.12 ± 0.97 *	
8-OH-DPAT (100)	21.63 ± 1.54 **	6.00 ± 0.15
paroxetine (0.1)	26.92 ± 1.99 **	8.41 ± 0.11
clomipramine (1)	24.00 ± 3.42 **	7.00 ± 0.07
flesinoxan (100)	5.14 ± 1.62	<5

Sharp, T., Bramwell, S.R., Clark, D. *et al.* (1989) *J. Neurochem.* 53, 234.
Sprouse, J.S., McCarty, D.R. & Dudley M.W. (1993) *Brain Res.* 617, 159.
Kreiss, D.S., Wiedland, S. & Lucki, I. (1993) *Neuroscience* 52, 295.

93P ASTROCYTES *IN VITRO* EXPRESS FUNCTIONAL 5-HT7 RECEPTORS

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Stimulatory cAMP responses have been reported for various 5-HT receptors including 5-HT_{4S} and 5-HT_{4L} (Gerald *et al.*, 1995), 5-ht_6 (Monsma *et al.*, 1993) and 5-ht_7 (Lovenberg *et al.*, 1993). Astrocytes have been shown to express various 5-HT receptors (Kimelberg, 1995), we have investigated whether astrocytes *in vitro* express 5-HT receptors positively coupled to adenylyl cyclase. Astrocytes were cultured from the thalamic/hypothalamic area of 2 day old Sprague-Dawley rat pups and maintained in serum containing DMEM media for 12 days. The media was changed to serum-free 24 hours prior to the cAMP assays. Intact cells were washed and preincubated in serum-free media containing 500μM isobutyl-1-methylxanthine (IBMX), 1μM paroxetine, 10μM pargyline and 1μM ascorbate for 30 minutes at 37°C. The cells were incubated with agonists ± antagonists for 10 minutes and then the reaction was terminated by addition of 50μl of 30% perchloric acid. Cells were solubilised and cAMP extracted into the aqueous phase of a 50:50 mixture of trichlorotrifluoroethane and trioctylamine. Cyclic AMP levels were measured using a NEN cAMP[^{125}I] RIA Flashplate™ kit (NEN DuPont).

5-HT elevated cAMP levels in a concentration dependent manner to 710 ± 134 % of basal levels, n=5 (basal level = 21.25 ± 1.34 pmol/mg protein, n=43) with a pEC_{50} of 6.68 ± 0.08. 5-carboxamidotryptamine (5-CT) and 5-methoxytryptamine (5-MeOT) were also full agonists, however a range of other serotonergic agonists were inactive. IC_{50} values were estimated by inhibition of 5-CT (100 nM) stimulated cAMP levels. These results are summarised in Table 1. The pharmacological profile thus observed is consistent with that of the 5-ht_7 receptor.

Table 1. Pharmacological profile of the 5-HT receptors positively coupled to adenylyl cyclase in cultured astrocytes (n = number of experiments).

Agonists	(n)	pEC_{50}	Antagonist	(n)	pIC_{50}
5-HT	5	6.68 ± 0.08	Methiothepin	6	7.10 ± 0.25
5-CT	4	7.81 ± 0.09	Clozapine	6	6.15 ± 0.19
5-MeOT	5	6.86 ± 0.36	Ritanserin	6	6.32 ± 0.24
8-OH-DPAT	5	<5	Mesulergine	4	6.70 ± 0.18
Cisapride	5	<5	Mianserin	4	5.53 ± 0.19
Sumatriptan	5	<5	WAY100635	5	<5
DOI	2	<5	GR127935	5	<5
RU24969	2	<5	Ketanserin	2	<5

In conclusion, we have shown that the functional 5-HT receptor positively coupled to adenylyl cyclase in cultured thalamic/hypothalamic astrocytes is of the 5-ht_7 receptor subtype. These data provide the first evidence for the presence of functional 5-ht_7 receptors in astrocytes.

Gerald, C., Adham, N., Kao, H-T. *et al.* (1995) EMBO J. 14, 2806-2815.
Kimelberg, H.K. (1995) Neurochem. Int. 26, 27-40.
Lovenberg, T.W., Baron, B.M., de Lecea, L. *et al.* (1993) Neuron 11, 449-458.
Monsma, F.J., Shen, Y., Ward, R.P. *et al.* (1993) Mol. Pharmacol. 43, 320-327.
Sebben, M., Ansanay, H., Bockaert, J. *et al.* (1994) Neuroreport 5, 2553-2557.

94P 5-HT_{1A} RECEPTORS ARE INVOLVED IN INHIBITION OF THE MIDBRAIN-EVOKED CARDIOVASCULAR DEFENCE RESPONSE BY NUCLEUS RAPHE OBSCURUS

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In anaesthetised rats, the cardiovascular "defence" response evoked by stimulation in the dorsolateral and lateral sectors of the periaqueductal grey matter (PAG) can be attenuated by selective activation of neurones in nucleus raphe obscurus (NRO) (Schenberg & Lovick, 1995). NRO sends serotonergic projections to the PAG (Clements et al, 1985) and electrophysiological studies have shown that 5HT has inhibitory $5HT_{1A}$-mediated effects on neuronal activity in this region (Lovick, 1993). We have therefore investigated whether inhibitory modulation of the PAG-evoked cardiovascular defence response by NRO involves a $5HT_{1A}$-like mechanism.

In 300-350g male Wistar rats anaesthetised with Saffan (9-12 $mg.kg^{-1}.hr^{-1}$ i.v.) electrodes were positioned in the PAG at sites where electrical stimulation (10s trains of 40-80 μA, 1 ms pulses, 80 Hz) evoked a "defence" response, ie an increase in blood pressure (BP) and heart rate (HR) together with an increase in femoral vascular conductance (FC), respiratory rate (RR) and amplitude (RA) but a decrease in renal vascular conductance (RC). Microinjection of 200nl 0.1M D,L-homocysteic acid (DLH, pH 7.4) into NRO to selectively stimulate neurones produced a significant reduction in all components of the defence response except the increase in RA. This reduction was maximal after 10 min. The inhibitory effects of stimulation in NRO were attenuated by the $5HT_{1A}$ antagonist, +WAY100135 (Fletcher et al., 1993; 0.05-0.5$mg.kg^{-1}$ iv) (Fig. 1).

These results indicate that a $5HT_{1A}$-like mechanism is involved in inhibitory modulation by the medullary raphe of the PAG-evoked cardiovascular defence response.

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Clements, J, Beitz, A, Fletcher, T et al. (1985) J. Comp. Neurol. 236, 60-70.
Lovick, TA (1993) Brain Res. 606 62-68.
Schenberg, LC & Lovick, TA (1995) Am. J. Physiol. In press.
Fletcher, A, Bill, D, Bill, S et al. (1993) Europ. J. Pharm. 237, 283-291.

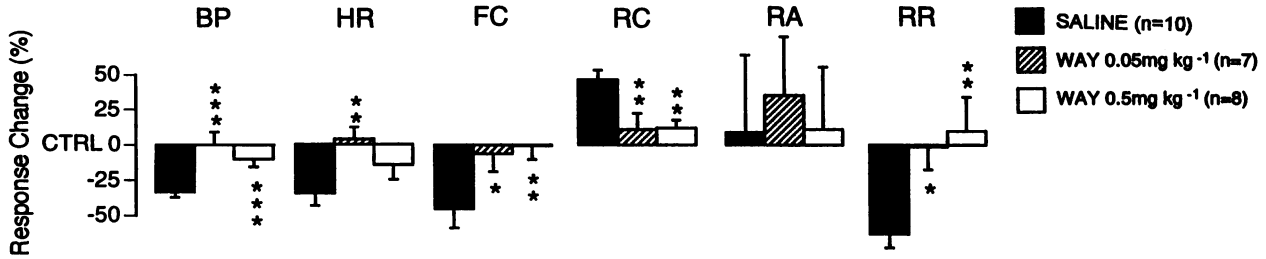

Fig 1. Effect of +WAY100135 on the depression of the PAG-evoked cardiovascular defence 10min after microinjection of DLH into NRO. Data were compared using Dunnett's t-test, * P<0.05, **P<0.001, ***P<0.005.

95P EFFECTS OF NMDA RECEPTOR ANTAGONISTS ON 5-HT RELEASE IN RAT SUBSTANTIA NIGRA

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There is a massive 5-HT projection from the dorsal raphe to the substantia nigra (SN) (Dray et al., 1976). Using fast cyclic voltammetry (FCV) it has been shown that electrical (Trout et al., 1992) or chemical (Rice et al., 1994) stimulation of the substantia nigra pars reticulata (SNr) releases a molecule with electrochemical characteristics of 5-HT. In this study we have characterised the identity of this monoamine as 5-HT, and studied the effects of a non-competitive and a competitive NMDA receptor antagonists dizolcipine (MK-801) and (±)-3-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) respectively, on electrically-evoked 5-HT release in this structure.

350 µm coronal male albino Wistar rat brain slices 4.6-4.8 mm posterior to bregma, containing the SN were set up in a brain bath superfused with artificial cerebrospinal fluid (aCSF), saturated with a 95% O_2 and 5% CO_2 mixture. 5-HT release was monitored by FCV (O'Connor & Kruk, 1991). Stimulation of the SNr was effected by either local pressure ejection of veratrine, or electrical stimulation with 50 pulses at frequencies ranging from 2-100 Hz. Drugs other than veratrine were dissolved in the superfusate.

Pressure ejection of veratrine 1mg.ml^{-1} (1s, 10 psi) from a micropipette in the SNr led to release of an electroactive substance with voltammetric characteristics of 5-HT. Pressure ejection of veratrine into the neighbouring ventral tegmental area yielded signals with electrochemical characteristics of dopamine (Iravani et al., 1995).

Electrical stimulation in the SNr with 50 pulses (25 volts; 0.2 ms pulse width) at frequencies greater than 10 Hz (50p/10 Hz) generated signals that were indistinguishable from those due to veratrine pressure ejection or exogenous 5-HT and were blocked by 1 µM TTX.

50p/10 Hz stimulation was used in all further experiments. 1 µM GBR 12909, 1µM benztropine or 1 µM sulpiride (each for 30 min.) had no effect on the signals.

1 µM fluvoxamine potentiated the 5-HT signal: 14.8 ± 2 nM, control; 63 ± 10 nM, fluvoxamine (n=6, P<0.01, paired t-test). 20 µM CPP did not affect the 5-HT signal (control: 14 ± 1.5 nM; CPP:15.3 ± 1.8 nM; n=6). 20 µM MK-801 potentiated 5-HT signal (control: 16 ± 5 nM; MK801: 31 ± 1.2 nM; n=5; P<0.05, paired t-test). In the presence of 20 µM MK-801, 1µM fluvoxamine failed to potentiate the 5-HT signal further (MK-801: 39 ± 9; MK-801 + fluvoxamine: 28 ± 6.5; n=4).

The results of the present study demonstrate that following either chemical stimulation with veratrine or trains of electrical pulses, release of 5-HT can be measured in the SNr using fast cyclic voltammetry. Potentiation of electrically-evoked 5-HT release by MK-801 but not CPP, argues against a role of NMDA receptors in modulation of the 5-HT release in this structure. Lack of potentiation of the 5-HT signal by fluvoxamine in the presence of MK-801 suggests that MK-801 and fluvoxamine may interact at 5-HT uptake sites.

Dray A., Gonye T.J., Oakley N.R. & Tanner T. (1976) Brain Res. **113**, 45-57.
Iravani M.M., Muscat R. & Kruk Z.L. (1995) *Neuroscience* In Press.
O'Connor J.J. & Kruk Z.L. (1991) *J. Neurosci. Methods* **38**, 25-33.
Rice M.E., Richards C.D., Nedergaard S., Hounsgaard J., Nicholson C. & Greenfield S.A. (1994) *Exp. Brain Res.* **100**, 395-406.
Trout S.J., Patel J. & Kruk Z.L. (1992) *Br. J. Pharmacol.* **107**, 217P.

96P AGONIST/ANTAGONIST RECOGNITION PROPERTIES OF THE 5-HT$_3$ RECEPTOR: IMPORTANCE OF GLUTAMATE 106

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The 5-HT$_3$ receptor is a member of a ligand-gated ion channel family. A single subunit (5-HT$_3$A$_L$) has been cloned from NG108-15 cells (Werner et al., 1994), where it forms a functional homomeric pentamer (Boess et al., 1995). The amino-acids responsible for the agonist/antagonist recognition properties of this receptor have been explored by mutagenic analysis and we report here on the importance of glutamate residue 106 (E106) to the agonist activation of the 5-HT$_3$ receptor.

The 5-HT$_3$A$_L$ cDNA was subjected to individual point mutations using the Altered Sites *in vitro* mutagenesis system (Promega). The altered cDNA was subcloned into the eukaryotic expression vector pRC/CMV and transiently expressed in HEK 293 cells by the calcium phosphate-DNA coprecipitation method. Pharmacological characterisation using radioligand binding and whole-cell patch clamp were used to determine the effect of the mutations on selective agonist and antagonist affinities.

Radioligand binding studies were performed on membrane homogenates of transiently transfected HEK 293 cells, essentially as described by Boess et al. (1992). In saturation studies with [^{3}H]GR65630 (0.04 - 12 nM), where non-specific binding was defined by metoclopramide (300 µM), the Kd for wildtype (WT, E106) was 0.27 ± 0.03 nM while that of E106N mutant was 0.42 ± 0.07 (mean ± SEM, n = 3-10). The E106D mutant showed a 14 fold decrease in affinity (Kd 3.69 ± 0.32; mean ± SEM, n = 3). In competition with [^{3}H]GR65630 (0.5-2 nM), the affinities of 5-HT, 2-methyl-5-HT and renzapride for E106D decreased by 132-fold, 246-fold, 22-fold and for E106N by 30-fold, 17-fold and 13-fold respectively, compared to WT (Table 1). Patch clamp studies, essentially according to Gill et al. (1995), showed that minimal changes in 5-HT EC_{50} values were observed for E106D (1.2 ±.0.2 µM) and E106N (8.7 ±.2.0 µM) compared to WT (1.2 ±.0.1 µM; n = 5), although the Hill coefficient of WT (2.0) decreased to unity for both mutants.

	WT	E106D	E106N
5-HT	15.6 ± 3.2 (10)	2056 ± 564 (3)	469 ± 25 (3)
2-methyl-5-HT	173 ± 18 (9)	42711 ± 9117 (3)	2947 ± 456 (3)
mCPBG	5.01 ± 1.09 (6)	8.37 ± 3.08 (3)	2.89 ± 0.37 (3)
Renzapride	1.29 ± 0.17 (4)	28.9 ± 10.0 (3)	17.0 ± 3.82 (3)

TABLE 1. The affinities (Ki, nM; mean ± SEM (n)) of various compounds competing for [^{3}H]GR65630 binding.

These data suggest that while E106 is an important determinant of agonist recognition by the receptor in its high affinity (desensitised) state, the resting state is not so compromised. However, in these mutants we find no evidence of functional positive cooperativity, which is present in the WT receptor.

Boess, F.G., Beroukhim, R. & Martin, I.L. (1995). J. Neurochem., 64, 1401-1405.
Boess, F.G., Lummis, S.C.R. & Martin, I.L. (1992). J. Neurochem., 59, 1692-1701.
Gill, C.H., Peters, J.A., & Lambert, J.J. (1995). Br. J. Pharmacol., 114, 1211-1221.
Werner, P., Kawashima, E., Reid, J., Hussy, N., Lundström, K., Bluell, G., Humbert, Y. & Jones, K.A., (1994). Mol. Brain Res., 26, 233-241.

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97P BINDING OF THE β-ADRENOCEPTOR ANTAGONIST RADIOLIGAND [^{125}I]-IODOCYANOPINDOLOL TO PARTICULATE PREPARATIONS FROM THE GUINEA-PIG CEREBRAL CORTEX AND CEREBELLUM

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In a recent communication to the Society, we described a propranolol-sensitive, isoprenaline-evoked cyclic AMP response in guinea-pig cerebellar slices (Wallace *et al.*, 1995). In contrast, isoprenaline failed to elicit a cyclic AMP response in the guinea-pig cerebral cortex, neostriatum or hippocampus. We have therefore investigated the binding characteristics of the β-adrenoceptor antagonist radioligand [^{125}I]-iodocyanopindolol (ICYP) to particulate preparations from the guinea-pig brain.

