1P A NOVEL sLe^x MIMETIC, CGP69669A, INHIBITS LEUKOCYTE ROLLING *IN VIVO*

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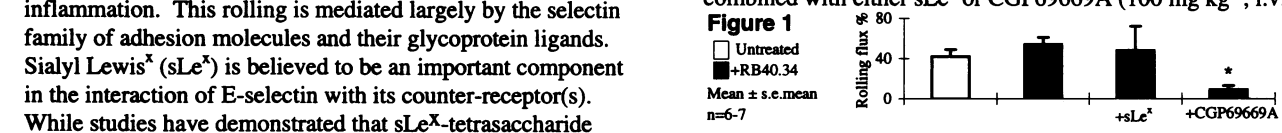
Rolling of leukocytes is the earliest observable event in their recruitment from the systemic circulation to sites of inflammation. This rolling is mediated largely by the selectin family of adhesion molecules and their glycoprotein ligands. Sialyl Lewis^x (sLe^x) is believed to be an important component in the interaction of E-selectin with its counter-receptor(s). While studies have demonstrated that sLe^x-tetrasaccharide and mimetics thereof can inhibit binding of leukocytes to E-selectin under static or flow conditions *in vitro* and can have beneficial effects in models of inflammatory disease, a direct effect of these molecules on E-selectin dependent rolling *in vivo* has not been described. Functional overlap between the selectins has been noted and the effect of anti-E-selectin antibodies in TNFα stimulated mouse cremaster is only apparent if P-selectin is absent or blocked (Kunkel *et al.*, 1996). Here we investigate the effects of sLe^x and an sLe^x mimetic, CGP69669A, on TNFα stimulated leukocyte rolling in mouse cremaster muscle.

Male C57/BL-6 mice (25-30 g) were stimulated with TNFα (500 ng, intrascrotal). 1.5 h later mice were anaesthetised i.p. with atropine (0.1 mg kg^{-1}) + pentobarbital (30 mg kg^{-1}) followed by ketamine (100 mg kg^{-1}). The trachea, jugular vein and carotid artery were cannulated and the cremaster muscle was exteriorised through a small scrotal incision, spread over a 10 mm glass cover slide and superfused with physiological bicarbonate buffer. Two hours after TNFα

Images of venules (20-40 μm) observed by light microscopy were recorded onto VHS video cassettes. Rolling data, expressed as rolling flux % and shown in figure 1, were collected from recordings made before and after treatments with anti-P-selectin antibody RB40.34 (0.3 mg kg^{-1}, iv) combined with either sLe^x or CGP69669A (100 mg kg^{-1}, i.v.).

Figure 1



Injected alone, neither sLe^x or CGP69669A reduced leukocyte rolling flux. However, when P-selectin was pre-blocked with RB40.34, CGP69669A but not sLe^x caused a significant reduction in leukocyte rolling flux % (Figure 1). Although sLe^x failed to reduce rolling flux % after P-selectin block, it did cause an increase in rolling cell velocity (Control velocity=13±2 μm s^{-1}; velocity after RB40.34+ sLe^x=55±13 μ m s^{-1}). CGP69669A had a still greater effect on velocity of rolling cells (velocity after RB40.34+CGP69669A =100±21 μ m s^{-1}). Circulating leukocyte counts, centerline red blood cell velocity and leukocyte surface expression of L-selectin measured by flow cytometry (FACS, Becton Dickinson) after direct staining of whole blood were not altered by the treatments given. Thus, although sLe^x disrupts E-selectin-ligand interaction *in vivo*, this is not sufficient to reduce rolling flux %. CGP69669A, an sLe^x mimetic with increased affinity for E-selectin, inhibits E-selectin dependent leukocyte rolling *in vivo*.

Kunkel *et al.*, (1996). *J. Exp.Med.* **183:** 57-65.

2P ANTISENSE NUCLEOTIDES TO LIPOCORTIN 1 AND PITUITARY FUNCTION *IN VITRO*

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Our previous studies have shown that the inhibitory actions of glucocorticoids (GC) on the secretion of adrenocorticotrophin (ACTH) and prolactin (PRL) *in vitro* and *in vivo* are dependent in part on the GC inducible protein lipocortin 1 (LC1:1, 2). Here we have utilised an antisense oligonucleotide to a unique sequence of cDNA which encodes the N-terminal to rat LC1 to investigate further the role of LC1 as a mediator of the inhibitory actions of glucocorticoids on the secretion of ir-ACTH and PRL by freshly dispersed rat anterior pituitary (AP) cells. Rat AP tissue was dispersed (collagenase and trituration) and the resulting cell suspension was then plated out (2.5x10^5 cells/well/ml) and incubated for 2.5h under controlled conditions. Where appropriate dexamethasone (Dex., 0.1μM) was included throughout this period and LC1 antisense, sense or a scrambled control nucleotide (50nM) was added at time 0, 1 and 2.5h. Cells were then challenged (1h) with submaximal concentrations of either corticotrophin releasing hormone (CRH-41;10nM), vasoactive intestinal peptide (VIP;100nM), forskolin (100nM) or the L-Ca^{++} channel agonist BAY K8644 (10nM). Medium was then collected and assayed for ir-ACTH and ir-PRL using radioimmunoassay and the tissue stored for histology. Results were analysed using Duncan's multiple range test, n=6. Electron microscopy of the cell suspension revealed that the morphology of the cells was well maintained following the dispersal procedure. In oligonucleotide-free groups the significant (P<0.01) increases in peptide release induced by CRH-41 vs. control (ACTH:- 240±20 vs. 131±16 pg/ml), VIP (PRL- 125±2 vs. 30±2 ng/ml) and forskolin (ACTH; 414±31 vs. 215± 20 pg/ml: PRL:- 128±6 vs. 29±5 ng/ml) were inhibited significantly (P<0.01) by Dex. LC1 antisense did not affect basal ir-ACTH or ir-PRL release in either the presence or absence of Dex.; however, it reversed (P<0.05) the inhibitory actions of Dex. on the secretagogue-induced release of both ir-ACTH and ir-PRL vs. antisense-free controls (CRH-41-induced ir-ACTH release-214±23 vs. 125±22 pg/ml; VIP induced ir-PRL release-127±4 vs. 23±3 ng/ml; forskolin: ACTH- 350±18 vs. 161±16 pg/ml; PRL-91±19 vs. 17±5 ng/ml). BAY K8644 also produced significant (P<0.01) increases in the release of ir-ACTH and ir-PRL in nucleotide free groups which were blocked (P<0.05) by Dex. The inhibitory actions of Dex. on the ir-ACTH responses were reversed specifically by the LC1 antisense (P<0.01; 215±8 vs. 83±10 pg/ml) but the Dex.-induced blockade of ir-PRL was not (35±2 vs. 41±5 ng/ml, N.S.). In all experiments LC1 sense and the scrambled control oligonucleotide were inert and the data obtained did not differ significantly from those of nucleotide-free controls. Confocal microscopy of the AP cells incubated with 5'-fluorocine labelled LC1 antisense (3.5h) demonstrated that the oligonucleotide readily passed into the cells and was concentrated in the nucleus. LC1 immunoprecipitation studies using ^{35}S-labelled cysteine/methionine as a probe demonstrated that in oligonucleotide-free groups Dex. (0.1μM, 3.5h) caused an increase in the expression of newly synthesised ir-LC1 on the outer surface of the pituitary cells when compared to control groups. The response to Dex. was unaffected by LC1 sense and the scrambled nucleotide (50nM); in contrast the steroid-induced appearance of LC1 on the cell surface was abolished by the LC1 antisense oligonucleotide (50nM). The results reinforce the importance of LC1 as a mediator of the actions of GC in the rat AP gland.

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1. Taylor A.D., Loxley H.D., Flower R.J., & Buckingham J.C. (1995a). Neuroendocrinology 62; 19-30.

2. Taylor A.D., Cowell A-M., Flower R.J., & Buckingham J.C. (1995b). Neuroendocrinology 62; 530-545.

3P A NOVEL METHOD FOR THE DETECTION AND ESTIMATION OF INTRACELLULAR LIPOCORTIN 1 IN THE ANTERIOR PITUITARY GLAND BY FAC-ANALYSIS/SORTING

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Lipocortin 1 (LC1) is an important protein mediator of glucocorticoid action on the anterior pituitary (AP) gland (1). However, although LC1 is readily detected in AP tissue by western blot analysis and ELISA (1), the cell types which express the protein remains unknown. We have therefore developed a method based on a combination of FAC-analysis/sorting and electron microscopy (EM) to detect and quantify intracellular LC1 and to identify the AP cell types in which it is expressed. In addition the influence of glucocorticoids on the expression of intracellular and cell-surface associated LC1 was investigated.

AP cells (collected post mortem from adult male rats) were dispersed into a single cell population (0.2% collagenase, 0.002% DNAse), washed in Ca^{2+} free medium, fixed and permeabilised. In initial experiments 3-cell fixation/permeabilisation protocols were examined: (a) paraformaldehyde (2%, 1h, 4°C) and Triton-X100 (0.2%, 10min, 4°C), (b) Zamboni's fluid (30min, 22°C) and Triton-X100 (0.4%, 18 h or 1h, 22°C) or (c) paraformaldehyde (2%, 15min, 4°C) and saponin (0.1%, 2h, 4°C). LC1 molecules were then detected by sequential incubation with a specific monoclonal anti-LC1 antibody (Ab, Zymed, dil 1:200) and a fluorescent-conjugated (FITC) second Ab (dil 1:100); control cells were exposed to a corresponding dilution of an isotype-matched control antibody. Cells displaying fluorescence for immunoreactive (ir) LC1 were quantified and separated by FAC-analysis/sorting and identified by EM. Surface LC1 was also detected on fixed cells that had not been rinsed in Ca^{2+}-free medium or permeabilised. Visualisation of the fixed/permeabilised cells at the EM level showed that protocol (a) provided optimal preservation of both cell ultrastructure and ir-LC1; this method was thus used subsequently. FAC-analysis revealed that ≈ 80% of the heterogeneous anterior pituitary cell population display fluorescence for intracellular ir-LC1 (46,200±7,200 LC1 molecules per cell vs control antibody 1,300±500; $P<0.01$, n=4; mean±s.e.mean). Morphological analysis (EM) of cells separated by FAC-sorting showed that corticotrophs, lactotrophs, somatotrophs and gonadotrophs are all included in the population which express LC1. Incubation of the AP cells with dexamethasone (Dex) or corticosterone (0.1μM-1.0μM, 3h) prior to fixation and analysis produced a significant ($P<0.01$) concentration-dependent decrease in intracellular ir-LC1 (Dex 0.1μM, 39,000±2,000, 1μM 34,000 ± 2,100 vs control 52,000±2,200, n=4). This was paralleled by a significant ($P<0.01$) concentration-dependent increase in ir-LC1 detected on the surface of cells (Dex 0.1μM 7,200±200, 1.0μM 8,000±300 vs control 6,000±200, n=4). The effects of Dex and corticosterone were indistinguishable. These data accord with our previous western blotting studies which demonstrated an exportation of LC1 by pituitary cells (1).

In conclusion, we have developed a novel method which permits (a) the detection and quantification of intracellular and surface ir-LC1 in AP cells and (b) the identification of the cell types which express the protein. Our initial experiments with glucocorticoids suggest that this method will provide a valuable tool for the investigation of factors which influence the expression and subcellular distribution of LC1. Further experiments using FAC-sorting and EM are now required in order to identify the pituitary cell types which export LC1 in response to glucocorticoid treatment.