A post-nuclear particulate preparation (15 minutes at 40 000 g of a supernatant layer derived from a 10 minute centrifugation at 1 000 g in 9 % w/v sucrose) was prepared from guinea-pig (Dunkin-Hartley, male, 350-650g) cerebral cortex or cerebellum. Binding using [^{125}I]-ICYP was carried out at 37°C for 60 minutes in a total volume of 250 µL, in the presence of 40-100 µg protein, in 50 mM Tris buffer, pH 8.0, containing 2 mM ascorbate. The presence of 100 µM isoprenaline was used to define non-displaceable binding. Data cited were derived from analysis of at least three different preparations using the computer program Prism (GraphPad, California, USA), while statistical analyses (P values) were performed using the Students t-test.

Analysis of saturation isotherms with increasing concentrations of [^{125}I]-ICYP in the presence of 150 mM NaCl showed similar binding density in the cerebral cortex and cerebellum (24 ± 3 and 34 ± 7 fmol.mg^{-1}, respectively; P=23.8%), while cerebellar preparations displayed a higher affinity (K_d values 20 ± 3 compared to 55 ± 10 pM; P=3.5%).

Competition for [^{125}I]-ICYP (ca. 100 pM) binding in the presence of 2 mM $MgCl_2$ elicited an estimate of the pK_i for isoprenaline in the cerebral cortex of 6.52 ± 0.07 (slope -0.96 ± 0.14), which was unchanged in the presence of 100 µM guanylyl-5'-imidodiphosphate (GppNHp) (pK_i = 6.46 ± 0.07; P=57.1%; slope = -1.03 ± 0.11). In the cerebellar preparations, isoprenaline competition for [^{125}I]-ICYP binding was of a more shallow slope (-0.63 ± 0.02). Fitting a two-site competition curve to these data allowed calculation of pK_i values of 8.41 ± 0.14 and 6.61 ± 0.09 with 36 ± 6 % of the binding sites fitted to the high affinity component. In the presence of 100 µM GppNHp, the pK_i values were not significantly altered (high affinity site 9.02 ± 0.36; P=18.9%; low affinity site 6.65 ± 0.14; P=81.7%), however, a significant reduction in the proportion of high affinity binding sites was observed (14 ± 5 %; P=4.8%).

These data indicate that the density of β-adrenoceptor-like binding sites is similar in guinea-pig cerebellum and cerebral cortex. However, agonist displaceable binding appears subject to regulation by guanine nucleotides in the cerebellum but not in the cerebral cortex. This raises the possibility that β-adrenoceptors present in the cerebellum are coupled to G-proteins, while those in the cerebral cortex are either "silent" or coupled to a distinct signal transduction mechanism.

Wallace K.E., Kendall D.A. & Alexander S.P.H. (1995) *Br.J.Pharmacol.* **116**, 137P

98P EVIDENCE FOR A PREFERENTIAL BLOCKADE OF INHIBITORY SYNAPTIC TRANSMISSION BY ω-AGATOXIN IVA IN THE *IN VITRO* SPINAL CORD OF THE NEONATAL RAT

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The spinal cord of the neonatal rat is a complex mixture of both excitatory and inhibitory neurones, many of the latter being GABAergic or glycinergic interneurones (Evans, 1989). At low concentrations, the peptide, ω-agatoxin IVA (ω-Aga-IVA) is a selective blocker of P-type, voltage-sensitive calcium channels (VSCCs; Dolphin, 1995) and we have examined its influence on dorsal root-evoked, ventral root potentials.

Dorsal root stimulation in the neonatal rat spinal cord essentially elicits a biphasic reflex in the corresponding ventral root (Evans, 1989). The initial phase is largely comprised of monosynaptic connections with latencies of 5-10 ms and durations of 7-10 ms. Polysynaptic connections elicit a potential of similar latency but of far longer duration (typically 2-20 s). In the present study, we used a grease-gap technique (Bufton *et al.*, 1995) to record from the ventral root of an L3, L4 or L5 segment of *in vitro*, hemisected spinal cords taken from Wistar rats (3-5 days old). The amplitudes, latencies and durations of evoked potentials were measured in response to single stimuli (0.5 ms, 8 x threshold) delivered at 5-min intervals to a dorsal root. Drugs were superfused at 0.5 ml min^{-1} in a Krebs-bicarbonate buffer maintained at 25°C and gassed with a 95% O_2/5% CO_2 mixture. ω-Aga-IVA was delivered in a carrier of bovine serum albumin (0.5 mg ml^{-1}). All data represent the mean ± s.e.mean.

In control cords, the short-latency (6.7 ± 0.3 ms), short-duration (9.7 ± 0.7 ms) potentials had a peak amplitude of 5.4 ± 0.2 mV (n=4). Although these potentials' parameters were unaltered, ω-Aga-IVA (100 nM) caused the appearance of one and sometimes two, intermediate polysynaptic potentials (IPPs) of initial latency 18.3 ± 2.0 ms and duration 6.7 ± 0.3 ms (n=3). The glycine receptor antagonist, strychnine (1 µM), induced similar IPPs with an initial latency and duration of 18.0 ± 1.8 ms and 7.8 ± 1.7 ms, respectively (n=4). The $GABA_A$ antagonist, bicuculline (10 µM), also produced IPPs of 19.3 ± 0.5 ms initial latency and 6.5 ± 0.3 ms duration (n=4) although these had a burst-like nature. The amplitudes of the first extra potential recruited by ω-Aga-IVA, strychnine and bicuculline were 67 ± 4%, 68 ± 9% and 42 ± 8% of the control, short-duration reflex, respectively. It is unlikely that the action of ω-Aga-IVA was due to a direct, post-junctional excitation since application of the postsynaptic neuronal excitant, *N*-methyl-D-aspartate (1 µM), failed to evoke any IPPs (n=3).

These data imply that P-type VSCCs regulate inhibitory connections within the spinal cord of neonatal rats. Even though 200 nM ω-Aga-IVA virtually abolishes glycine-mediated transmission in spinal cord slices (Takahashi & Momiyama, 1993) and its action on IPP amplitude and number was mimicked more closely in the present study by strychnine than by bicuculline, a functional role of P-type channels on GABAergic spinal neurones cannot be discounted.

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Bufton, H.R., Lodge, D. & Kilpatrick, I.C. (1995) *Br. J. Pharmacol.* **116**, 437P.
Dolphin, A.C. (1995) *Exp. Physiol.* **80**, 1-36.
Evans, R.H. (1989) *Prog. Neurobiol.* **33**, 255-279.
Takahashi, T. & Momiyama, A. (1993) *Nature* **366**, 156-158.

99P EFFECT OF DANTROLENE ON THE PERTURBATIONS IN IONIC HOMEOSTASIS INDUCED BY COMBINED OXYGEN AND GLUCOSE DEPRIVATION IN THE RAT HIPPOCAMPUS *IN VITRO*

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Cerebral anoxia causes a characteristic loss of ionic homeostasis - during phase 1 the extracellular K^+ concentration ($[K^+]_e$) rises from ca. 3 to 10 mM with little change in $[Ca^{2+}]_e$ and during phase 2 $[K^+]_e$ increases rapidly, reaching >60 mM, and $[Ca^{2+}]_e$ falls dramatically. A negative extracellular d.c. potential is coincident with these phase 2 changes - likely reflecting cellular depolarisation (see Hansen, 1985). Dantrolene is known to inhibit intracellular Ca^{2+} release and has been reported to reduce anoxia-induced hippocampal cell hyperpolarisation (Belousov *et al.*, 1995); it has also been reported to be neuroprotective against ischaemia *in vivo* (Zhang *et al.*, 1993). Therefore, we wished to determine whether dantrolene might attenuate extracellular ionic changes resulting from oxygen and glucose deprivation in the rat hippocampus *in vitro*.

Transverse hippocampal slices (350 μm) were prepared from pentobarbitone-anaesthetised male Sprague-Dawley rats (100-160g). Slices were submerged in a chamber and superfused with an aqueous solution at 30°C containing (mM): NaCl 125, KCl 2, KH_2PO_4 1, $MgSO_4$ 1, glucose 5, $NaHCO_3$ 25, $CaCl_2$ 2.5 gassed with 5% CO_2 in O_2. Oxygen and glucose deprivation was induced by gassing the aqueous solution with 5% CO_2 in N_2 and omitting glucose. Ion concentrations were measured by ion-selective, double-barrelled microelectrodes based on ionophore cocktails (K^+-Fluka 60031 & Ca^{2+}-Fluka 21048). Recording electrodes were placed in the CA1 stratum pyramidale and near-maximal field potentials were evoked by a stimulating electrode placed in the CA3 stratum radiatum, with both the evoked potential and extracellular d.c. potential being recorded via the reference barrel of the ion-selective electrode. Each slice was subjected to a single period of oxygen and glucose deprivation, with or without dantrolene pretreatment (75 min), until a few minutes after the maximum change (10-25 min). Data were analyzed with the unpaired Mann-Whitney U-test and are presented as median (range, number).

Combined oxygen and glucose deprivation resulted in a biphasic increase in $[K^+]_e$. During phase 1 $[K^+]_e$ rose gradually from 3 mM to 7.1 mM (6.0 to 8.9, n=4). In phase 2 $[K^+]_e$ rose sharply to 17.8 mM (13.1 to 19.1, n=4), peaking at 9.4 min (8.5 to 10.2). Coincident with phase 2 only, there was a rapid fall in the $[Ca^{2+}]_e$, from 2.5 mM to 1.1 mM (0.7 to 1.2, n=6). Dantrolene (20 μM) abolished the evoked field potential after ca. 25 min of perfusion. In its presence, the maximum change in $[K^+]_e$ produced by oxygen and glucose deprivation was smaller, rising to 9.1 mM (7.4 to 10.0, n=4) (P=0.01), and phases 1 and 2 of this response were much less distinct. Dantrolene had no clear effect on the peak fall in $[Ca^{2+}]_e$, it dropped to 1.4 mM (1.3 to 1.6, n=5) (P=0.05), but in 3 out of 5 slices the rate of this fall was reduced. Dantrolene reduced the negative extracellular d.c. potential change, coincident with phase 2, from 1.4 mV (0.8 to 3.5, n=10) to <0.2 mV (<0.2 to 1.9, n=6) (P=0.03). Dantrolene did not alter the basal levels of $[K^+]_e$, $[Ca^{2+}]_e$ or the d.c. potential.

These results suggest that intracellular Ca^{2+} release may be involved in the loss of K^+ and Ca^{2+} homeostasis seen in the CA1 pyramidal cell layer as a result of oxygen and glucose deprivation.

Belousov, A.B., Godfraind, J-M. & Krnjevic, K. (1995) *J.Physiol.*, 486, 547-556
Hansen, A.J. (1985). *Physiol. Rev.*, 65, 101-148
Zhang, L., Andou, Y., Masuda, S., *et al.* (1993). *Neurosci. Lett.*, 158, 105-108

100P STEROID SULPHATASE INHIBITION AMELIORATES COLLAGEN-II ARTHRITIS IN MICE

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The hypothalamic-pituitary-adrenal axis is central in the response to many diverse physiological challenges, including immune and inflammatory reactions. Dehydroepiandrosterone (DHEA, an endogenous anti-glucocorticoid), and its inactive sulphated form (DHEAS), are major circulating steroid hormones. *In vitro* DHEA potentiates lymphocyte activation (Padgett *et al*, 1994) and augments DTH-type reactions *in vivo* (Suitters *et al*, 1995). *In vivo* serum DHEAS in rheumatoid arthritis (RA) patients is reduced compared to normal controls whilst there is only a small reduction in serum DHEA (Feher *et al*, 1986), suggesting increased turnover to the active form. We postulated therefore that inhibition of steroid sulphatase may be beneficial in RA. To test this, we used CT2251 (3-estrone sulphamate), a synthetic non-reversible inhibitor in the collagen-II (C-II) based model of RA in DBA/1 mice. This is induced by *s.c.* injection of chick sternal C-II in Freunds' Complete Adjuvant (100ug/ 100uL) on day 0, and in Incomplete Adjuvant on day 15. Around day 25, paw swelling develops, and each mouse is scored for paws with swelling (0-4) and each paw for severity (0-3), giving a maximum severity of 12 per mouse. Efficacy was tested in two experiments. In both, CT2251 was given at 10 $mg.kg^{-1}$ *p.o.* in olive oil weekly, a dose previously shown to be effective at inhibiting contact sensitisation in mice (Suitters *et al*, 1995). Firstly, 20 mice were immunised with C-II on day 0. On day -1, 10 were given CT2251 and 10 vehicle. This was repeated weekly. Mice were scored from day 25 for symptoms, and at day 35 killed and their feet removed for histology. In a separate experiment, mice were immunised with C-II and after symptoms of inflammation developed were allocated in turn to CT2251 or vehicle, given weekly. These were followed for a further 35 days. After this they were killed and their feet removed for histology. This was performed on formalin fixed, decalcified sections, and each paw graded on a scale of 0-4 for inflammation and bone erosion by a blinded observer.

In the first experiment, by day 35, 50% of animals showed symptoms in the vehicle group, whereas in the CT2251 treated group, no mouse had developed any signs of inflammation, either macro- or microscopically ($P<0.05$, Fishers Exact Test). In the second experiment, where animals were treated with CT2251 or vehicle after the onset of symptoms, CT2251 halted any further progression of swelling, or increase in the number of paws involved. By day 35 post-appearance there was a difference in the number of paws inflamed (CT2251 2.3 ± 0.28 vs. vehicle 3.15 ± .22; $P< 0.05$, Students' T-test) and the mean severity of each mouse (CT2251 2.31 ± 0.93 vs. vehicle 7.16 ± 0.63; $P<0.005$). To demonstrate overall efficacy, the AUC was calculated for both groups. This also displayed a clear difference (CT2251 31.62 ± 20.6 vs. vehicle 144.4 ± 19.8; $P<0.001$, Students T-test).

In summary, we have shown that CT2251, an irreversible inhibitor of the steroid sulphatase enzyme is capable of reducing the symptoms of C-II induced arthritis in the mouse both when throughout the experiment, and when given therapeutically after the onset of symptoms. This suggests that DHEA is pro-inflammatory in this model of rheumatoid arthritis, and that this type of inhibition may be of potential use in the treatment of this disorder in man.

Feher, K.G. *et al* (1986). *Exp.Clin.Endocrinol.* 87, 38-42.
Padgett, D.A. & Loria, R.M. (1994). *J.Immunol.* 153, 1544-1552.
Suitters, A.J. *et al.* (1995). *Brit.J.Pharmacol.* 116, 15P.

101P ENANTIOSELECTIVE INHIBITION OF THE FORMALIN PAW LATE PHASE BY THE NK_1 RECEPTOR ANTAGONIST L-773,060 IN GERBILS

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Intrathecal injection of the NK_1 receptor antagonist CP-96,345, but not its less active stereoisomer CP-96,344, inhibits the late phase behavioural response to intraplantar injection of formalin in rats (Yamamoto & Yaksh, 1991). However, the same compounds when administered systemically produce antinociceptive effects in the formalin paw test but only at relatively high doses, and without enantioselectivity (Nagahisa et al, 1992). These findings suggest that the central NK_1 receptor-specific antinociceptive effects of CP-96,345 may be masked by nonspecific actions (e.g. ion channel blockade) in peripheral tissues. It was hypothesised that a greater separation between the NK_1-specific and nonspecific antinociceptive effects might be acieved using an NK_1 receptor antagonist with a long central duration of action since the response to formalin could be observed when peak plasma levels, and the likelihood of peripheral nonspecific effects, had subsided. We now describe the effect of such a compound, L-773,060 ((2S,3S)-3-((3,5-bis(trifluoromethyl)phenyl)methyloxy)-2-phenylpiperidine; Harrison et al, 1994), and its 2R,3R enantiomer L-733,061, on the early and late phase response to formalin in gerbils.

The CNS penetration and duration of action of L-733,060 was determined by the inhibition of GR73632-induced foot tapping in gerbils (Rupniak & Williams, 1994). Under isoflurane anaesthesia, L-733,060 (0.1-1 mg/kg), L-733,061 (10 mg/kg) or vehicle (0.1M HCl) were injected i.v. in male or female Mongolian gerbils (40-70 g) either immediately before, or up to 8 h prior to, i.c.v. infusion of GR73632 (3 pmol in 5µl). On recovery from anaesthesia, the duration of foot tapping was recorded for 5 min (n=3-6 per group). The duration of paw licking following intraplantar injection of formalin (2%) was recorded in separate animals 3 h after i.v. injection of L-733,060 (0.01-10 mg/kg), L-733,061 (3 or 10 mg/kg) or vehicle (n=6-14 per group).