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1 Buckingham, JC (1996) Br J Pharmacol, 118, 1-19

4P EOTAXIN RECRUITS EOSINOPHILS IN SENSITISED MICE

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Blood and tissue eosinophilia is a characteristic feature of a number of allergic disease states including asthma and atopic dermatitis. Eotaxin, a newly discovered member of the C-C family of chemokines, and is a specific activator of eosinophils (Eøs) (1,2). Eotaxin is more effective in recruiting Eøs to a specific site *in vivo* in the presence of a blood eosinophilia (3) which may reflect the situation in sensitised individuals. In the present study, we have tested this hypothesis by investigating eotaxin-induced Eø recruitment in mice sensitised to ovalbumin (OA) as these mice exhibit a blood eosinophilia (4).

Balb/c mice were sensitised to OA (sc injection of 100 μg OA adsorbed to 3.3 mg aluminium hydroxide gel) on days 0 and 7. On day 15, sensitised or naive mice were injected i.p. with either vehicle or rm-eotaxin (0.25 - 1.0 μg) and sacrificed at different timepoints after the treatments. The peritoneal cavities were washed out with 3ml phosphate buffered saline containing 10 mM EDTA. Total and differential cell counts were performed on the lavaged cells. Blood cell numbers were determined from samples collected by cardiac puncture. In some experiments sensitised mice were pretreated with different drugs: dexamethasone (DEX) was administered s.c. at doses of 10, 5 and 1 μg 1 h prior to the administration of rm-eotaxin; the H_1 antagonist tripolidine (0.5mg/kg, i.p.) and the PAF antagonist WEB2086 (10mg/kg, i.p.) were administered 30 min prior to the rm-eotaxin injection. Data is presented as the mean ± sem. Statistical differences between two groups were analysed using the Mann-Whitney test and the Kruskal-Wallis test was used for multiple comparisons.

A dose-dependent increase in Eø migration was observed at 6 h following i.p. administration of rm-eotaxin to sensitised mice. 0.5 μg rm-eotaxin elicited a significant and specific peritoneal eosinophilia (vehicle injected, n=15: 2.47± 0.44x10^5 Eøs, rm-eotaxin injected, n=17: 7.07±0.86x10^5 Eøs; $P<0.01$). When rm-eotaxin was administered to naive, unsensitised mice (which have 3-fold less blood eosinophil numbers than sensitised mice) the extent of eosinophilia was not as marked (vehicle injected, n=7: 1.13+0.24x10^5 Eøs; rm-eotaxin injected, n=8: 2.48±0.44x10^5 Eøs; $P<0.05$). The effect of the anti-inflammatory glucocorticoid dexamethasone (DEX) was examined on rm-eotaxin-induced peritoneal eosinophil influx in sensitised animals. DEX induced a dose-dependent inhibition of the eosinophil infiltration (rm-eotaxin alone, n=11: 8.57±0.99x10^5 Eøs; rm-eotaxin + 10μg or 5μg or 1μg DEX, n=6-7: 0.91±0.28x10^5 Eøs, $P<0.01$; 0.92±0.13x10^5 Eøs, $P<0.01$; and 5.27±0.94x10^5 Eøs per mouse respectively). The PAF antagonist WEB2086 had no effect on rm-eotaxin-induced eosinophil recruitment. However, the H_1-antagonist tripolodine significantly reduced the Eø migration (-65%, n=6; $P<0.05$).

In conclusion, we have demonstrated that rm-eotaxin is effective in recruiting Eøs *in vivo* and that action is dependent on the sensitisation status of the animal. We have also shown that this chemokine is sensitive to the actions of DEX. Further, we have shown for the first time that eotaxin appears to be acting by causing the release of endogenous histamine.

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(1) Jose et al (1994) *J.Exp.Med.* **179**,881
(2) Elsner et al (1996) *Eur.J.Immunol.* **26**,1919
(3) Collins et al (1995) *J.Exp.Med.* **182**,1169
(4) Das et al *Br.J.Pharmacol.* (1996) **119**,58P.

5P MOLECULAR DETERMINANTS OF CRYSTAL-INDUCED INFLAMMATION

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There is a renewed interest in the inflammatory reponse induced by gout-related crystals (monosodium urate, MSU) especially in light of the recent advances in understanding the leucocyte-endothelium interaction [1]. Here we have defined the molecular determinants responsible for neutrophil extravasation in response to MSU injection.

Male Swiss Albino mice (28-32g) were treated i.v. with fucoidin (300 µg), specific anti-CD62P and anti-CD62E monoclonal antibodies (mAb) (100 µg of each) or control rat IgG (100 µg) 1 h prior to i.p. injection of 3 mg MSU crystals. In other cases, mice received the platelet-activating factor (PAF) antagonist WEB2086 (10 mg kg^{-1}) or the H_1 antagonist triprolidine (0.5 mg kg^{-1}) together with the crystals. In other experiments, mice received an anti-CD11b mAb (250 µg i.v.) 1 h prior to MSU crystals. The lipocortin 1-derived N-terminus peptide (Ac2-26) was given s.c. (200 µg) 30 min prior to MSU crystals. Six h after crystal injection peritoneal cavities were washed with 3 ml of PBS + 3 mM EDTA and 25 U ml^{-1} heparin. Differential cell counts were performed using a Neubauer haematocytometer following staining in Turk's solution. MSU dose and time-point were selected on the basis of preliminary experiments. Data are reported as 10^6 cells per mouse (mean ± s.e. mean), and statistical differences were assessed by ANOVA followed by the Bonferroni test.