L-733,060 (0.1-1.0 mg/kg), but not L-733,061 (10 mg/kg), caused a dose-related and complete inhibition of foot tapping when administered immediately before GR73632 (ANOVA $F_{4,22}$=33.29, $p<0.001$; ID_{50}=0.25 mg/kg i.v.). Foot tapping remained inhibited by ≥ 80 % after pretreatment for up to 4 h with 1.0 mg/kg i.v. of L-733,060.

In gerbils habituated to the testing environment for 3 h following i.v. injection of vehicle, two discrete periods of paw licking were induced by intraplantar injection of formalin (early phase 0-15 min, late phase 20-40 min after formalin). The duration of licking in early phase was approximately halved by 10 mg/kg i.v. of L-733,060, but not by L-733,061, administered 3 h previously ($F_{9,78}$=2.51, p=0.014). In contrast, the late phase was dose-dependently and fully inhibited by L-733,060 (0.01-10 mg/kg i.v., 3 h previously; $F_{9,78}$=4.26, $p<0.001$; ID_{50}=0.17 mg/kg i.v.). Only the highest dose only of L-733,061 (10 mg/kg i.v.) attenuated the late phase, by approximately 50%, indicating an enantiomeric separation of greater than 50-fold in this assay.

The demonstration of an NK_1 receptor-mediated antinociceptive effect of a long acting, brain penetrant NK_1 receptor antagonist in the formalin paw assay supports the preclinical rationale for the clinical use of such compounds as centrally-acting analgesics.

Harrison, T., Williams, B., Swain, C. and Ball, R. (1994) *Bioorg.Med.Chem.Letts.*,4,2545.
Nagahisa, A., Asai, R., Kanai, Y. *et al* (1992) *Br. J. Pharmacol.*,107,273.
Rupniak,N. & Williams, A. (1994) *Eur J. Pharmacol.*, 265,179.
Yamamoto , T. & Yaksh, T. (1991) *Life Sci.*, 491, 955.

102P SENSORY NERVE ACTIVATION OF RAT SMALL MESENTERIC VEINS

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Activation of capsaicin sensitive primary afferent neurones (CSPANs) causes an inflammatory response characterised by vasodilation and oedema formation. It has been suggested that within the microcirculation CSPANs are located in small arteries, and that nerve activation induces the release of calcitonin gene-related peptide (CGRP), which is responsible for arteriolar dilatation and substance P (SP), which increases venular permeability. It is generally considered that sensory nerves do not exist in close contact with small veins of the microcirculation and that neuropeptides have minmal effect on the tone of these vessels, however this has not been explored directly. We have now investigated the activity of CSPANs in small veins of the rat mesentery microcirculation using the tension myograph.

Male Wistar rats (240-290g) were sacrificed by cervical dislocation. The mesentery was removed and placed in cold (4°C) Krebs solution. Small veins (110-420 µm diameter) were dissected free and 1-2 mm lengths were cut and mounted in a myograph for the measurement of isometric tension in Krebs solution at 37°C bubbled with 5 % CO_2 in O_2. Vessels were left to equilibrate for 45 min prior to stretching to determine the relationship between the passive tension and internal circumference of each vessel using the Laplace equation. From this the internal diameter was determined (Mulvany & Halpern, 1977). These vessels were stretched to an internal circumference 90 % of that when under a transmural pressure of 20 mmHg.

Vessels were contracted repeatedly with the thromboxane A_2-mimetic, U-46619 (11α,9 α-epoxymethano-PGH_2) (0.1 µM) until the response was constant. After this the vessels were submaximally (EC50) contracted using U-46619 (0.01-0.1 µM) and endothelium integrity determined by measuring the response to BK (1 µM) (>10 % relaxation designated endothelium intact.

In veins not preconstricted with U-46619, capsaicin had no tension effects, however in precontracted tissues capsaicin caused a concentration-dependent relaxation response which was attenuated in the presence of ruthenium red (30 µM, n=5) indicating that capsaicin was activating sensory neurones. The NO synthase inhibitor L-NMMA (100µM, n=5) had no effect on the responses to capsaicin, however denudation of endothelium significantly attenuated capsaicin-induced relaxation. Neither SP (0.1 -1µM) or CGRP (0.1 µM) had any significant relaxant effects in precontracted tissues (n=3). However SP (0.001-10 µM) did cause a concentration-dependent contraction of tissues. The response curve to SP was shifted to the right by the NK1 receptor blocker, RP67,580, in a concentration-dependent fashion to give a pA_2 value of 6.88. RP 68,651 (1 µM), the inactive enantiomer, had no effect on the response curve to SP (CR=1.6 ± 0.4 (n=4)).

These results demonstrate the existence of sensory nerve fibres in small mesenteric veins of the rat, activation of which causes relaxation. This relaxation appears to be independent of NO,SP and CGRP but is dependent on an intact endothelium. SP causes a contraction of tissues that is mediated by activation of NK1 receptors. Together these results suggest that within the microcirculation there may be functional C-fibres in both venular aswell as the arteriolar side, activation of which contributes to changes in reactivity observed within the microcirculation in response to neurogenic stimuli.

Mulvany, M.J. & Halpern, W. (1977). *Circ. Res.* **41**, 19-25.

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103P ACTIVATION OF PERIPHERAL SENSORY NEURONES IN THE NEONATAL RAT TAIL BY ATP

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Several workers have suggested a role for adenosine 5'-triphosphate (ATP) in the perception and processing of somatosensory information. The proposal that ATP may be an important mediator of primary afferent sensation is supported by clinical data showing that ATP produces pain when injected intradermally into human volunteers (Bleehen & Keele, 1977). However, little is known about the precise mechanisms by which ATP may activate sensory nerve endings and the nature of the receptor(s) mediating these actions remains unknown. In the present study, we have used the neonatal rat spinal cord-tail preparation (Dray *et al.*, 1990), in which activation of exposed sensory nerve endings in the tail is recorded indirectly via measurement of spinal ventral motoneuron reflexes, to examine the effects of ATP on cutaneous afferents.

Recordings *in vitro* of spinal L_3-L_5 ventral root d.c. potentials were made from 1-2 days old rats using low impedance glass microelectrodes essentially as described by Dray *et al.* (1990). The spinal cord and attached tail were placed in a recording chamber and independently superfused at 2-3ml min^{-1} with a modified Krebs solution at 22-28°C, gassed with 95% O_2/5% CO_2. Viability of the preparation was assessed by determining depolarisation responses to 5-hydroxytryptamine (5-HT, 100μM, 30s to the cord) and to bradykinin (BK, 10-1000nM; 15s to the tail); if responses did not exceed 500μV or very unstable baseline recordings were obtained preparations were rejected. Application of ATP (1-100μM) to the tail for 30s evoked concentration-related depolarisation responses of the ventral root which were rapid in onset (<5s) and faded in the continued presence of the drug. Reproducible responses to ATP could be obtained if periods of 20-30min were left between drug applications. The EC_{50} value (95% confidence limits) for ATP was 4.0μM (1.4-11.7, n=4) and the maximal response amounted to 95.0±6.7% of the peak amplitude of the response to bradykinin (BK, 1μM). The ATP analogue, αβmethylene ATP (αβmeATP), also evoked ventral root depolarisation and was effective at much lower concentrations than ATP (EC_{50} value 0.43μM (0.27-0.67) and maximal response 117.0±3.8% of BK; n=4). 2-Methylthio-ATP was approximately equieffective to ATP (mean EC_{50} value 3.1μM), but UTP (1-100μM, n=2) was inactive. Preincubation with the P_2 purinoceptor antagonist pyridoxal-5-phosphate (P-5-P, 100μM) reduced responses to αβmeATP (1μM) to 16.4±8.5% of the pre-antagonist control value (n=4). Responses to capsaicin (100nM) were not modified by P-5-P (93.1±3.5%; n=3). No cross desensitisation between αβmeATP (10μM), bradykinin (1μM) and low concentrations of capsaicin (200nM) was observed (each n=4).

These preliminary observations illustrate that ATP and certain related purines activate sensory, possibly nociceptive, neurones in the neonatal rat tail. An indirect mechanism of action of ATP cannot be excluded at the present stage, but evidence for the existence of distinct P_2 purinoceptors on dorsal root ganglion and other sensory neurone cell bodies would suggest that ATP excites sensory nerve endings directly (see Bean *et al.*, 1992 for review). The inability of bradykinin or capsaicin to produce heterologous desensitisation of responses to αβmeATP in the present study supports this contention. The high agonist potency of αβmeATP indicates that ionotropic P_{2X} purinoceptors may be involved (see Humphrey *et al.*, 1995 for review) although further studies are required to confirm this.

Bean, B.P. (1992). *Trends Pharmacol. Sci.*, **13**, 87-90.
Bleehen, T. & Keele, C.A. (1977). *Pain*, **3**, 367-377.
Dray, A. *et al.* (1990). *Br. J. Pharmacol.*, **101**, 727-733.
Humphrey, P.P.A. *et al.* (1995). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **352**, 1-12..

104P CHARACTERISATION OF P_{2X}-PURINOCEPTORS IN GUINEA-PIG ISOLATED VAS DEFERENS USING AGONISTS, ANTAGONISTS AND THE ECTO-ATP-ase INHIBITOR ARL 67156

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The receptors through which adenosine 5'-triphosphate (ATP), P^1, P^5-di-(adenosine-5') tetraphosphate (AP_4A) and P^1, P^5-di-(adenosine 5') pentaphosphate (AP_5A) act to evoke contraction of the guinea-pig isolated vas deferens were studied using the three P_2-purinoceptor antagonists suramin, pyridoxal 5'-phosphate (P-5-P) and pyridoxal phosphate-6-azo-phenyl-2, 4-disulphonic acid (PPADS). The effect of the ecto-ATPase inhibitor 6-N,N,diethyl-D-β,γ-dibromo methyleneATP (ARL 67156) on agonist potency was also assessed.

Guinea-pig isolated vasa deferentia were mounted in a modified Krebs solution at 36°C and bubbled with 95% O_2/5% CO_2. Concentration effect curves to ATP, AP_4A and AP_5A showed no clear maximum, so it was not possible to measure EC_{50} concentrations. Therefore, equi-effective concentrations of each agonist were estimated as the concentration of agonist which gave a contractile response of about 50% of that obtained to 1000 μM ATP. These concentrations, ATP 300 μM, AP_4A 30 μM and AP_5A 3 μM, were used in subsequent experiments to obtain the IC_{50} values shown in Table 1.

PPADS and P-5-P were effective antagonists against all three of the agonists, PPADS being up to 50 times more potent than P-5-P. Suramin was an effective antagonist against AP_4A and AP_5A, and at 100 μM suramin abolished responses to both agonists. However, responses to ATP were not antagonised by suramin, which produced a concentration-dependent increase in responses to ATP of up to 40%. Obviously calculation of an IC_{50} was not applicable in this case (denoted as N.A. in table 1). The contrasting effects of suramin on the rapidly hydrolyzable agonist ATP and the more stable diadenosine compounds may be due to inhibition of ecto-ATPases by suramin, preventing the breakdown of ATP, thus enhancing its effect as an agonist. This possibility was examined using the novel ecto-ATPase inhibitor ARL 67156 (Crack *et al.*, 1995). ARL 67156 (100 μM) significantly enhanced responses to exogenous ATP (from 3.35 ± 0.25 g to 4.68 ± 0.41 g) and AP_4A (from 3.58 ± 0.27 g to 5.71 ± 0.39 g), whereas responses to AP_5A were significantly reduced (from 3.37 ± 0.15 g to 2.54 ± 0.24 g). All comparisons are by Students' t-test for paired data, and considered significant if $P<0.05$, n = 6-8.

These results imply that the ability of suramin to inhibit responses to AP_4A and AP_5A, but not those to ATP cannot be explained on the basis of inhibition of ecto-ATPase by suramin.

Table 1. IC_{50} values (μM) for P_2-purinoceptor antagonists in guinea-pig vas deferens. (n = 6).

	ATP	AP_4A	AP_5A
PPADS	1.3 ± 0.1	2.8 ± 0.1	3.3 ± 0.1
P-5-P	25.7 ± 12.1	55.8 ± 1.2	161 ± 4.0
Suramin	N.A.	5.8 ± 0.1	13.5 ± 0.1

Crack, B.E., Pollard, C.E., Beukers, M.W. *et al.* (1995) *Br. J. Pharmacol.*, 114, 475-481.

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105P TRANSFECTED P_{2U}- AND P_{2Y}-PURINOCEPTORS: DIFFERENTIAL SENSITIVITY TO SURAMIN

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We have previously reported that the two co-existing receptors for ATP and ADP found in bovine aortic endothelial cell cultures and aortic collaterol artery rings show differential sensitivity to suramin. The response to the P_{2Y}-purinoceptor agonist 2-methylthioATP (2MeSATP), but not that of the P_{2U}-purinoceptor UTP, was clearly shifted to the right in a manner consistent with action as a competitive antagonist (Wilkinson et al, 1993;1994). Here we report on the differential sensitivity to suramin of cloned P_{2U}- and P_{2Y}-purinoceptors transfected into a common host cell, and ask whether the results are affected by ectonucleotidase breakdown of the agonists.

We used cloned human P_{2U}-purinoceptors and turkey P_{2Y}-purinoceptors, transfected into a 1321N1 subclone which showed no native response to the nucleotide agonists (Parr et al, 1994; Filtz et al, 1994). The accumulation of total [^{3}H]inositol (poly)phosphates in the presence of lithium was measured in cells in 24 well multiwells labelled for 24h with [^{3}H]inositol, and stimulated with the agonists for 15 min. Suramin was also present for a 10 min preincubation. The accumulation of mass inositol (1,4,5)trisphosphate was measured by a standard protein binding assay, after a stimulation of 15 seconds.

The position of the concentration response curve to UTP at the transfected P_{2U}-purinoceptor was affected by the presence of suramin. Data from 3 experiments, each in triplicate, expressed as -log EC_{50} ± s.e.mean: control, 5.93 ± 0.19; 30 μM suramin, 5.68 ± 0.11; 100 μ suramin, 5.27 ± 0.15; 300 μM suramin, 4.96 ± 0.14. In each case there was no change in the maximum response (to 100 μUTP). By contrast the effect of suramin on the transfected P_{2Y}-purinoceptor response was more profound: with 100 μM suramin the response was shifted 2 orders of magnitude to the right (with no effect on the maximal response). Suramin is known to inhibit the breakdown of agonists by ectonucleotidase seen in other preparations: this could be contributing to the differential effect of suramin on the 2 receptors. The EC_{50} for the 15 min UTP stimulation measuring total [^{3}H]inositol phosphates was not significantly different from that with the 15 sec response measuring inositol (1,4,5)trisphosphate, suggesting that breakdown has little effect on the concentration response curves. The ectonucleotidase inhibitor ARL 67156 (Crack et al, 1995) at 100 μM caused a small shift in the UTP dose response curve to the left in the absence, but not in the presence, of suramin. In these experiments 100 μM suramin in the absence of ARL 67156 moved the -log EC_{50} for UTP from 5.85 to 5.23, and in the presence of ARL 67156 from 6.17 to 5.22. While these results show an influence of breakdown on the responses, they also confirm the low sensitivity to suramin of the P_{2U}- compared to the P_{2Y}-purinoceptor

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Crack, B.E. et al. (1995) Br. J. Pharmacol. 114, 475-481.
Filitz, T.M. et al. (1994) Molec. Pharmacol. 46, 8-14.
Parr, C.E. et al (1994) Proc. Natl. Acad Sci USA 91, 3275-3279.
Wilkinson, G.F. et al (1993) Br. J. Pharmacol. 111, 723-728.
Wilkinson, G.F. et al (1994) J.Pharmac.Exper.Ther. 268, 881-887.

106P ATP-STIMULATED ^{45}Ca UPTAKE AS A MEANS OF MEASURING P_{2X} PURINOCEPTOR ACTIVATION IN PC12 CELLS

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Recently a P_{2X} purinoceptor has been cloned from the PC12 cell line (Brake et al., 1994). To date the majority of the pharmacological characterisation of both the endogenous and the recombinant PC12 P_{2X} purinoceptor has been carried out electrophysiologically. This work was part of a study aimed at measuring directly the permeation of ions through the channel, in order to characterise further the endogenous PC12 receptor. Due to the high calcium permeability of the P_{2X} purinoceptor channel we were able to measure radiolabelled calcium (^{45}Ca) influx as a consequence of receptor activation.

PC12 cells, grown in suspension in RPMI supplemented with 10% fetal calf serum and 5% horse serum, were harvested and resuspended in assay buffer: (mM), Hepes (20), KCl (5.6), Glucose (10), $MgCl_2$ (1), N-methyl-D-glucamine (139), $CaCl_2$ (2) EDTA (0.1) pH 7.4. Reactions were carried out in a volume of 500μl containing 1μCi of ^{45}Ca and 3-5 x 10^5 cells for 4 min and were terminated by vacuum filtration. Filters were washed with ice cold 100mM NaCl for 5 secs. Antagonists were preincubated with the cells for 30 min. ^{45}Ca uptake stimulated by 100μM ATP was optimal at 22°C. Specific uptake ocurred over a 2 min period and thereafter remained steady for 8 min. All data are mean ± s.e.mean ($n \geq 3$).

Various purine agonists were able to elicit ^{45}Ca uptake in PC12 cells with the following rank order of potency: (log EC_{50} ± s.e.m. in parenthesis) ATP-γ-S (4.75 ± 0.05) > ATP (4.5 ± 0.03) > 2Me-S-ATP (4 12 ± 0 09) > dibenzovl-ATP (3.54 ± 0 25) >> UTP, diA5P αβMeATP and adenosine which were virtually inactive at concentrations up to 1mM. Purinoceptor antagonists were also shown to inhibit ATP stimulated uptake with pyridoxalphosphate-6-azophenyl-2'-,4'-disulfonic acid (PPADS) and pyridoxal-5-phosphate (P-5-P) exhibiting potent insurmountable antagonism. Thus PPADS at 1, 3, 10 and 30μM inhibited maximal ATP-stimulated ^{45}Ca uptake by 3.7 ± 7.6, 34 ± 10.4, 69.4 ± 11.8 and 73.3 ± 14.3 %, respectively, and P-5-P at the same concentrations inhibited uptake by 27.7 ± 4.6, 33.6 ± 2.6, 49.7 ± 1.8 and 80 ± 5.4 %, respectively. D-tubocuraraine (dTC) was also an insurmountable antagonist, although approximately 10-fold weaker than PPADS and P-5-P. Both suramin and cibacron blue surmountably inhibited uptake at concentrations of 100 and 300μM, while DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) only inhibited uptake at concentrations > 300μM. ATP-stimulated uptake was not affected by nifedipine (10μM) but was reduced by sodium, choline and tris and inhibited by guanidinium (EC_{50} = 14.6 ± 1.8mM).

The ability of ATP, ATP-γ-S and 2Me-S-ATP to elicit ^{45}Ca influx along with the ability of suramin, cibacron blue and dTC to antagonise the ATP-stimulated uptake is consistent with the pharmacological profile of the recombinant PC12 P_{2X} purinoceptor (Brake et al., 1994), suggesting that the ^{45}Ca influx described here reflects P_{2X} purinoceptor activation. The blockade of the ^{45}Ca uptake by guanidinium adds further corroboration as it has been reported that this ion is very permeant at the recombinant PC12 P_{2X} purinoceptor (Evans, 1995). In addition, we have demonstrated that the P_{2X} antagonists, PPADS and P-5-P (see Humphrey et al., 1995), were able to potently antagonise this uptake. In conclusion, measurement of ATP-stimulated ^{45}Ca influx may provide a robust and rapid assay system to determine the operational characteristics of P_{2X} purinoceptors.

Brake A.J *et al* (1994) *Nature* 372 519-523
Evans R.J. (1995). *J. Physiol.* 482P, (Oxford meeting)
Humphrey P.P.A. *et al.*, (1995). *Naunyn-Schmiedeberg's Arch Pharmacol.* 352, 1-12

107P COMPARISON OF THE FUNCTIONAL MUSCARINIC RECEPTOR SELECTIVITY OF DARIFENACIN WITH TOLTERODINE AND OXYBUTININ

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Darifenacin ((S)-2-{1-[2-(2,3-dihydrobenzofuran-5-yl)ethyl]-3-pyrrolidinyl}-2,2-diphenylacetamide) is a novel M_3-selective muscarinic antagonist (Wallis *et al.*, 1995). The aim of the present study was to compare the muscarinic receptor pharmacology of darifenacin with that of tolterodine (Gillberg *et al.*, 1994) and the clinical agent oxybutynin.

Muscarinic receptor affinity was determined in tissue bath studies with guinea-pig bladder (M_3) using acetylcholine as the agonist, guinea-pig atria (M_2) using carbachol as the agonist and the rabbit vas deferens (M_1) using McN-A-343 as the agonist. pA_2 values were determined using Schild regression analysis (Table 1). The potency of compounds to inhibit carbachol (10^{-5}M) stimulated ^{86}Rb efflux from guinea-pig submandibular gland *in vitro* (Newgreen *et al.*, 1995) was measured and expressed as a pIC_{50} (Table 1).

All studies were carried out in Krebs-Henseleit solution gassed with 95%O_2/5%CO_2 at 37°C (atria 32°C).

Darifenacin had a higher affinity for the bladder than the atria or vas deferens, confirming the previously reported M_3 selectivity. In contrast, tolterodine and oxybutynin had similar profiles to atropine with highest affinity for M_1 receptors and no selectivity for M_3 over M_2. When pIC_{50} values on submandibular gland are compared - relative to atropine - to pA_2 values on bladder, tolterodine and oxybutynin are essentially non-selective whereas darifenacin shows 6-fold bladder selectivity. The novel profile of darifenacin may confer a therapeutic advantage in the treatment of urge incontinence.

Gillberg, P.G., Modiri, A.R. & Sparf, B. (1994). *Neurourol. Urodyn.*, 13, 435.
Newgreen, D.T., Leahy, D.J. & Naylor, A.M. (1995). *Br. J. Pharmacol.*, 116, 146P
Wallis, R. M., Burges, R.A., Cross, P.E. *et al.* (1995) *Pharmacol. Res.*, 31S, 54

Table 1: Functional potencies (pA_2/pIC_{50}), mean ± s.e.mean (n = 4)

Compound	Bladder	Atria	Vas Deferens	Salivary Gland	Bladder:Salivary Gland
	pA_2 value	(slopes not significantly different from 1)		pIC_{50}	Relative to atropine
Darifenacin	8.66 ± 0.14	7.3 ± 0.10	7.80 ± 0.10	7.0 ± 0.10	6
Tolterodine	7.77 ± 0.06	7.68 ± 0.25	8.49 ± 0.17	6.56 ± 0.07	2
Oxybutynin	7.44 ± 0.16	7.12 ± 0.29	7.90 ± 0.14	6.31 ± 0.16	1
Atropine	8.78 ± 0.08	8.80 ± 0.08	9.48 ± 0.04	7.87 ± 0.14	1

108P MUSCARINIC M_2 RECEPTORS MODULATE RELAXANT RESPONSES TO 5-HT RECEPTOR AND β_3-ADRENOCEPTOR AGONISM IN ISOLATED OESOPHAGEAL MUSCULARIS MUCOSAE OF RAT

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Activation of muscarinic M2 receptors inhibits relaxant responses to β-adrenoceptor agonists in guinea-pig isolated ileum (Thomas & Ehlert, 1993), by attenuating elevations in intracellular cAMP (Reddy *et al.*, 1995). In the present study, the role of muscarinic M2 receptors in reversing relaxations to 5-HT (via 5-HT4 receptors) or isoprenaline (via β3 adrenoceptors) has been assessed in isolated oesophageal muscularis mucosae of rat (Ford *et al.*, 1991; Ford *et al.*, 1992; de Boer et al., 1995). All values quoted are mean ± s.e.mean, n=4-8 animals.

Muscarinic receptor characterization studies. The muscarinic receptor subtype mediating contraction to oxotremorine M (pEC50=7.1±0.1) was assessed by determining the affinities of several antagonists. The rank order of affinity estimates (pKB values in parentheses) was atropine (9.1±0.2), 4-diphenyl-acetoxy *N*-methylpiperidine methiodide (4-DAMP 8.7±0.1), zamifenacin (8.6±0.3), *p*-fluorohexahydrosiladifenidol (7.5±0.1), himbacine (7.2±0.2), pirenzepine (6.8±0.3) and methoctramine (6.2±0.2). This affinity profile is consistent with activation of a muscarinic M3 receptor (Eglen *et al.*, 1994).

Relaxation studies. To study relaxations to 5-HT or isoprenaline, tissues were precontracted with 0.7 μM, of the TP receptor agonist, U46619 (approximate EC90). Responses to U46619 were unaffected by either methoctramine (1 μM) or exposure to 4-DAMP mustard (40 nM). In these tissues, the relaxant potencies (pEC50) of 5-HT and isoprenaline were 8.1±0.2 and 8.0±0.1, respectively. Methoctramine (1 μM) did not antagonize responses to either agonist, suggesting that muscarinic M2 receptors did not tonically inhibit relaxations to these agonists. Moreover, in tissues precontracted with oxotremorine M (1 μM), the relaxant potencies of both agonists were unaffected by methoctramine (1 uM), suggesting that even in the presence of a muscarinic acgonist, no role for muscarinic M2 receptors was disclosed.

Selective alkylation studies. To explore further a putative role for M2 receptors, selective muscarinic M2 receptor protection (using methoctramine, 1 μM), in the face of muscarinic M3 receptor alkylation (using 4-DAMP mustard, 40 nM, 60 min exposure) was undertaken (Reddy *et al.*, 1995). The contractile responses to oxotremorine M were virtually abolished by this procedure (before alkylation pEC50=7.0±0.2; Emax =1.4±0.08g; post alkylation pEC50=5.3±0.1; Emax =0.25±0.06g), suggesting that muscarinic M2 receptors did not play a direct role in contraction.

Recontraction studies. Demonstration of a role for muscarinic M2 receptors requires both depletion of the muscarinic M3 population *and* elevation of adenylyl cyclase (Thomas et al., 1993). Thus, following selective alkylation of muscarinic M3 receptors, tissues were precontracted with U46619 (0.7 μM), and maximally relaxed with either 5-HT (0.1 μM) or isoprenaline (0.1 μM). Under these conditions, oxotremorine M induced concentration-dependent re-contraction (pEC50=5.7±0.2; Emax=0.64±0.09g). These recontractions were surmountably antagonized by methoctramine (10 μM), with pKB values of 7.4±0.2 and 7.3±0.2 (5-HT or isoprenaline relaxed, respectively). These affinity estimates are consistent with activation of muscarinic M2 receptors (Eglen *et al.*, 1994; Reddy *et al.*, 1995).

Taken together, these data suggest that activation of muscarinic M2 receptors reverses relaxation to both 5-HT4 receptor and β3-adrenoceptor activation in oesophageal muscularis mucosae of rat.

de Boer, R.E.P., Brouwer, F., & Zaagsma, J., (1995) *Br. J. Pharmacol.*, 116, 1945-1947.
Eglen, R.M. *et al.*, (1994) *Trends Pharmacol. Sci.*, 15, 114-119.
Ford, A.P.D.W. *et al.*, (1991) *Br.J. Pharmacol.*, 105, 230P.
Ford, A.P.D.W. *et al.*, (1992) *Eur. J. Pharm.*, 211, 117-120.
Thomas, E.A. *et al.*, (1993) *J. Pharm. Exp. Ther.*, 14, 102-110.
Reddy, H. *et al.*, (1995) *Br. J. Pharmacol.*, 114, 49-56

109P THE α_1-ADRENOCEPTOR ANTAGONIST SB216469 (REC 15/2739) DISCRIMINATES BETWEEN α_{1A}-ADRENOCEPTORS AND THE α_1-ADRENOCEPTORS OF THE HUMAN PROSTATE

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It has been postulated that the contractile responses of the human prostate are mediated via the α_{1A}-adrenoceptor subtype (Forray et al., 1994; Marshall et al., 1995). SB216469-S (REC 15/2739) is an α_1-adrenoceptor antagonist which has been shown in radioligand binding experiments to have a high affinity for native α_{1A}- and cloned α_{1c}-adrenoceptors (Testa et al., 1995). The present study compares the actions of SB216469 at the functional α_1-adrenoceptors of several tissues with those in the human prostate.

Epididymal vas deferens, hemi-spleen, anococcygeus muscle and denuded aorta from rats, and human prostate strips were isolated and set up in gassed Krebs-bicarbonate solution at 37°C. Concentration-response curves to phenylephrine or noradrenaline were constructed in the absence and presence of SB216469 after a 30 min antagonist equilibration period. All experiments were performed in the presence of cocaine (10μM), corticosterone (10μM) and propranolol (1μM). Affinity values were compared using t-test following ANOVA.

On all tissues examined, SB216469 produced dextral shifts of agonist concentration response curves without altering maximum responses. Schild plots had slopes of unity except for the anococcygeus muscle ($P<0.05$) where the value was low (Table 1).

Table 1: Affinity values for SB216469. pA_2 values were determined from Schild plots and mean (±sem) pK_B values from individual shifts of curves.

Tissue	pA_2	slope	pK_B	n
Vas deferens	10.3	0.8±0.2	10.0±0.1	12
Anoc. Muscle	9.7	0.7±0.1	9.5±0.1	5
Spleen	6.8	0.8±0.1	6.5±0.1	14
Aorta	8.4	1.5±0.3	8.8±0.1	15
H. Prostate	8.1	1.0±0.2	8.4±0.1	15

SB216469 exhibited highest affinity for the α_{1A}-adrenoceptors of the rat vas deferens and anococcygeus muscle (only an apparent value for anococcugeus), lowest affinity for the α_{1B}-adrenoceptors of the rat spleen and intermediate affinity for the α_{1D}-adrenoceptors of the rat aorta. SB216469 had a significantly lower affinity at the human prostatic receptor than at the vas deferens or anococcygeus α_{1A}-adrenoceptor ($P<0.0001$).

Thus, SB216469 discriminates between the functional α_{1A}-adrenoceptors of the vas deferens and the α_1-adrenoceptors of the human prostate providing further evidence that the human prostate possesses a novel α_1-adrenoceptor subtype.

Forray et al. (1994) Mol. Pharmacol. 45, 703-708.
Marshall et al. (1995) Br. J. Pharmacol. 115, 781-786.
Testa et al. (1995) Pharmacol. Commun. 6, 79-86.

110P DIFFERENCES IN AFFINITY FOR THE ANTAGONIST RS 17053 AT α_{1A}-ADRENOCEPTORS BETWEEN RAT TISSUES

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Three α_1-adrenoceptor subtypes have been characterized in rat tissues (α_{1A}-, α_{1B}-, α_{1D}-) which correlate well with the three cloned α_1-adrenoceptor subtypes. RS 17053 is a novel selective α_{1A}-adrenoceptor antagonist in rat tissues and for the α_{1a} clone (Ford et al., 1995). In this study the selectivity of RS 17053 has been examined in functional experiments for the α_{1A}-adrenoceptors mediating contraction of the rat epididymal vas deferens (Burt et al., 1995) and rat hepatic portal vein (Lepretre et al., 1994) and compared with the α_{1B}- and α_{1D}- adrenoceptors mediating contraction of the rat spleen (Burt et al., 1995) and aorta (Kenny et al., 1995) respectively.

Epididymal vas deferens, portal vein, splenic strips and endothelium denuded thoracic aortic rings from male Sprague-Dawley rats (350-500g) were set up in Krebs solution (except for the portal vein where a high 50mM K^+ Krebs was used to suppress spontaneous phasic activity) at 37°C, and gassed with 95%O_2/5%CO_2. Cocaine and β-oestradiol (both 10^{-5}M) were added to the Krebs for the vas deferens. Tissues were placed under 0.5g tension except for the spleen (1g).

Non-cumulative concentration-contraction curves were measured in the vas deferens and cumulative curves were measured in the spleen, portal vein and aorta. Noradrenaline was used as the agonist in the vas and aorta and phenylephrine was used in the portal vein and spleen. The curves in all tissues were measured in the absence and presence of RS 17053 (equilibrated for 2 hr) from which concentration ratios were measured.