Polymorphonuclear leucocytes (PMN) represented the major cell type (>90%) recovered in the peritoneal fluids 6 h post-MSU injection: 8.60±0.19 x10^6 cells per mouse, n=10. Fucoidin significantly reduced MSU-induced PMN infiltration to 3.10±0.35 x10^6 cells per mouse (n=11, $P<0.01$). The involvement of selectins was investigated in more detail. Treatment with control rat IgG resulted in a MSU-induced PMN influx of 10.2±0.54 x10^6 cells (n=17), and this value was reduced to 6.3±0.51 x10^6 cells after administration of anti-CD62P mAb (-38.2%, n=12; $P<0.01$), and to 5.2 ± 0.35 x10^6 PMN by the anti-CD62E mAb (-49%, n=10; $P<0.01$). Co-injection of both mAbs gave a higher degree of inhibition with a PMN accumulation of 3.4±0.24 x10^6 cells per mouse (-66.7%, n=13 , $P<0.01$). A similar degree of inhibition was achieved with the anti-CD11b mAb: -67.6%, n=12, P <0.01 WEB 2086 and triprolidine were also able to significantly attenuate PMN influx (-61% and -45%, respectively; n=10). Finally, systemic administration of peptide Ac2-26 prior to MSU inhibited cell accumulation at the 6 h time-point by 63% (n=6; $P< 0.05$).

In conclusion, we found a functional role for endogenous PAF and histamine in MSU-induced inflammation. In addition, a distinct and additive action for the endothelial selectins, CD62P and CD62E, was also observed. Amongst the $ß_2$-integrins, CD11b appears to play a major role. Similarly to other models of cell recruitment, the lipocortin 1 pharmacophore, peptide Ac2-26, was effective in reducing PMN accumulation.

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[1] Chapman *et al.*, Br. J. Rheumatol. **35**, 323-334, 1996.

6P MOLECULAR CLONING AND EXPRESSION OF THE HUMAN HYPOTHALAMIC TYPE I INTERLEUKIN-1 RECEPTOR

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Interleukin-1 (IL-1) acts directly on the central nervous system, probably within the hypothalamus (Rothwell, 1991). Studies using either radiolabelled ligand or polymerase chain reaction (PCR) analysis have previously failed to demonstrate the presence of IL-1 receptor type I (IL-1RI) binding sites or mRNA in this region of the brain (Rothwell, 1991). It is possible however, that levels of IL-1RI message or protein may have been beyond the limits of detection of these particular techniques. We decided to attempt to clone the brain IL-1RI cDNA from human hypothalamus, utilising a polymerase chain reaction (PCR) amplification of a commercially available human hypothalamal cDNA library (CLONtech) as a template. Primers complementary to the 5' and 3' regions flanking the coding IL-1RI were designed, based on the published sequence of human fibroblastic IL1-RI cDNA (Chua and Gubler, 1989).

A PCR was isolated and DNA sequencing confirmed its identity as a full length IL1-RI cDNA clone. The IL1-RI clone was subcloned into a mammalian expression vector, which harbours a neomycin resistance marker. The resulting construct (pchIL-1rI) was initially expressed transiently in green monkey kidney COS7 cells. Specific binding of iodinated IL-1α was readily detected in cells transfected with pchIL-1RI but not in a control cell line transfected with empty vector. The receptor was subsequently expressed in Chinese Hamster Ovary (CHO) cells to facilitate analysis of interleukin-1 receptor mediated signal transduction.

CHO cells exhibiting stable expression of the IL1-RI were isolated using G418 (a neomycin analogue) resistance. Scatchard analysis using [^{125}I]IL-1α revealed that a clone from a single cell line expressed 3469±933 receptors per cell with a K_D of 0.41±0.12 nM (n=3). This clone, CHO.hIL-1RI, was used in all further analysis of IL-1 signal transduction. Northen blotting analysis of total RNA, using the full length hIL-1RI cDNA as a probe, revealed a single RNA species of 2 kB present in the transfected clone but not the parental cell line.

In order to demonstrate that the cell line CHO.hIL-1RI expressed functional receptors, the cells were assessed for their ability to activate the nuclear transcription factor, NFκB, in response to IL-1 stimulation using an electrophoretic mobility assay. Activation of NFκB occurred (and was maximal) after 5 minutes stimulation with 10 ng/ml IL-1α in transfected , but not parental, cells.The presence of the Rel protein p65 as the transcriptionally active member of the IL-1-stimulated NFκB dimer complex was confirmed by the production of "supershifts" with an anti-p65 antibody in the mobility assay. In preliminary studies the MAP kinase kinase inhibitor, PD 098059, did not block the IL-1 stimulated NFκB response. Furthermore, the IL-1-induced acidification response of CHO.hIL-1RI cells in the Cytosensor (Smart et al., This meeting) was not inhibited by 50 µM PD098059: untreated cells exhibited a response of 386±36µvolts $sec^{-1}min^{-1}$ when stimulated with IL-1α compared to 400±27µvolts sec^{-1} min^{-1} in cells treated with 50 µM PD098059. These data imply that the classical MAP kinase cascade is not required for the IL-1 induced NFκB response in CHO cells.

To conclude, a stable CHO cell line expressing human hypothalamic IL-1 Type I receptors is now available facilitating a thorough analysis of the signalling mechanisms mediated by interleukin-1 via this receptor

Rothwell, N. J. (1991) Trends Pharmacol. Sci. 12, 430-436.
Chua, A. O. and Gubler, U. (1989) Nucleic Acids Res. 17, 10114-10119.

7P CHARACTERISATION USING THE CYTOSENSOR MICROPHYSIOMETER OF RECOMBINANT HUMAN TYPE I INTERLEUKIN-1 RECEPTOR PHARMACOLOGY

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The pharmacology of the Type 1 interleukin-1 (IL-1) receptor (IL-1RI) remains to be fully elucidated (Bankers-Fulbright *et al.*, 1996; Mantovani *et al.*, 1996). The Cytosensor microphysiometer measures the cellular acidification rate, as a reliable index of the integrated functional response to receptor activation, and is well suited to the pharmacological study of stably expressed recombinant receptors (Jordan *et al.*, 1995). Therefore, having recently cloned the human IL-1RI from the hypothalamus (Hammond *et al.*, 1996), we have used the Cytosensor to examine the pharmacology of the IL-1RI stably expressed in CHO (CHO-IL) cells.