RS 17053 produced rightward shifts in the concentration-contraction curves in all tissues. In the epididymal vas deferens RS 17053 3×10^{-8}M caused a reduction in maximum response. Concentration-ratios from lower concentrations of RS 17053 (which did not reduce the maximum response) gave a pA_2 of 9.5, (slope of Schild plot 1.07±0.08). In the spleen and aorta RS 17053 gave pA_2 values of 7.2 (slope 1.04±0.04) and 6.6 (slope 1.96±0.36) respectively. However in the portal vein RS 17053 antagonized the phenylephrine contractions by only 3 fold at 10^{-7}M but around 100 fold at 10^{-6}M.

RS 17053 was selective for α_{1A}-adrenoceptors in the vas compared with α_{1B}- and α_{1D}-adrenoceptor mediated responses. However the antagonist appeared to have a 100-fold lower affinity in the rat portal vein (similar to that in the human prostate, Ford et al., 1995) compared with the vas deferens, both α_{1A}- mediated contractions. This difference, consistent with two subtypes of the α_{1A}-adrenoceptor, requires further investigation.

We thank Roche Bioscience for RS 17053.

Burt, R.P. *et al.* (1995) *Br. J. Pharmacol.*, **115**, 467-475.
Ford, A.P. *et al.* (1995) *Br. J. Pharmacol.*, **114**, 24P.
Kenny, B.A. *et al.* (1995) *Br. J. Pharmacol.*, **115**, 981-986.
Lepretre *et al.* (1994) *J. Pharmacol. Exp. Ther.*, **268**, 167-174.

111P HYPORESPONSIVENESS TO α-ADRENOCEPTOR AGONISTS IN CIRRHOTIC PATIENTS IS NOT EVIDENT WHEN HUMAN HEPATIC AND MESENTERIC ARTERIES ARE STUDIED *IN VITRO*

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Patients with established cirrhosis exhibit hyporesponsiveness to infused vasoconstrictors (MacGilchrist et al. 1991). It is unclear whether this is secondary to a chronic defect induced in the vascular wall or to a circulating factor released by the cirrhotic liver. This study aimed to determine whether hyporesponsiveness to α-adrenoceptor agonists is shown when hepatic arteries from cirrhotic patients are studied *in vitro*. Hepatic and mesenteric arteries from non-cirrhotic subjects were used as controls.

Hepatic arteries from patients with cirrhosis (5 male, 5 female, age 51.1±3.2 years), and hepatic and mesenteric arteries from (non-cirrhotic) donors (4 male, 7 female, age 27.6±6.62 years), were obtained at transplantation. Donor hepatic arteries were kept in University of Wisconsin solution (UWS) for up to 12 hours (as they were required during transplantation), all other vessels were placed directly into Krebs solution at 4°C. Within 24 hours, 2mm long arterial rings were mounted in organ baths containing Krebs solution at 37°C, perfused continuously with 95%O_2: 5% CO_2. Measurement of isometric force was performed with vessels at a resting force of 4g. Cumulative concentration response curves to phenylephrine (PE; 10^{-9}-3x10^{-4}M), noradrenaline (NA; 10^{-9}-3x10^{-4}M) and potassium chloride (KCl; 2.5-120mM) were obtained. Endothelial cell (EC) integrity was assessed using acetylcholine (ACh;10^{-5}M) and SIN-1 (10^{-5}M) after constriction with NA (10^{-5}M).

Contractions of the donor hepatic arteries were relatively small when compared with those produced by cirrhotic hepatic and donor mesenteric arteries, but differences were not significant when analysed using oneway analysis of variance (Table 1). There were no significant differences in sensitivity (EC_{50}) to any of the agonists. SIN-1, but not ACh, induced relaxation in all vessels (Table 1).

Use of SIN-1 and ACh indicated that, whilst able to relax in response to NO, the vessels lacked a functional endothelium. The lack of any difference in sensitivity to PE, NA or KCl suggests hyporesponsiveness in cirrhotic patients *in vivo* is not due to an intrinsic defect in the vascular smooth muscle. However, absence of ECs in these preparations precludes investigation of their role in mediation of hyporesponsiveness *in vivo*. The relatively small contractions of the donor hepatic artery may be due to manipulation and storage in UWS during transplantation.

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MacGilchrist, A. et al. (1991) *Hepatology* 13, 689-694.

Table 1. Maximum contraction and -logEC_{50} responses to vasoconstrictors and maximum relaxation values to vasodilators.

	Maximum Contraction (g)			-LogEC50				Max. relaxation (% Reversal of Tone)		
	CH (10)	DH (5-7)	DM (10-11)	CH (10)	DH (5-7)	DM (10-11)		CH (6)	DH (6)	DM (8)
PE	10.2±1.5	7.2±1.4	11.5±1.5	5.86±0.26	5.85±0.07	5.74±0.17	ACh	-8.1±10.2	-3.4±6.9	11.6±5.8
NA	9.3±1.3	6.1±1.5	11.3±1.6	5.92±0.18	6.13±0.18	5.99±0.21	SIN-1	73.7±6.4	72.5±16.7	67.3±12.4
KCl	8.6±0.9	5.8±1.4	10.8±1.4	1.68±0.10	1.55±0.10	1.61±0.07				

Results are given as mean ± s.e.mean, n numbers (indicating different individuals) in brackets. CH = cirrhotic hepatic, DH = donor hepatic, DM= donor mesenteric arteries. A negative relaxation value indicates contraction.

112P α-ADRENOCEPTOR-MEDIATED RESPONSES IN THE CAUDA EPIDIDYMIS OF THE GUINEA-PIG

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Multiple subtypes of α_1-adrenoceptor exist in the vas deferens of the rat (Hanft and Gross, 1989). In this study we investigate the α-adrenoceptor subtypes of the cauda epididymis of the guinea-pig.

Preparations of cauda epididymis were set up under 0.25g resting force in modified Krebs solution (37°C, bubbled with 5% CO_2 in O_2). Contractions were recorded using a Grass polygraph. Mean pEC_{50}, pK_B (both -log molar), and maximum response (Emax in mg force) values are shown (with 95% confidence limits). Statistical significance ($P<0.05$) was determined with an F-test.

The α_1-adrenoceptor agonist, phenylephrine, but not the α_2-adrenoceptor agonist, xylazine (up to 10μM), elicited concentration-dependent contractions from preparations of cauda epididymis (pEC_{50} 5.59 (5.28, 5.91); Emax 231 (193, 268); n = 12). Preincubation of the tissues with the α_{1B}-adrenoceptor-alkylating agent, chloroethylclonidine (CEC; 50μM, 30min), did not affect either the pEC_{50} (5.42 (5.19, 5.65)) or Emax (211 (185, 269); n = 12). The Ca^{2+} channel antagonist, nifedipine (10μM), significantly reduced the Emax, but not the pEC_{50} of phenylephrine-concentration-response curves (to 80 (53, 107) and 5.24 (4.54, 5.94), respectively; n = 12). In preparations incubated with nifedipine (10μM), CEC significantly reduced the maximal response to phenylephrine (Emax 29 (22, 35)) compared to tissues incubated with nifedipine only (53 (48, 57); n = 6).

Compared to control preparations xylazine (1μM) significantly potentiated responses to phenylephrine 4-fold (pEC_{50} s 5.63 (5.35, 5.91) and 6.22 (5.83, 6.63) respectively, n = 6). This effect could be blocked by the α_2-adrenoceptor antagonist, idazoxan (100nM).

Following the incubation of preparations with CEC and in the presence of idazoxan (100nM), the α_{1A}-adrenoceptor antagonist, 5-methyl-urapidil (10 and 100nM), shifted phenylephrine concentration-response curves to the right (pK_B values 8.41 (7.81, 9.02) and 7.94 (7.41, 8.47) respectively). Schild analysis revealed a slope of 0.65 (95% confidence limits 0.12, 1.17); n = 6).

These studies demonstrate that the epididymis of the guinea-pig contains post-junctional α_1- and α_2-adrenoceptors. The α_1-adrenoceptor mediated response may be divided into nifedipine sensitive and nifedipine-insensitive components.

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Hanft, G. & Gross, G. (1989). *Br. J. Pharmacol.*, 97, 691-700.

113P CHEMOKINE PRODUCTION BY HUMAN VASCULAR SMOOTH MUSCLE CELLS: MODULATION BY IL-13

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Atherosclerotic lesions are characterized by an accumulation of monocytes and lymphocytes in the vessel wall. This migration is mediated by a series of co-ordinated signals, probably including the production of chemotactic factors by smooth muscle cells. The chemokines, a family of structurally similar 8-10 kDa chemoattractant proteins, have relatively narrow selectivity for specific populations of inflammatory cells and may be important in this setting.

We have investigated the ability of the cytokines IL-1α and TNFα to induce chemokine production in cultured human vascular smooth muscle cells (SMC) obtained by explant or collagenase digestion from saphenous vein and aorta (Chamley-Campbell *et al.* 1976). Since no difference was noted between responses obtained with either venous or arterial SMC all data have been pooled. Production of the chemokines interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and RANTES were measured in the supernatants from quiescent cultures (about $1.5x10^4$ cells, p3-7) stimulated with IL-1α or TNFα. Over 24h, unstimulated cells secreted less than 0.2 ng/ml of IL-8 or RANTES protein and between 0.3-4.0ng/ml of MCP-1 as determined by double ligand ELISA (Yoshimura *et al.* 1991, Jordan *et al.* 1995). There was a marked differential effect of IL-1α and TNFα on the expression of the different chemokines. After 24h incubation with optimal concentrations of IL-1α (3ng/ml) or TNFα (30ng/ml) levels of 56 ± 11 and 19 ± 4 ng/ml IL-8 were measured respectively (mean $\pm$ sem, n=6 cultures). At these concentrations IL-1α induced less than 1ng/ml RANTES, whereas TNFα induced 10 ± 3ng/ml (n=4). IL-1α and TNFα were equally efficacious stimuli for MCP-1 inducing respectively 18 ± 3 and 17 ± 3 ng/ml over 24h (n=6). The time course of chemokine gene expression also varied, as measured by Northern blotting. IL-1α or TNFα induced MCP-1 mRNA expression by 1h with a maximum expression at 2h which was sustained up to 96h. Peak IL-8 mRNA expression was detected at 4-6h post stimulation and decreased by 24h. RANTES mRNA could not be detected before 14h and it increased up to 48h following stimulation with TNFα, little induction was seen with IL-1α.

We also investigated whether the T-cell derived cytokines IL-10 and IL-13 could modulate SMC chemokine secretion. Alone, these cytokines did not induce chemokine production. IL-13, but not IL-10 caused a concentration dependent (1-10ng/ml) increase in both IL-8 and MCP-1 protein induced by TNFα. For example, IL-13 (10ng/ml) induced a $210\pm19\%$ (n=6) increase in IL-8 and a $193\pm33\%$ (n=6) increase in MCP-1 production induced by TNFα (30ng/ml). These increases were significant ($p<0.01$, Dunnett's test on transformed raw peptide levels). Thus in addition to its 'anti-inflammatory' properties, IL-13 may play a role in potentiating monocyte infiltration in atherosclerosis.

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Chamley-Campbell J., Campbell G.R. & Ross R. (1974) Phys. Rev. **59** p1-61.
Jordan N.J., Watson, M.L & Westwick, J. (1995) Biochem. J. **311** p89-95.
Yoshimura, T. Takeya, M. Takahashi, K. *et al.* (1991) J. Immunol. **147** p2229-2233.

114P MECHANISMS INVOLVED IN THE ACTIVATION OF EOSINOPHIL AGGREGATION BY ARACHIDONIC ACID

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The activation of phospholipase A_2 and the subsequent release of arachidonic acid (AA) plays an important role in leukotriene B_4 (LTB_4)-induced respiratory burst of guinea pig eosinophils (Lindsay et al, 1995). However, AA-induced activation of the NADPH oxidase was independent of leukotriene or prostanoid production (Lindsay et al, 1995). In this study, we evaluated whether AA induced eosinophil homotypic aggregation and whether lipoxygenase and/or cyclooxygenase products played any role in this response.

Eosinophils elicited in the peritoneal cavity of horse serum-treated guinea pigs were purified on a discontinuous Percoll gradient. Cells which were over 95% pure were diluted (5×10^6 eosinophils/ml) in PBS and 300 μl aliquots placed in aggregation cuvettes at 700 rpm, 37°C for 3 min. The stimuli (AA, LTB_4 or C5a) were added and aggregation assessed as changes in light transmission using a platelet aggregometer (Teixeira et al, 1995). Eosinophil aggregation is presented as % maximal aggregation, which is defined as the aggregation response to 1 μM PMA. Intracellular calcium levels after activation with AA or LTB_4 were measured using FURA-2 as previously described (Jose et al, 1994).

AA (1 to 10 μM) induced a concentration-dependent eosinophil aggregation (1 μM, $2.7 \pm 1.7\%$; 3 μM, $10.4 \pm 3.7\%$; 10 μM, $26.5 \pm 3.2\%$ maximal aggregation). The 5-lipoxygenase inhibitor ZM230487 concentration-dependently inhibited AA-induced eosinophil aggregation with an IC_{50} of approximately 0.6 μM. In contrast, aggregation induced by C5a or LTB_4 were unaltered by ZM230487. Similarly, the LTB_4 antagonist LY255283 (1 μM) effectively inhibited AA- but not C5a-induced responses. At this concentration, LY255283 inhibited 10 nM LTB_4-induced aggregation by 71% (n= 4). The cyclooxygenase inhibitor flurbiprofen (1 μM) had no effect on AA-induced eosinophil aggregation (AA 10 μM, $25.8 \pm 3.6\%$; AA + flubiprofen, $23.4 \pm 3.0\%$ maximal aggregation). Pretreatment of eosinophils with increasing concentrations of AA (1 to 10 μM) desensitized the cells to further aggregation in response to LTB_4, but not C5a. However, when eosinophils were pre-incubated with ZM230487 prior to AA, the aggregation response to LTB_4 was preserved. In contrast to its ability to induce an aggregation response, AA induced no increase in intracellular calcium. In addition, AA had no inhibitory effect on calcium influx in response to eosinophil activation with LTB_4.

Although AA may activate the eosinophil respiratory burst independent of leukotriene production, our results suggest that LTB_4 is generated after eosinophil activation with AA and that the generated LTB_4 plays an important role in mediating AA-induced eosinophil aggregation. The reasons why AA fails to induce a calcium response and to desensitize for LTB_4-induced calcium responses are under investigation.

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Jose, P.J.; Griffith-Johnson, D.A.; Collins, P.D. et al (1994) *J.Exp.Med.* 179: 881-887.
Lindsay, M.A.; Perkins, R.S.; Barnes, P.J. & Giembycz, M.A. (1995) *Am.J.Respir.Crit.Care Med.* 151: A678.
Teixeira, M.M.; Williams. T.J., Au, B.T.; Hellewell, P.G. & Rossi, A.G. (1995) *J.Leuk.Biol.* 57: 226-234.

115P STIMULATION OF POTASSIUM (^{86}RUBIDIUM) EFFLUX FROM HUMAN PLATELETS BY EXTERNAL POTASSIUM AND ITS INHIBITION BY BUMETANIDE: EVIDENCE FOR Na/K/Cl CO-TRANSPORT

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The sodium/potassium/chloride (Na/K/2Cl) co-transport system, which mediates a bi-directional, passive, electrically neutral co-transport of Na, K, and Cl ions across the cell membrane, is present in a wide variety of animal tissues. It appears to serve a number of important physiological functions, such as the absorption and secretion of salt and water, regulation and maintenance of cellular volume, and regulation of cytoplasmic pH (Chipperfield, 1986). The co-transporter may be operationally defined by the mutual acceleration of Na movements by K and of K movements by Na when both ions are present at the same side of the membrane, its susceptibility to inhibition by loop diuretics such as bumetanide, and chloride dependence (Chipperfield, 1986). In addition to Na/K/2Cl co-transport, the system can support K/K and Na/Na exchange (Canessa *et al.*, 1986). The presence of Na/K/Cl co-transport in platelets has been inferred but not demonstrated (Kimura & Aviv, 1993). We have therefore studied Na/K/2Cl co-transport in platelets from healthy subjects.

We preloaded platelets with ^{86}Rb (as a tracer for K; Chipperfield, 1986), immobilized them on a filter, and measured K (^{86}Rb) efflux using a superfusion technique (Carver *et al.*, 1994) in the following medium (mM): NaCl (119), KCl (4.6), $CaCl_2$ (1.5), NaH_2PO_4 (1.2), $NaHCO_3$ (15), glucose (11), pH 7.4. For experiments using increasing concentrations of KCl, the concentration of NaCl was reduced to maintain constant osmolality. The cells were allowed to stabilize for 20 min at the start of each experiment.

External KCl (15–60 mM) dose-dependently increased ^{86}Rb efflux; data for 30 mM and 60 mM KCl are shown in Figure 1 and Table 1. This effect was completely inhibited by bumetanide (10 μM) (Figure 1, Table 1), which on its own had no effect, or may have slightly inhibited ^{86}Rb efflux (Table 1). Ouabain (10^{-5} M) had no effect on either spontaneous or KCl-induced ^{86}Rb efflux (not shown).

Thus, K efflux from human platelets is stimulated by high concentrations of external K; this K-stimulated efflux is inhibited by bumetanide and is insensitive to ouabain.

We conclude that in human platelets external K in high concentrations stimulates K efflux via the Na/K/2Cl co-transport, acting in a mode of K/K exchange.

Table 1 Cumulative ^{86}Rb efflux (pmol)

	Control (KCl 5 mM)	KCl (30 mM)	KCl (60 mM)	KCl (30 mM) + Bumetanide (10 μM)	Control + Bumetanide (10 μM)
^{86}Rb efflux	86.6	104.2*	117.5*	72.0	76.4
SEM	3.5	3.3	5.0	2.6	6.3
n	18	19	12	5	5

*Significantly different from control (ANOVA plus Dunnett's test) P<0.01

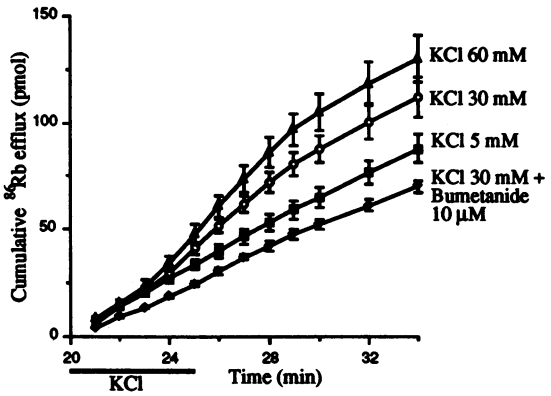

Figure 1 Cumulative Rb efflux stimulated by external KCl in the presence (closed symbols) and absence (open symbols) of bumetanide, added at time zero (n=5; mean, s.e.mean)

Canessa, M., Brugnara, C., Cusi, D., Tosteson, D.C. (1986) *J. Gen. Physiol.* 87, 113-42.
Carver, J.G., Grahame-Smith, D.G., Johnson, E.S., Madgwick, Z. (1994) *Br. J. Clin. Pharmacol.* 35, 473-8.
Chipperfield, A.R. (1986) *Clin. Sci.* 71, 465-76.
Kimura, M., Aviv, A. (1993) *Pflugers Arch.* 422, 585-90.

116P BIOPHYSICAL ANALYSIS OF THE INTERACTION OF Ro 405967 WITH K$^+$ CHANNELS IN THE RAT NG108-15 CELL-LINE

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Ro 405967 was originally described as an L-type Ca^{2+} channel antagonist. However more recent experimental data has demonstrated that this compound has activity against a wide range of Ca^{2+} channels as well as both Na+ and K+ channels. This communication describes our recent analysis of the interaction of this compound with delayed rectifier K^+ channels. We have utilised standard whole patch clamp methods to record K+ currents from the NG108-15 cell line. The extracellular solution consisted of (mM) NaCl, 119; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; D-glucose, 30; HEPES-NaOH, 25; pH 7.3. Na^+ currents were blocked with TTX (1 μM) and Ca^{2+} currents with La^{3+} (10 μM). Electrodes were filled with $KMeSO_4$, 110; NaCl, 10; $MgCl_2$, 5; EGTA, 5; ATP, 2; GTP, 0.2; HEPES-KOH, 35; pH 7.3. All data are presented as mean ± S.E.M.

K^+ currents (I_K) in the NG108-15 cell activated in a voltage-dependent fashion. In control conditions the currents exhibited little inactivation during 120 ms test pulses to any potential. Application of 0.3 to 30 μM Ro405967 caused a dose-dependent reversible block of I_K However, in the presence of the drug, I_K developed an appearance of stong inactivation, the higher the dose of drug the faster the inactivation. As a consequence the apparent IC_{50} for inhibition depended on the where in the test pulse the current was measured. Application of Ro 405967 at up to 100 μM inside cells (n=4) had no obvious activity, and did not occlude the activity of the compound applied externally n=3). To further investigate the drug-channel interaction, we carried out an extendend series of experiments utilising a dose of 10 μM Ro405967. In the steady state, at a test potential (V_t)= +50 mV,applied every 5s, from a holding potential (V_h)= -80 mV, this dose inhibited the peak of I_K by 74 ± 2% (n=11). This block developed with a time constant of 9.4s. Upon removal of the drug I_K recovered with a time constant of 112 s (n=9). At V_h=-120 mV(n=6), both the blocking rate (9.5 s) and degree of steady state block (69 ± 5%) were similar. However there was somewhat less block (58 ± 3%, n=6) at V_h=-40 mV. Analysis of pooled wash-out data revealed that the lower affinity at V_h=-40 mV resulted from a higher off-rate at this potential (Vh=-40 mV, 0.0241 s^{-1}; Vh=-80 mV, 0.0089 s^{-1}; Vh=-120 mV, 0.0094 s^{-1}). The steady state block recorded was also dependent on the frequency of test pulse application (n=6). For V_h=-80 mV and V_t=+50 mV I_K decreased 2.7± 0.2 fold as the test frequency was increased stepwise from 0.02 Hz to 1Hz.

Current-voltage relationships constructed in the presence and absence of Ro 405967 revealed that there was slightly less block at test potentials near the foot of the activation curve, however normalized peak conductance-voltage relationships were indistinguishable in control ($V_{1/2}$=11.08 mV, k=13.09mV, n=13) and drug treated ($V_{1/2}$=11.01, k=13.85 mV, n=13) cells. The voltage-dependence of block was further characterised utilising paired test-pulse protocols. When, in the presence of Ro 405967 (n=9), a variable amplitude first test pulse was proceeded (with a constant latency of 30 ms) by a second test pulse to +50 mV, the relationship between prior channel opening and subsequent channel block was obtained. The depression of the second current with respect to the amplitude of the first test pulse was well fit by a modified Boltzman function of $V_{1/2}$=0.6 mV and k=9.6 mV, this was leftward shifted compared to the current activation curve determined in both the presence and absence of drug (see above). The frequency- and potential-dependence of the actions of Ro 405967 on K^+ channels has interesting implications for the mechanisms underlying its useful therapeutic actions in conditions such as congestive heart failure and hyper-tension. Previously these have been thought to arise solely from actions of Ro 405967 on Ca^{2+} channels.

117P CHARYBDOTOXIN AND APAMIN INHIBIT RELAXATIONS MEDIATED BY EDHF IN THE GUINEA-PIG BASILAR ARTERY

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It has been proposed that acetylcholine releases an endothelium-derived hyperpolarizing factor (EDHF), distinct from nitric oxide (NO), in the guinea pig basilar artery (Nishiye *et al.*, 1989). The aim of the present study was to examine the effects of K^+ channel inhibitors on the non-NO/non-prostanoid-mediated relaxation in this artery. Tension experiments were carried out on ring preparations from guinea pig basilar arteries suspended in organ baths.

Acetylcholine (0.01 - 100 μM) and A23187 (0.1 - 3 μM) elicited endothelium-dependent relaxations in the presence of N^G-nitro-L-arginine (L-NOARG, 0.3 mM) and indomethacin (10 μM). The relaxations were abolished in the presence of a 60 mM K^+ Krebs solution. Inhibitors of ATP-sensitive K^+ channels (glibenclamide, 10 μM), voltage-sensitive K^+ channels (dendrotoxin, 0.1 μM or 4-aminopyridine, 1 mM), small (apamin, 0.1 μM) and large (iberiotoxin, 0.1 μM) conductance Ca^{2+}-activated K^+ channels did not affect the L-NOARG/indomethacin-resistant relaxation induced by acetylcholine. However, charybdotoxin (0.1 μM), another inhibitor of large conductance Ca^{2+}-activated K^+ channels, caused a rightward shift of the concentration response curve for acetylcholine ($-\log EC_{50}$ = 5.42 ± 0.16 vs $-\log EC_{50}$ = 6.46 ± 0.09 in controls; n = 10 - 11; $P < 0.05$) and reduced the maximal relaxant response (E_{max} = 53 ± 13 % vs E_{max} = 98 ± 2 % in controls; $P < 0.05$). In contrast, the relaxation induced by A23187 was not significantly inhibited after treatment with charybdotoxin. However, relaxations induced by acetylcholine as well as those elicited by A23187 were abolished in the presence of charybdotoxin (0.1 μM) and apamin (0.1 μM) combined.

The results suggest that the endothelium-dependent L-NOARG/indomethacin-resistant relaxations induced by acetylcholine and A23187 are mediated by activation of K^+ channels which can be inhibited by a combination of K^+ channel inhibitors. The synergistic effect of charybdotoxin and apamin may indicate that more than one type of K^+ channel is involved in the responses.

Nishiye, E., Nakao, K., Itoh, T. *et al.* (1989). *Br. J. Pharmacol.*, 96, 645-655.

118P ROLE OF POTASSIUM CHANNELS IN ENDOTHELIUM-DEPENDENT RELAXATION RESISTANT TO NITROARGININE IN THE RAT HEPATIC ARTERY

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In the presence of indomethacin (10 μM) and N^G-nitro-L-arginine (L-NOARG, 0.3 mM), acetylcholine (ACh, 10 nM - 10 μM) induces an endothelium-dependent smooth muscle hyperpolarization and relaxation in the rat isolated hepatic artery (Zygmunt *et al.* 1994). The underlying mechanism behind these responses has not been clarified.

The potassium channel inhibitors tetrabutylammonium (TBA, 1 mM) and to a lesser extent 4-aminopyridine (4-AP, 1 mM) inhibited the ACh-induced relaxation, whereas apamin (0.1 - 0.3 μM), charybdotoxin (0.1 - 0.3 μM), iberiotoxin (0.1 μM), dendrotoxin (0.1 μM) and glibenclamide (10 μM) each had no effect. When combined, apamin (0.1 μM) plus charybdotoxin (0.1 μM), but not apamin (0.1 μM) plus iberiotoxin (0.1 μM) or a triple combination of 4-AP (1 mM) plus apamin (0.1 μM) plus iberiotoxin (0.1 μM), inhibited the ACh-induced relaxation. Apamin plus charybdotoxin each at a concentration of 0.3 μM completely abolished the relaxation. The L-NOARG/indomethacin-resistant relaxation induced by A23187 (0.1 - 10 μM) was also abolished by this toxin combination. However, in the absence of L-NOARG, charybdotoxin (0.3 μM) plus apamin (0.3 μM) failed to inhibit the ACh-induced relaxation.

The gap junction inhibitors halothane (2 mM) and 1-heptanol (2 mM), or replacement of NaCl with sodium propionate did not affect the ACh-induced relaxation. Similarly, inhibition of Na^+/K^+-ATPase by ouabain (1 mM) had no effect on the response. Exposure to a K^+-free Krebs solution, however, caused a small reduction of the maximal ACh-induced relaxation without affecting the sensitivity to ACh; E_{max} (mean ± s.e.) was reduced from 97 ± 1 % (control) to 84 ± 6 % (K^+-free).

The results suggest that the L-NOARG/indomethacin-resistant relaxation in the rat hepatic artery is mediated by activation of K-channels sensitive to TBA and a combination of apamin and charybdotoxin. Chloride channels, Na^+/K^+-ATPase and gap junctions are probably not involved in the response. It is proposed that endothelial cell activation induces secretion of a single or a family of chemical factor(s), distinct from NO and cyclo-oxygenase products, which activate(s) more than one type of potassium channel to elicit endothelium-dependent hyperpolarization and vascular relaxation

Zygmunt, P.M., Waldeck, K. & Högestätt, E.D. (1994). *Acta Physiol. Scand.* 152, 375-384.

Key words: Vascular endothelium, endothelium-derived hyperpolarizing factor, potassium channels

119P *IN VITRO* PHARMACOLOGICAL EFFECTS OF TWO NOVEL 5-HT_{1D}-RECEPTOR AGONISTS L-741,519 AND L-741,604: COMPARISON WITH SUMATRIPTAN AND BW311C

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5-HT_{1D}-receptor agonists such as sumatriptan and BW311C are effective in the treatment of migraine headache. The vasoconstrictor properties of these compounds are believed to be important in their therapeutic action. We compared the vasoconstrictor properties of two novel compounds L-741,519 (N-methyl-4-[5-(1,2,4-triazol-4-yl)-1H-indol-3-yl]piperidine) and L-741,604 (N,N-dimethyl-2-[5-(1,2,4-triazol-4-yl)-1H-indol-3-yl]ethylamine) with those of sumatriptan and BW311C. We also examined the potential sympatholytic properties by determining the effects of these compounds on sympathetic nerve function in vitro.

5-HT_{1D}-receptor mediated vasoconstrictor responses were assessed using the rabbit isolated saphenous vein (male New Zealand white rabbits 2-2.5kg see Razzaque et al., 1995). 5-HT_{2A}-receptor mediated responses were assessed using the rat isolated tail artery and effects on sympathetic nerve function were assessed using rat isolated vas deferens (male Sprague Dawley rats 175-225g). The tissues were mounted for isometric tension recording in organ baths containing Kreb's physiological salt solution (37°C, aerated with 95%O_2 / 5%CO_2, pH 7.4). In rat vas deferens contractions were evoked by electrical field stimulation (0.1Hz, 0.5msec, 30-40V). EC_{50} values are given as geometric means (95% C.L.).

In rabbit saphenous L-741,519 and L-741,604 were approximately 10-fold more potent in causing contraction than sumatriptan or BW311C (EC_{50} values were 36.3nM (20.9-63.1nM, n=4), 25.7nM (21.4-39.8nM, n=8), 630.2nM (309-900nM, n=6) and 184.9 (115-302nM, n=5) for L-741,519, L-741,604, sumatriptan and BW311C respectively. The maximum contractions evoked by L-741,519, sumatriptan and BW311C were not significantly greater than that evoked by 5-HT itself, however, L-741,604 was significantly more efficacious (E_{max} = 135 ± 10, Students' t-test $P < 0.005$). In rat tail artery, BW311C caused a contraction (EC_{50} = 3136nM (1059-9226nM); E_{max} relative to 5-HT was 53 ± 5.8%, n = 3), however, L-741,519, L-741,604 and sumatriptan were devoid of contractile effects in concentrations up to 100μM. In rat vas deferens, L-741,519, L-741,604, sumatriptan and BW311C inhibited electrically evoked contractions with EC_{50} values of 141nM (110-182nM, n=7), 239nM (76-741nM, n=5), 4000nM (2900-5200nM, n=6) and 660nM (354-1230nM, n=4) respectively. L-741,604 abolished electrically evoked contractions and L-741,519, sumatriptan and BW311C partially inhibited these contractions.

L-741,519 and L-741,604 were more potent than sumatriptan and BW311C in causing 5-HT_{1D}-receptor mediated contractions of rabbit isolated saphenous vein and devoid of 5-HT_{2A}-receptor stimulating properties. Impairment of sympathetic nerve function was a general property shared by 5-HT_{1D}-receptor agonists.

Razzaque, Z., Longmore, J., & Hill, R.G. (1995) Eur. J. Pharmacol., 283, 199-206.