CHO-IL cells were cultured in Hams F-12 medium and seeded into Cytosensor capsule cups as described previously (Jordan *et al.*, 1995). The cups (~0.6×10^6 cells per cup) were perfused in the Cytosensor at 120µl min^{-1} with bicarbonate-free Hams F-12 (pH 7.4), and the acidification rate taken every 2min by stopping the perfusion for 15s. IL-1α (0.1-100ng ml^{-1}) was added to the perfusate for various intervals (3-20min), in the presence or absence of the antagonist IL-1ra (5-200ng ml^{-1}). Responses were measured as area under the curve, and are presented as mean±s.e.mean unless otherwise stated.

Exposure of the CHO-IL cells to IL-1α (10ng ml^{-1} for 5min) caused a delayed (onset 4-6min after exposure began, n=15) broad monophasic increase in the acidification rate, which peaked at 16-22min and returned to basal levels within 36-44min (n=15). Surprisingly, varying the length of exposure (3-20min) had no effect on either the magnitude or time course of the response (n=4). However, the IL-1α (0.1-100ng ml^{-1} for 5min)-induced acidification response was dose-dependent, with a pEC_{50} of 9.93±0.08 (n=6). The IL-1RI antagonist, IL-1ra (5-200ng ml^{-1}) had no effect on the basal acidification rate, but dose-dependently inhibited the IL-1α (10ng ml^{-1} for 5min)-induced response, with an IC_{50} of 19.7ng ml^{-1} (n=4-8) and complete blockade (98.8±1.2%) occurring at 200ng ml^{-1} Activation of the IL-1RI by IL-1α (10ng ml^{-1} for 5min) caused profound desensitisation of the receptor, with a subsequent challenge eliciting a reduced (<20%, n=4) response even after a 12h interval. This desensitisation was homologous and did not involve a reduction in cell viability, as the acidification response to activation of the endogenously expressed P_{2U} receptor with 3µM UTP was not effected (n=3).

Both the EC_{50} (2.0ng ml^{-1}) for IL-1α and the complete blockade of the IL-1α response with a ~20-fold excess of IL-1ra are consistent with established IL-1RI pharmacology (Bankers-Fulbright *et al.*, 1996; Mantovani *et al.*, 1996), further confirming the identity of our clone (Hammond *et al.*, 1996). Moreover, we report here for the first time that there is a profound and prolonged desensitisation of the IL-1RI following its activation with IL-1α. In conclusion, these data clearly demonstrate the utility of the Cytosensor for studying the pharmacology of the IL-1RI.

Bankers-Fulbright, J.L., Kalli, K.R. & McKean, D.J. (1996) Life Sci. 59, 61-83.
Hammond, E.A., Smart, D., Webdale, L.J. *et al.*, (1996) This meeting.
Jordan, R.E., McKnight, A.T. & Woodruff, G.N. (1995) Br. J. Pharmacol. 116, 445P.
Mantovani, A., Muzio, M., Ghezzi, P. *et al.*, (1996) Int. J. Clin. Lab. Res. 26, 7-14.

8P EFFECT OF CRF ON THE FEBRILE RESPONSE TO IL-1β OR LPS IN RATS

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Corticotrophin-releasing factor (CRF) is a neuropeptide involved in diverse responses to stress and infection, including fever and sickness behaviour by direct actions on the brain. Cytokines such as interleukin (IL)-1β are intimately involved in these changes and act via the release of CRF (Rothwell, 1989). However, the direct effect of CRF on body temperature, and the relationship between IL-1β and CRF in the development of fever remains unclear. We investigated the effects of CRF in the brain on core body temperature, and its role in fever.

Unrestrained, male, Sprague-Dawley rats (275-325g) were injected (2µl) intracerebroventricularly (i.c.v.) with either CRF (3µg) or vehicle, subsequent to the following pre-treatments: CRF receptor antagonist, α-helical CRF_{9-41} (30µg, i.c.v., 5min before); IL-1β (5ng, i.c.v., 50min before); or intraperitoneal injection of the peripheral immune stimulus, lipopolysaccharide (LPS; 100µg/kg, 90min before). The effects of these molecules on core body temperature were measured at 10min intervals using remote radiotelemetry via pre-implanted, abdominal transmitters. Data were analysed using either MANOVA for comparison over the experimental time-course, or ANOVA for individual time-points.

Injection (i.c.v.) of 3µg CRF (n=7) produced significant ($P<0.01$) hypothermia, declining to a minimum temperature (36.2±0.2°C) 1h post-injection. α-helical CRF significantly ($P<0.01$) inhibited CRF hypothermia (36.4±0.1°C; n=8), but when injected alone (n=5) caused significant ($P<0.05$) hyperthermia (38.6±0.2°C) 1h post-injection. Both IL-1β and LPS fever (38.9±0.1°C, 38.8±0.3°C) were significantly ($P<0.01$, $P<0.001$) attenuated by a previously determined (Gardner *et al.*, 1996) subthreshold (non-hypothermic) dose (0.3µg) of CRF (n=6).

The development of hypothermia in response to i.c.v. injection of CRF is an observation contrary to those made in previous studies (Rothwell, 1989; 1990). The inhibition and reversal of the hypothermic effect of CRF by α-helical CRF suggests a direct receptor-mediated mechanism involving the displacement of endogenous CRF from its receptors. The attenuation of IL-1β and LPS fever by CRF may also be a direct mechanism to limit fever. Alternatively, these effects may be caused indirectly, by CRF-stimulated release of endogenous antipyretic molecules such as corticosterone, or may result from changes associated with cardiovascular actions of CRF. These results suggest that CRF is neither pyrogen or cryogen, but probably acts as an effector involved in general thermoregulation.