120P EFFECTS OF CYTOCHROME P450 INHIBITORS ON ENDOTHELIUM-DEPENDENT AND LEVCROMAKALIM-INDUCED RESPONSES IN RAT BLOOD VESSELS

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Segments of rat hepatic artery were contracted with phenylephrine (1 - 10 μM) under isometric conditions. In the presence of 0.3 mM N^G-nitro-L-arginine (L-NOARG) plus 10 μM indomethacin, acetylcholine (ACh, 1 nM-10 μM) relaxed the segments *via* the release of 'endothelium-derived hyperpolarising factor' (EDHF), an action which was glibenclamide-insensitive (Zygmunt et al., 1994). Levcromakalim (10 nM - 1 μM) relaxed the tissues in a glibenclamide-sensitive manner *via* the opening of the ATP-sensitive K-channel, K_{ATP} (Zygmunt et al., 1994). In the additional presence of the cytochrome P450 inhibitors proadifen (10 μM) or clotrimazole (3 μM), the relaxant effects of ACh and levcromakalim were antagonised (Table 1). The action of ACh was not modified by the addition of the cytochrome P450 suicide inhibitor, 17-octadecaynoic acid (17-ODYA, 50μM).

In the presence of 30 mM K^+ plus 10 μM indomethacin, ACh induced an L-NOARG-sensitive relaxation mediated *via* NO release. Under these conditions, 10 μM proadifen (but not 3 μM clotrimazole) shifted the concentration-response curve to ACh rightward without affecting the maximal relaxation (-log EC_{50} = 6.7 ± 0.1, test; 7.5 ± 0.1, control: n = 6, $P < 0.001$). The relaxant actions of the NO donor, 3-morpholino-sydnonimine, were unaffected by 10 μM proadifen (n = 5).

In freshly-isolated cells from rat portal vein held under voltage-clamp conditions, exposure to either 10 μM proadifen or 30 μM clotrimazole inhibited the delayed rectifier current $I_{K(V)}$ (holding potential, -90 mV, inhibition at +50 mV test potential; proadifen, 68 ± 6%: clotrimazole, 85 ± 5%; n = 4-5) and totally inhibited $I_{K(ATP)}$ induced by 3 μM levcromakalim (holding potential, -10 mV; n = 5).

Collectively, these studies show that the antagonism of EDHF by cytochrome P450 inhibitors probably involves inhibition of the underlying K-channel rather than reduction of EDHF formation.

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Zygmunt, P.M., Waldeck, K. & Högestätt, E.D. (1994) *Acta Physiol. Scand.* 152, 375-384.

Table 1

	ACh		Levcromakalim	
	-log EC_{50}	% inhibition of PhE	-log EC_{50}	% inhibition of PhE
Control (n = 6)	7.8 ± 0.3	98 ± 1	7.2 ± 0.0	100 ± 0
Proadifen 10 μM (n = 6)	6.3 ± 0.2*	89 ± 4*	Complete inhibition	0
Control (n = 7)	7.5 ± 0.1	94 ± 1	7.3 ± 0.0	100 ± 0
Clotrimazole 3 μM (n = 7)	7.0 ± 0.1*	73 + 11	6.4 ± 0.0*	70 ± 9*

*$P < 0.05$, compared to control (Student's t-test, two-tailed unpaired). PhE = phenylephrine.

121P PROGRESSIVE REDUCTION OF MAXIMAL ACTIVE WALL STRESS IN RAT MESENTERIC RESISTANCE ARTERIES FOLLOWING MYOCARDIAL INFARCTION

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After myocardial infarction (MI), a variety of neurohumoral mechanisms are activated to maintain systemic perfusion pressure. We evaluated whether the peripheral vasculature adapts to this condition. In 2 groups of male Wistar rats (n=8 in each group), MI was induced by permanent ligation of the left coronary artery while anaethetized with sodium pentobarbital (60mg kg^{-1} i.p.) (Schoemaker et al, 1990). 3 and 5 weeks post-MI, thoracic aorta (Ao) and mesenteric resistance arteries (MrA) were isolated, chemically sympathectomized (0.3 mM 6-OHDA, 10 min) and mounted in a myograph for recording of isometric tension development. Maximal active wall tension (AWT) induced by various vasoconstrictor agonists was determined by constructing concentration response curves. Also AWT induced by a depolaring stimulus (125 mM K^+) was determined. At the end of the experiments, arteries were fixed and processed for structural analyses. Findings were compared to those in sham operated animals (SHAM, n=10). Data are expressed as precentage decrease in maximal AWT compared with SHAM animals. At 3 and 5 weeks post-MI, media cross-sectional area (CSA) and maximal contractile responses to various vasoconstrictor agonists were not altered in Ao. In MrA, CSA and optimal lumen diameter were not modified at 3 and 5 weeks post-MI. However, maximal contractile responses to noradrenaline (NA, 10nM-10μM), phenylephrine (PHE, 10nM-10μM) and 125 mM K^+ were reduced 3 weeks post-MI (resp. by 25, 29 and 25%). This reduction in contractility was even more pronounced at 5 weeks post-MI (NA: 43%, PHE: 47% 125mM K^+: 38%). Furthermore, contractile responses to serotonin (5-HT, 10nM-3μM) and vasopressin (AVP, 10pM-30nM) were reduced (5-HT: 36%, AVP: 25%). All observations were significantly different between groups ($p<0.05$, Student's t-test). Since the decrease in contractility was most pronounced for NA and PHE, we evaluated whether this was due to a decrease in α_1-adrenoceptor (α_1-AR) density. Saturation binding-curves were determinded with ^{3}H-prazosin in intact MrA segments. Despite the reduction of contractile responses to NA and PHE, no alterations in α_1-AR density were observed (Bmax: 1.95±.19 (MI) vs 1.86±.47 (SHAM) fmol/μg DNA, n.s.). These findings indicate that MI leads to a progressive, receptor independent, reduction of maximal active stress development of smooth muscle cells in resistance arteries. Such a mechanism may be involved in the transition from compensated to decompensated heart failure.

Schoemaker, R.G., Urquhart, J., Debets, J.J.M. et al., (1990). Basic. Res. Cardiol., 85, 9-20

122P KINETICS OF L-DOPA UPTAKE IN LLC-PK_1 CELLS IN CULTURE

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The cellular uptake of L-3,4-dihydroxyphenylalanine (L-DOPA) has been suggested to rate limit the synthesis of dopamine (DA; an endogenous natriuretic agent) in tubular epithelial cells, but information concerning the mechanism(s) involved and the nature of the process(es) of cell membrane transport of the amino acid precursor in epithelial cells of renal tubules is lacking (Soares-da-Silva, 1994). The present work reports on the kinetics and characteristics of cellular uptake of L-DOPA and DA in LLC-PK_1 cells in culture. LLC-PK_1 cells (ATCC 1392-CRL) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Medium 199 supplemented with 3% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 130 μg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, LLC-PK_1 cells were preincubated (30 min) with Hanks medium with added pargyline (100 μM), tolcapone (1 μM) and benserazide (50 μM) in order to inhibit the enzymes monoamine oxidase, catechol-O-methyltransferase and aromatic L-amino acid decarboxylase, respectively. Determination of initial rate of uptake was performed in experiments in which LLC-PK_1 cells were incubated with L-DOPA or DA (500 nM) during 1, 3, 6, 12, 30, 60 and 120 min; using this procedure the compounds were applied from the apical cell border. L-DOPA, but not DA, was rapidly accumulated in LLC-PK_1 cells. The parameters of an exponential saturation equation ($C_i/C_o=k_{in}/k_{out}.(1-e^{-k_{out}.t})$) were fitted to the experimental data; C_i and C_o represent the intracellular and extracellular concentration of the substrate, and t the incubation time. The analysis revealed for L-DOPA a rate constant of total inward transport (k_{in}) of 17.3±1.1 μl mg $protein^{-1}$ min^{-1}, a rate constant of total outward transport (k_{out}) of 2.3±0.4 μl mg $protein^{-1}$ min^{-1} and an equilibrium factor of accumulation (C_i/C_o) of 65.3±3.0 (n=5). Saturation experiments were performed in LLC-PK_1 cells incubated for 6 min with concentrations of L-DOPA which ranged from 0.5 to 250 μM; non-linear analysis of the saturation curve revealed for L-DOPA a K_m of 59 μM (46, 75; 95% confidence limits) and a V_{max} of 486±27 pmol mg $protein^{-1}$ min^{-1}. Accumulation of DA and D-DOPA (5 to 250 μM) in LLC-PK_1 cells applied from the apical border was found to be non-saturable, the amount accumulated corresponding to less than 10% obtained with the L-DOPA. In another series of experiments, LLC-PK_1 cells were cultured on permeable membranes (Costar Transwell 3413) and the substrates were applied to the cells only from the basal border. The kinetics (V_{max} in pmol mg $protein^{-1}$ min^{-1} and K_m in μM) of L-DOPA uptake through the basal border were as follows: V_{max}=6.5±1.1 and K_m=54 (4, 112). The accumulation of DA was non-saturable. It is concluded that L-DOPA is taken up into LLC-PK_1 cells through both the apical and basal borders, but the apical border appears to be the most important cell surface for uptake of the DA precursor.

Soares-da-Silva, P. (1994) News in Physiol. Sci., 9,128-134.

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123P EFFECTS OF SHORT- AND LONG-TERM EXPOSURE OF LLC-PK$_1$ CELLS TO CYCLOSPORINE A ON THE FORMATION AND OUTFLOW OF DOPAMINE

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In the rat, chronic administration of cyclosporine A (CsA) is accompanied by antinatriuresis, hypertension and a reduction in daily urinary excretion of dopamine. This appears to result from a reduction in the amount of L-DOPA made available to the kidney which in turn may be responsible for the upregulation of the L-DOPA uptake system in renal tubules obtained from CsA-treated rats (Pestana et al., 1995). The present work has examined the effects of short- (30 min) and long-term (14 hours) exposure to CsA on the uptake of L-DOPA, its decarboxylation to dopamine and the cellular outflow of the newly-formed amine in monolayers of LLC-PK$_1$ cells in culture. LLC-PK$_1$ cells (ATCC 1392-CRL) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Medium 199 supplemented with 3% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 µg ml^{-1} amphotericin B and 100 µg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 130 µg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In conditions of decarboxylase inhibition (benserazide 50 µM), the initial rate of uptake (6 min) of L-DOPA (0.5 to 250 µM) was a saturable process with the following kinetics: K_m=59 (46, 75; 95% confidence limits), V_{max}=486±27 pmol mg protein^{-1} min^{-1}. The effect of a 30 min exposure of 0.3, 1.0 and 3.0 µg ml^{-1} CsA was a concentration dependent decrease on the uptake of 0.5 µM L-DOPA (25%, 31% and 39% reduction). In the absence of benserazide, 61±4% of total L-DOPA taken up in to the cells is converted to dopamine; 28±5% of the amine escaped out of the cells. Short-term exposure of cells to 0.3, 1.0 and 3.0 µg ml^{-1} CsA produced a concentration dependent increase in the total formation of dopamine (31%, 50% and 59% increase); the fractional outflow of newly-formed dopamine increased from 28±5 up to 56±5% in the presence of 3.0 µg ml^{-1} CsA (P=0.02). The long-term exposure to increasing concentrations of CsA (0.01 to 1.0 µg ml^{-1}) produced a concentration dependent decrease in the formation of dopamine from added L-DOPA (0.5 µM); the highest concentration of CsA reduced by 28% the formation of dopamine. The fractional outflow of the newly-formed dopamine increased up to 35% (P=0.01) long-term exposure to 1.0 µg ml^{-1} CsA. In conclusion, both the short- and long-term exposure of LLC-PK$_1$ cells to CsA increases the ability of newly-formed dopamine to leave the cellular compartment; however, in contrast to the increased formation of dopamine observed after short-term exposure to CsA, the long-term exposure to the imunossupressant reduced the availability of L-DOPA to the cells.

Pestana et al., (1995) Br. J. Pharmacol., 115:1349-1358.

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124P CO-LOCALISATION OF DOPAMINE D_{1A} RECEPTOR PROTEIN AND mRNA IN RAT COLON

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Peripheral dopamine (DA) receptors have been classified on pharmacological grounds as DA-1 and DA-2 (Goldberg et al., 1986). The renal D_{1A} receptor has recently been shown to be homologus to the central D_{1A} receptor (O'Connell et al., 1995). Furthermore, the D_{1A} receptor has also been identified in the rat small intestine raising the possibility that it may be present in other gastrointestinal sites (Marmon et al., 1993).

Employing immunohistochemistry for receptor protein and in situ hybridization for receptor mRNA we sought to determine if the recently cloned central DA D_{1A} gene was expressed in the rat colon. Immunohistochemical detection of receptor protein was carried out using polyclonal antisera raised against synthetic peptide sequences derived from the putative rat DA D_{1A} receptor amino acid sequence corresponding to epitopes ^{241}CQTTAGNGNPVE^{252} and ^{299}GSEETQPFC^{307}. Specificity was verified by attenuation of the ELISA response following preincubation of the antisera with its immunization peptide and by the recognition of the native receptor expressed in a transfected murine fibroblast (LTK$^-$) cell-line. Immunoreactive D_{1A} receptor was detected using a standard avidin-biotin immunoperoxidase method. D_{1A} mRNA was detected using a novel transcription-based isothermal in situ amplification system (O'Connell et al., 1994). The amplified product was detected using non-radioactive in situ hybridisation using a digoxigenin-labelled oligonucleotide probe based on the sequence of rat D_{1A} cDNA. The probe was subsequently detected using an alkaline phosphatase NBT/BCIP technique.

D_{1A} mRNA had a transmural distribution with an intense hybridisation signal in the muscularis mucosa. A weaker signal was present in the epithelium surrounding goblet cells, and in the muscularis externa. Control sections processed with sense probe did not reveal any signal. Immunohistochemical detection of D_{1A} receptor colocalised with the in situ hybridisation signal (Table 1). This study is the first to identify expression of the central D_{1A} receptor in normal rat colon.

Table 1. Distribution of D_{1A} protein and mRNA in colon

	mRNA		Protein
Epithelium	+		+
M. Mucosa	++		+
M. Externa	+		+
Signal:	Intense ++	Present +	Absent -

Goldberg, L. I., J. D. Kohli, and D. Glock (1986) in *Dopaminergic Systems and Their Regulation* ed.Woodruff, GN. p. 195-212. London: Macmillan.

O' Connell D.P., S.J. Botkin, S.I. Ramos et al. (1995) *Am. J. Physiol.* 268: F1185-F1197.

Marmon L.M., F. Albrecht, L.M. Canessa et al. (1993) *J. Surg. Res.* 54: 616-620.

O'Connell, D.P., Botkin, S.J., Felder, R.A., et al. *J. Hypertension* 12 (Suppl 3): A 731, 1994.

125P IMMUNOHISTOCHEMICAL MAPPING OF A NOVEL DOPAMINE D_{1B} RECEPTOR IN RAT KIDNEY

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Dopamine (DA), modulates a variety of functions in the kidney including vasodilation, natriuresis and diuresis through it's interaction with two pharmacologically distinct receptor families. Renal DA receptors have been classified into DA-1 and DA-2 subtypes on the basis of their synaptic localisation (Goldberg *et al.*, 1986). In recent years an unexpected heterogeniety has arisen in the molecular biology of DA receptor subtypes with the cloning and purification of several DA receptors. Two D1-like receptors have been identified (D_{1A} and D_{1B} in rat, or D_1 and D_5 in man) and are coupled to the stimulation of adenylyl cyclase (Weinshank *et al.*, 1991). The distribution of the renal D_{1A} receptor has recently been described (O'Connell *et al.*, 1995). The aim of this work was to investigate the hypothesis that the D_{1B} subtype is also expressed in the kidney.

Immunohistochemical detection of D_{1B} receptor in rat was carried out using polyclonal antisera directed against D_{1B} receptor protein. The antisera were raised against a synthetic peptide sequence derived from the predicted rat D_{1B} DA receptor amino acid sequence, corresponding to epitopes located on both the third extracellular ^{216}GWELEGRTEN^{225} and third intracellular loop ^{282}EHAQSCRSRG^{291} of the receptor. The specificity of the antisera was verified by attenuation of the ELISA response following preincubation of the antisera with its immunisation peptide and by the ability of the anti-peptide antisera to recognise the native receptor expressed in a murine fibroblast (LTK$^-$) cell-line stably transfected with a full-length rat D_{1B} cDNA. Controls included omission of primary antibody, and use of preimmune serum.

Immunoreactivity was detected in the renal cortex and medulla. Immunostaining was present in the proximal tubule, and pronounced in distal tubule and collecting ducts. Immunoreactive D_{1B} receptor protein was not detectable in the glomeruli or renal vasculature. All control sections were negative.

Table 1.	D_{1A}	D_{1B}
Glomerulus	-	-
Proximal tubule	++	+
Distal tubule/CD	+	++
JGA	++	-
Arterial	++	-
Signal:	Intense ++ Present + Absent -	

The newly cloned D_{1B} receptor is expressed in the rat kidney in sites previously labelled as DA-1, but has a distinct distribution compared to the D_{1A} subtype (Table 1).

Goldberg, L. I., J. D. Kohli, & D. Glock (1986) in *Dopaminergic Systems and Their Regulation* ed.Woodruff, GN. p. 195-212. London: Macmillan.
Weinshank, R.L., N. Adham, M. Macchi, et al. (1991) *J. Biol. Chem* 266:22427-35.
O' Connell D.P., S.J. Botkin, S.I. Ramos et al. (1995) *Am. J. Physiol.* 268:F1185-F1197.

126P SB 207266: THE FIRST POTENT AND SELECTIVE 5-HT_4 RECEPTOR ANTAGONIST AMIDE WITH ORAL ACTIVITY

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Full evaluation of the functional role of 5-HT_4 receptors in both the CNS and periphery has been hampered by the lack of an antagonist with good oral bioavailability. We now report on a novel, selective, potent, orally active amide 5-HT_4 receptor antagonist, SB 207266 (N-(1-Butyl-4-piperinylmethyl)-3,4-dihydro-2H-[1,3] oxazino [3,2-a] indole-10-carboxamide hydrochloride). The antagonistic effects of SB 207266 were investigated *in vitro* in the guinea-pig distal colon (Wardle and Sanger, 1993) and *in vivo* in the dog Heidenhain gastric pouch (Bermudez *et al.*, 1990; Bingham *et al.*, 1995).

In the presence of 1 μM granisetron and 0.1 μM methiothepin, 5-HT (10 pM - 10 nM) evokes a 5-HT_4 receptor-mediated contraction of the guinea-pig distal colon with a pEC_{50} of 9.1 ± 0.1 (n = 6). SB 207266, at low concentrations (0.1 - 10 nM, n = 6) produced a concentration-dependent rightward displacement of the curve (apparent pA_2 10.6 ± 0.1, slope confined to unity) and an additional reduction in the maximum response at higher concentrations (30 nM and above). When examined against the partial 5-HT_4 receptor agonist BIMU 1, SB 207266 produced a reduction in maximum at all concentrations tested (0.1 - 10 nM, n = 4). This decrease in the maximum response was not due to irreversible antagonism since the effects were reversed by washing.

In the conscious dog Heidenhain gastric pouch model, bolus injections of 5-HT (5 - 10 μgkg^{-1}, i.v.) evoked a 5-HT_4 receptor-mediated contractile response. Intravenous (0.1 - 100 μgkg^{-1}, n = 4 - 8) or oral (0.1 - 100 μgkg^{-1}, n = 3 - 6) administration of SB 207266 produced a dose-dependent reduction of 5-HT-evoked contractions with estimated ID_{50} values of 1.3 μgkg^{-1} (CL 0.1 - 14.0 μgkg^{-1}) and 9.6 μgkg^{-1} (CL 0.7 - 128 μgkg^{-1}) respectively. Following i.v. administration, the antagonistic effects of low (0.1 - 1 μgkg^{-1}) doses of SB 207266 were reversible with time. With higher doses, the duration of the antagonism exceeded the 105 min observation period. The duration of the antagonism observed with 10 - 100 μgkg^{-1} p.o was also in excess of 105 min. At all doses tested, SB 207266 had no effect on baseline activity and no adverse effects on the behaviour of the dogs were observed.

In binding studies, SB 207266 had a high affinity for 5-HT_4 receptors in piglet caudate nucleus (pK_i 9.5 ± 0.1) but bound only weakly (pK_i < 6) to a range of receptors including 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3 and dopamine D_2 and D_3.

These results suggest that SB 207266 is a highly potent, orally active 5-HT_4 receptor antagonist.

Bermudez, J., Dunbar, A., Sanger, G.J. and Turner, D.H. (1990). *J. Gastrointest. Motility*, 2, 281 - 286. Bingham, S., King, B.F., Rushant, B., Smith, M.I., Gaster, L.M. and Sanger, G.J. (1995). *J. Pharm. Pharmacol.*, 47, 219-222. Wardle, K.A. and Sanger, G.J. (1993). *Br. J. Pharmacol.*, 110, 1593-1599.

127P MPTP IS N-DEMETHYLATED BY CYPs 2D6, 1A2 AND 3A4: IMPLICATIONS FOR SUSCEPTIBILITY TO PARKINSON'S DISEASE

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MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induces a Parkinson-like syndrome through biotransformation by monoamine oxidase B to the neurotoxic metabolite MPP^+ (1-methyl-4-phenylpyridine). Neuroprotection may be provided by parallel N-demethylation and N-oxidation pathways mediated by the microsomal cytochrome P450 and flavin monooxygenase systems, respectively.

The aims of this study were to characterise the N-demethylation of MPTP by human liver microsomes over a wide range of concentrations, and to identify the cytochrome P450 enzymes involved in this reaction.

The kinetics of the N-demethylation of MPTP (1 μM - 3 mM) by microsomes from the liver of an extensive CYP2D6 metaboliser (EM) activity were biphasic (Table 1). The high affinity activity was abolished in the presence of quinidine (Qd) (1 μM) and was absent in microsomes from a genotypically poor CYP2D6 metaboliser (PM) (Table 1). Yeast microsomes containing heterologously expressed CYP2D6 N-demethylated MPTP with a K_m value similar to that for the high affinity site in EM liver microsomes (Table 1). There was a high correlation between the quinidine-inhibitable N-demethylation of MPTP (50 μM) and the α-hydroxylation of metoprolol in microsomes from 11 human livers (r_s = 0.92; $p<0.001$). At 2mM MPTP, N-demethylase activity in human liver microsomes was inhibited by furafylline (10 μM) and ketoconazole (2 μM) (mean inhibition 51% and 24%, respectively; n = 11 livers). Yeast microsomes containing heterologously expressed human CYP1A2 N-demethylated MPTP with a K_m closer to that for the low affinity site in liver microsomes (Table 1). These findings indicate that CYP2D6, and to a lesser extent, CYP1A2 and CYP3A4, may have a role in protecting against Parkinson's disease induced by MPTP and other potential environmental neurotoxins. The data are consistent with evidence suggesting that genotypically PM are over-represented in some populations of Parkinson's patients, and that smokers (induced CYP1A2?) are under-represented.

Table 1 Kinetic parameters for N-demethylation of MPTP by human liver and yeast microsomes (data are mean values ±S.D., n=6).

	Human liver microsomes				Yeast microsomes		
Source	K_{m1} (μM)	V_{max1} (pmol min^{-1} mg $protein^{-1}$)	K_{m2} (μM)	V_{max2} (pmol min^{-1} mg $protein^{-1}$)		K_m (μM)	V_{max} (pmol min^{-1} pmol $P450^{-1}$)
HL6 (EM)	48±2	25±1	2882±665	89±12	CYP2D6	39±3	3.0±0.1
HL6 (+Qd)	-		1274±171	34±3	CYP1A2	2246±136	1.5±0.1
HL3 (PM)	-		831±60	51±3			

128P MODULATION OF CYP2E1 ACTIVITY BY ISONIAZID IN FAST AND SLOW N-ACETYLATORS

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CYP2E1 is important in the metabolic activation of various toxic chemicals including environmental carcinogens like N-alkylnitrosamines, industrial solvents, and other low molecular weight compounds. Levels of CYP2E1 are readily modulated by a variety of physiological and pharmacological factors, and it has been speculated that this might account for differences in individual susceptibility to toxicity/carcinogenicity.

An *in vivo* probe to measure CYP2E1 activity has recently been developed based on the 6-hydroxylation of chlorzoxazone [1]. Using this approach, isoniazid pretreatment for 7 days resulted in a biphasic effect in slow acetylators - inhibition while isoniazid was present in the body but modest induction subsequent to the anti-tubercular agent's elimination [2]. The present study was designed to determine if this response was dependent on the N-acetylator phenotype.

Studies were performed in young (18-37 yr) healthy men, 7 slow and 8 fast acetylators of dapsone, who received 300 mg isoniazid daily for 14 days. After an overnight fast, a single 250 mg oral dose of chlorzoxazone was administered prior to isoniazid pretreatment (Day-1), following the last dose (Day 14), 48 hr later (Day 16) and 2 weeks afterwards (Day 30). Plasma and urinary levels of chlorzoxazone and its 6-hydroxy metabolite were determined by HPLC [1].

The biphasic effect of isoniazid pretreatment was confirmed in slow acetylators; CYP2E1-mediated metabolism was reduced to about 20% of baseline on Day 14 but 2 days after discontinuing isoniazid there was a 60% increase in catalytic activity (Table 1). Induction was not present 2 weeks later. However, in fast acetylators the initial inhibition was much less (40% of baseline) and induction was not observed after stopping isoniazid (Table 1).

Table 1. Effect of daily 300 mg isoniazid administration for 14 days on chlorzoxazone's CYP2E1-mediated metabolism.

	6-Hydroxychlorzoxazone formation clearance, $ml.min^{-1}.kg^{-1}$, mean ± SD			
	Day -1	Day 14	Day 16	Day 30
Slow	3.70±0.98	0.67±0.31**	5.92±2.29*	3.77±1.87
Fast	3.41±0.53	1.32±0.65**	3.64±1.01	3.46±1.78

*p <0.05 compared to Day -1
**p <0.001 compared to Day -1

Thus, the *in vivo* effect of isoniazid on human CYP2E1 activity is dependent on the N-acetylator phenotype. The interphenotypic differences in both inhibition and induction probably reflect differences in pretreatment exposure to isoniazid and/or its metabolites. Regardless, N-acetylator phenotype would appear to be an important factor in drug interactions involving isoniazid and CYP2E1 substrates.

Kim RB, *et al.* (1995) *Clin Pharmacol Ther* 57, 645.
Zand R, *et al.* (1993) *Clin Pharmacol Ther* 54, 142.

129P STRIATAL DOPAMINE D_2 RECEPTOR BINDING ESTIMATED *IN VIVO* IN OLANZAPINE-TREATED SCHIZOPHRENIC PATIENTS BY ^{123}I IBZM SINGLE PHOTON EMISSION TOMOGRAPHY (SPET)

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Striatal dopamine D2 receptor binding was studied *in vivo* in schizophrenic patients treated with the novel compound olanzapine. It was hypothesized that D2 receptor binding in olanzapine treated patients (n=6) would be similar to that observed in clozapine treated patients (n=10) and higher than typical antipsychotic (n=10), or risperidone treated patients (n=6) from a previously ascertained database. 123I iodobenzamide (IBZM) single photon emission tomography (SPET) estimated striatal D2 receptor binding *in vivo*. Dynamic single slice SPET using a brain dedicated tomographic SME 810 detector (resolution 7-9mm in plane) was performed immediately after intravenous injection of 185MBq IBZM at a slice chosen to include the basal ganglia (BG) and frontal cortex (FC). All subjects were scanned during the plateau portion of the time/activity curve (60-80 minutes post injection). Computerised region of interest analysis was performed on the images obtained. An index approximating to the saturable component of D2 receptor binding was obtained by the mean ratio of BG density (representing total activity)/FC (representing background). Olanzapine treated patients had similar levels of striatal D2 binding *in vivo* (1.41 SE 0.06) as those treated with clozapine (1.49 SE 0.04), reflecting lower levels of D2 receptor occupancy by both drugs. Mean striatal D2 binding was significantly lower in typical antipsychotic (1.25 SE 0.05) and risperidone (1.24 SE 0.04) treated patients (Mann-Whitney U test, $p<0.05$). Symptom severity in olanzapine treated patients was prospectively rated by the Brief Psychiatric Rating Scale (BPRS), blind to scanning data. Mean %BPRS improvement was 49% (SD 44). Thus we provide confirmation for another atypical, clozapine like drug in which therapeutc response is not contingent upon a high degree of striatal D2 occupancy *in vivo*.

Busatto G.F., Pilowsky L.S., Costa D.C. et al (1995), Psychopharmacology, 117, 55-61
Pilowsky L.S., Costa D.C., Ell P.J. et al (1992), The Lancet, 340, 199-202
Pilowsky L.S., Costa D.C., Ell P.J. et al (1993), Psychological Medicine, 23, 791-799

130P THE BINDING PROFILE OF THE NOVEL MUSCARINIC RECEPTOR ANTAGONIST DARIFENACIN AGAINST THE FIVE CLONED HUMAN MUSCARINIC RECEPTORS EXPRESSED IN CHO CELLS

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Darifenacin ((S)-2-{1-[2-(2,3-dihydrobenzofuran-5-yl)ethyl]-3-pyrrolidinyl}-2,2-diphenylacetamide) is a novel muscarinic antagonist shown to be selective for the M_3 receptor in isolated tissue studies (Wallis *et al.*, 1995). In the present study the affinity of darifenacin for the 5 human cloned muscarinic receptors was investigated together with standard muscarinic antagonists and the clinical agents oxybutynin and dicyclomine.

The binding affinities of test compounds for human muscarinic receptors (m_1-m_5) in stably transfected CHO cells were determined by displacement of 0.1 nM [^{3}H]quinuclidinyl benzilate (QNB) using 12 concentrations of antagonist. Specific binding was defined using atropine (1 μM). The pK_i values are listed in Table 1. Equilibrium binding parameters (K_D, B_{max} (fmol/mg protein)) were m_1 (0.24 ± 0.027, 916 ± 67.92); m_2 (0.21 ± 0.031, 265 ± 45.8); m_3 (0.43 ± 0.058, 1169 ± 69); m_4 (0.26 ± 0.037, 635 ± 50); m_5 (0.73 ± 0.052, 248 ± 29).

The binding affinities and receptor selectivities of standard muscarinic antagonists were comparable to those obtained in previous studies (Dörje *et al.*, 1991; Buckley *et al.*, 1989). Darifenacin showed higher affinity for the m_3 receptor than the other 4 muscarinic receptor subtypes. In contrast, oxybutynin had similar affinity for m_1, m_2, m_3 and m_5 receptors with slightly lower affinity on m_4 receptors whilst dicyclomine had similar affinity for m_1, m_3, m_4 and m_5 receptors with lower affinity on m_2. The unique profile of darifenacin may confer a therapeutic advantage in the treatment of smooth muscle disorders associated with increased cholinergic drive.

Buckley, N.J., Bonner, T.I., Buckley, C.M. *et al.* (1989). *Mol. Pharmacol.*, 35, 469-476.
Dörje, F., Wess, J., Lambrecht, G. *et al.* (1991). *J. Pharmacol. Exp. Ther.*, 256(2), 727-733.
Wallis, R. M., Burges, R.A., Cross, P.E. *et al.* (1995) *Pharmacol. Res.*, 31S, 54

Table 1 pK_i values for muscarinic antagonists for cloned human muscarinic receptor subtypes (mean ± s.e. mean)

COMPOUND	m_1	m_2	m_3	m_4	m_5
Darifenacin (n=8-12)	7.46 ± 0.06	7.38 ± 0.06	8.42 ± 0.10	7.99 ± 0.09	7.93 ± 0.10
Oxybutynin (n=4)	8.03 ± 0.18	7.7 ± 0.17	8.31 ± 0.37	7.43 ± 0.43	7.99 ± 0.05
Dicyclomine (n=6)	8.38 ± 0.04	7.44 ± 0.08	8.45 ± 0.10	8.50 ± 0.167	8.44 ± 0.07
Atropine (n=8-12)	9.01 ± 0.05	9.05 ± 0.08	8.89 ± 0.05	9.16 ± 0.05	8.94 ± 0.12
Pirenzepine (n=7)	7.62 ± 0.06	6.47 ± 0.10	6.56 ± 0.06	7.54 ± 0.08	6.58 ± 0.06
Methoctramine (n=6)	6.17 ± 0.07	7.05 ± 0.26	5.94 ± 0.13	6.5 ± 0.19	6.07 ± 0.06
4-DAMP (n=4)	8.27 ± 0.06	7.6 ± 0.08	8.2 ± 0.27	8.11 ± 0.34	7.55 ± 0.13