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Gardner, J.D., Rothwell, N.J. & Luheshi, G.N. (1996) This meeting.
Rothwell, N.J. (1989) *Am. J. Physiol.* 256, E111-115.
Rothwell, N.J. (1990) *Neurosci. Biobehav. Rev.* 14, 263-271.

9P CONTROL OF GENE EXPRESSION BY ELEVATION OF CELL cAMP CONTENT IN PRIMARY CULTURES OF HUMAN AIRWAY SMOOTH MUSCLE CELLS (HASM) TRANSFECTED WITH A cAMP-RESPONSIVE REPORTER CONSTRUCT

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Many genes important for the control of responses in airway smooth muscle cells contain cyclic AMP response elements (CREs) which can upregulate gene expression following elevation of cell cyclic AMP (cAMP) content. However, because many other regulatory motifs are also present, indirect effects of changes in cell cAMP may also occur. To investigate the control of gene expression by cAMP elevation in cultured HASM cells, we transfected cells with a reporter construct containing 6 CRE motifs upstream of the gene for firefly luciferase (p6CRE/luc).

Primary cultures of HASM cells were grown as previously described (Hall et al 1992). We used the pGL3 control reporter construct to optimise transfection conditions in these cells grown in 6 well plates. Highest levels of luciferase activity were observed using cationic liposomal transfection with 4μg DNA at a ratio of 1μg DNA:1.8μl Transfectam, with cells harvested at 48h (mean luciferase activity 249 ± 44 Turner light units (TLU)/mg soluble protein, n=22). cAMP responses were measured as previously described (Hall et al 1992).

Luciferase activity in lysates from HASM cells transfected with the p6CRE/luc construct after 48h was 143 ± 35 TLU/mg protein, (n=23). Following incubation with isoprenaline (10μM) for 24h a 5.9 ± 1.1 fold increase (cf control) in luciferase activity was observed ($p<0.0001$, n=19). This effect was concentration related and was inhibited by the β2 selective antagonist ICI 118551 (50nM) (Hall et al 1992). In parallel experiments the time course of cAMP levels was studied following exposure to 10μM isoprenaline: cAMP rose rapidly, was maximal at 4h and then slowly fell, although was still significantly above baseline at 12h. The nonselective phosphodiesterase inhibitor IBMX (50μM) also increased luciferase activity (3.0 ± 0.4 fold, n=4), but did not induce a further increase in the response to isoprenaline alone, implying that the response to isoprenaline was maximal. Isoprenaline, IBMX and ICI 118551 produced no significant effects upon luciferase activity in HASM cells transfected with pGL3 (all n=4).

These results demonstrate that pharmacological manipulation of cAMP levels in HASM cells produces changes in gene expression of a reporter gene under CRE control.

Hall, I.P., Widdop S., Townsend P. et al, *Br. J. Pharmacol.* 1992; 107: 422-428.
Hall, I.P.& Kotlikoff, M. *Am J Physiol.* 1995; 268 : L1-11.

I.P.H is a National Asthma Campaign Senior Research fellow.

10P THE TYPE IV PHOSPHODIESTERASE INHIBITOR ROLIPRAM INHIBITS DNA SYNTHESIS IN PRIMARY CULTURES OF HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Proliferation of airway smooth muscle (ASM) is a feature of remodelling of the airways seen in chronic asthma. Agents which elevate cAMP levels are potentially able to prevent mitogenic responses in these cells. The aim of this study was to determine whether inhibition of individual phosphodiesterase (PDE) isozymes in human ASM cells was able to inhibit DNA synthesis in response to the mitogen platelet derived growth factor (PDGF-BB). Primary cultures of human ASM cells were grown as previously described (Hall et al 1992). DNA synthesis was assessed by determining incorporation of [^{3}H]-thymidine (Danielpour et al 1989) over 16h in the presence or absence of agonists (added 8h prior to thymidine) following 48h serum deprivation.

PDGF-BB produced concentration related [^{3}H]-thymidine incorporation in human ASM cells (EC_{50} 8.3 ± 1.5 ng/ml, n=4, maximum response to 20ng/ml PDGF-BB 5.3 ± 0.6 fold over control). Prior exposure to the nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Hall et al 1992) (100μM) for 30min did not significantly alter basal thymidine incorporation but resulted in a 30 ± 6% inhibition of the response to PDGF-BB (20ng/ml) (n=12, p=0.003). We have previously demonstrated that cAMP levels in these cells can also be elevated by the type IV selective PDE inhibitor rolipram (Hall et al 1992). Rolipram (EC_{50} 20 ± 4 μM, n=8) induced concentration related inhibition of the [^{3}H]-thymidine response to PDGF (20ng/ml), the maximum inhibition seen with 100μM rolipram being 49 ± 5% (n=4, p=0.004). Rolipram (100μM) also inhibited basal thymidine incorporation by 48 ± 6% (n=4, p=0.01) in these cells. SK&F 94836 (Hall et al 1992) (100μM), a selective type III PDE inhibitor, inhibited [^{3}H]-thymidine incorporation in these cells by 43 ± 9% (n=7, p=0.007).

These results demonstrate that inhibition of type III and type IV PDE isoforms in cultured human ASM cells inhibits DNA synthesis in response to the mitogen PDGF-BB. PDE inhibitors of these isoforms may potentially prevent the ASM remodelling that occurs in asthmatic lungs.

Danielpour, D., Dart L.L., Flanders, K.C. et al, *J. Cell Physiol.* 1989; 138: 79-86
Hall, I.P., Widdop, S, Townsend, P, et al, *Br J Pharmacol* 1992; 107: 422-428.

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11P PROSTAGLANDINS CONTRIBUTE TO THE ANTI-PROLIFERATIVE EFFECT OF ISOENZYME-SELECTIVE PHOSPHODIESTERASE 4 INHIBITORS BUT NOT THEOPHYLLINE IN HUMAN MONONUCELAR CELLS

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Lymphocyte proliferation plays a key role in the inflammatory response in bronchial asthma and other allergic diseases (Azzawi *et al.*, 1992). Agents which can elevate cAMP including phosphodiesterase (PDE) inhibitors and some prostaglandins, e.g. PGE_2 can inhibit the activation of a variety of inflammatory cell types including lymphocytes (Torphy & Undem, 1991). In the present study we tested whether the anti-proliferative effects of three PDE inhibitors are due, in part, to stimulation of endogenous PGE_2 release. Peripheral venous blood (25ml) was drawn from healthy volunteers and mononuclear cells (10^5 per well) stimulated to proliferate with phytohaemagglutinin (PHA) (2μg ml^{-1}) in the absence or presence of the PDE 4 inhibitors, rolipram (0.1-10μM), R-(+)-4-[2-(3-cyclopentoxy-4-methoxyphenyl)-2-phenylethyl]pyridine (CDP 840; 0.03-10μM), or the non-selective PDE inhibitor, theophylline (10-1000μM) for 24h at 37°C in a 95% air, 5% CO_2 atmosphere. [^{3}H]-thymidine (0.1μCi per well) was then added and cells incubated for a further 24h before being harvested onto glass fibre filters for scintillation counting of β emission. IC_{50} values for inhibition of proliferation were determined. Each drug was tested alone and with the addition of indomethacin (3μM). Each drug was examined on 6 or 7 blood samples (each from a separate individual) and each concentration of PDE inhibitor was examined in triplicate. Data were analysed by ANCOVA.

Indomethacin alone had no effect on basal or PHA stimulated proliferation ($P>0.05$, ANCOVA). Theophylline, rolipram and CDP 840 produced concentration related inhibition of proliferation ($P<0.05$, ANCOVA). Indomethacin reduced this effect, significantly increasing the IC_{50} value for CDP 840 from 0.81μM (95% confidence limits 0.23 and 3.16) to 2.82μM (0.19-4.27) and for rolipram from 2.51μM (1.29-4.89) to > 10μM ($P<0.05$, ANCOVA), but did not affect the IC_{50} for theophylline which was 282μM (186-427) without and 288μM (202-411) with indomethacin ($P>0.05$, ANCOVA). These data suggest that CDP 840 and rolipram inhibit proliferation, in part, by causing the release of endogenous prostaglandins whereas theophylline does not. To confirm this we shall need to replicate studies with measurement of prostaglandin production. Furthermore, these results suggest that the anti-proliferative effect of theophylline may be distinct from that induced by selective PDE 4 inhibitors.

Azzawi, M., Johnston, P.W., Majamdar, S. *et al.* (1992). *Am. Rev. Respir. Dis.* 145, 1477-1482.
Banner, K.H., Roberts, N.M. & Page, C.P. (1995). *Br. J. Pharmacol.* 116, 3169-3174.
Torphy, T.J. & Undem, B.J. (1991). *Thorax*, 46: 512-523.

12P EFFECTS OF PHOSPHODIESTERASE (PDE) INHIBITORS ON cAMP PDE ACTIVITY IN HUMAN LUNG MAST CELLS AND BASOPHILS

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Previous studies have shown that cyclic nucleotide phosphodiesterase (PDE) exists as multiple molecular forms (Barnes, 1995). PDE 4 has been found to be the predominant isozyme involved in the regulation of inflammatory cell function. Indeed, it has been shown that selective inhibitors of PDE 4 attenuate mediator release from human basophils (Peachell *et al*, 1992). However, we have found PDE 4 inhibitors to be ineffective at inhibiting mediator release from human lung mast cells (HLMC) (Weston & Peachell, 1996).

In the present study, we have examined the effects of non-selective and isozyme-selective PDE inhibitors on cAMP hydrolytic activity in extracts prepared from purified basophils and HLMC. Mean purities were 90±4% (basophils, n=10) and 86±5% (HLMC, n=12). Hydrolysis of cAMP by PDE in extracts of purified basophils and HLMC was determined essentially according to the method of Reeves *et al* (1987). Extracts derived from both HLMC and basophils hydrolysed cAMP (pmol cAMP hydrolysed min^{-1} by 10^6 cell equivalents : 1.6±0.5, basophils, n=10; 1.6±0.2, HLMC, n=12). The cAMP hydrolytic activity present in both cell types was inhibited dose-dependently and equipotently by the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX). The IC_{50} for IBMX was 0.04 mM in both basophils (n=5) and HLMC (n=6). In basophil extracts (n=5), the isozyme-selective PDE inhibitors (all at 10 μM) 8-methoxymethyl IBMX (PDE 1 inhibitor), siguazodan (PDE 3), and zaprinast (PDE 5) were ineffective at inhibiting cAMP hydrolysis, whereas rolipram (PDE 4 selective) and Org 30029 (mixed PDE 3/4) were both effective inhibitors of PDE activity (56±8% and 47±6% inhibition respectively; $p<0.05$). These results parallel the effects of these compounds on IgE-mediated histamine release from basophils, where only PDE 4-active compounds were found to be effective inhibitors (40±8% inhibition by rolipram; 32±7% by Org 30029, n=4; $p<0.05$). In contrast, PDE activity in HLMC (n=6) was inhibited to a significant ($p<0.05$) and similar extent by siguazodan (35±5% inhibition), rolipram (26±6%), Org 30029 (25±6%), zaprinast (23±4%), and 8-methoxymethyl IBMX (16±6%). All of these isozyme-selective inhibitors were ineffective (≤10±4% inhibition, $p>0.05$) at attenuating anti-IgE-mediated histamine release from HLMC.

In summary, we have shown that PDE 4-active compounds act selectively to inhibit both histamine release and PDE activity in basophils. In HLMC, isozyme-selective PDE inhibitors had no effect on histamine release. Moreover, PDE 4-active compounds were no more active than alternative isozyme-selective inhibitors at attenuating cAMP PDE activity in HLMC extracts. These data provide further evidence that PDE 4 is important in the regulation of human basophil responses, whereas an association of the PDE 4 isozyme with HLMC function remains uncertain.

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Barnes, P.J. (1995) *Eur. Respir. J.* 8, 457-462.
Peachell, P.T. *et al* (1992) *J. Immunol.* 148, 2503-2510.
Reeves, M.L. *et al* (1987) *Biochem. J.* 241, 535-541.
Weston, M.C. & Peachell, P.T. (1996) *Br. J. Pharmacol.* 118, 115P.

13P PROTECTION BY DEXAMETHASONE OF THE FUNCTIONAL DESENSITIZATION TO BETA ADRENOCEPTOR AGONIST RESPONSES IN HUMAN LUNG MAST CELLS

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Beta adrenoceptor agonists continue to be important in the therapeutic management of asthma. Long-term treatment with ß adrenoceptor agonists, however, may induce tolerance. Corticosteroids, in addition to well recognized anti-inflammatory properties, have been shown to reverse tolerance to ß adrenoceptor agonists *in vivo* (Holgate *et al.* 1977). We have previously reported that long-term (4-24 h) pretreatment of human lung mast cells (HLMC) with ß adrenoceptor agonists leads to a subsequent functional desensitization to ß adrenoceptor agonists (Chong *et al.* 1995). The aim of the present study was to establish whether glucocorticoids could prevent the functional desensitization to ß adrenoceptor agonists.

HLMC were incubated (24 h) with or without isoprenaline (ISO, 10^{-6}M), washed and then exposed (10 min) to ISO for a second time before challenge with anti-IgE to induce histamine release. ISO (10^{-7}M) inhibited histamine release by 56±4% in untreated cells, whereas in treated cells (10^{-6}M ISO for 24 h), ISO (10^{-7}M) inhibited histamine release by 23±7%, a statistically significant (ANOVA $p \leq 0.001$, n=7) reduction in the ability of ISO to inhibit histamine release. In these same experiments, HLMC were exposed (24 h) either to DEX (10^{-7}M) alone or HLMC were co-incubated with DEX (10^{-7}M) and ISO (10^{-6}M). Incubation of DEX had no effect on the subsequent ability of ISO (10^{-7}M) to inhibit histamine release (53±4% inhibition). Although the co-presence of DEX did protect against the desensitization induced by ISO, this effect, apparently, was not statistically significant (23±7% inhibition by 10^{-7}M ISO following desensitizing conditions; 30±9% inhibition by 10^{-7}M ISO with DEX present during the desensitization step). Further experiments were performed in which HLMC were exposed for longer periods to DEX. Thus, HLMC were either incubated a) in buffer for 48 h, b) with DEX (10^{-7}M) for 48 h, c) in buffer for the first 24 h and then with ISO (10^{-6}M) for 24 h or d) for 48 h with DEX (10^{-7}M) and together with ISO (10^{-6}M) for the final 24 h. Following these four different conditions, ISO (10^{-7}M) inhibited histamine release by 54±5% (buffer), 56±6% (DEX), 8±4% (ISO) and 19±5% (ISO + DEX). Thus, the presence of DEX protected against the desensitization and this effect was statistically significant (ANOVA $p \leq 0.001$, n=7). In a further experiment, HLMC were incubated either a) for 72 h in buffer, b) 72 h with DEX (10^{-7}M), c) 48 h with buffer and then for 24 h with ISO (10^{-6}M), or d) for 72 h with DEX (10^{-7}M) and for the final 24 h with ISO (10^{-6}M). Under these conditions, a second exposure to ISO (10^{-7}M) inhibited histamine release by 56±5% (buffer), 65±9% (DEX), 14±8% (ISO) and 37±13% (ISO + DEX). The DEX protection of desensitization was statistically significant (ANOVA $p \leq 0.001$, n=4). Therefore, pretreatment of HLMC with DEX for either 24 h, 48 h or 72 h provided protection against the functional desensitization to ISO by 21%, 24% and 55%, respectively.

To conclude, prolonged treatment with DEX protects against desensitization to ß adrenoceptor agonist responses in HLMC.

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Chong, L.K. *et al.* (1995) *Am. J. Respir. Cell Mol. Biol.* **13**, 540-546.
Holgate, S.T. *et al.* (1977) *Lancet* **2**, 375-377.

14P EOSINOPHIL ADHESION TO HUMAN BRONCHIAL EPITHELIAL CELLS: MODULATION BY EOTAXIN

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Eotaxin, a C-C chemokine, is chemoattractant on human eosinophils [1] and also increases eosinophil adhesion to human lung microvascular endothelial cells [2]. Eosinophil/epithelial adhesion may, in part, mediate airway epithelial injury seen in asthma. In the present study we have compared the effects of eotaxin (10, 30, 100ng ml^{-1}) on eosinophil adhesion to unactivated, or cytokine-activated normal human bronchial epithelial cells (NHBEC), with the effects of two other C-C chemokines, RANTES or MIP-1α (30, 100 ng ml^{-1}) and also C5a (10^{-7}M) or phorbol-12-myristate-13-acetate (PMA; 10^{-8}M).

NHBEC (Clonetics, San Diego, USA) were maintained in basal epithelial growth medium supplemented with antibiotics. Confluent monolayers of NHBEC grown on 96-well plates were incubated for 24h with IFNγ (0.01-100ng ml^{-1}) alone or in combination with tumour necrosis factor-α (TNFα), interleukin (IL)-1β, IL-4 (0.1, 1 or 10 ng ml^{-1}) or lipopolysaccharide (LPS; 0.1, 1 or 10μg ml^{-1}). A specific enzyme-linked immunosorbant assay was used to measure ICAM-1 expression [3] and results were expressed as mean optical density (OD_{405}) ± s.e. mean of 5 experiments. Human eosinophils isolated from peripheral blood of 5 mildly atopic adult donors and labelled with a fluorescent dye (Calcein-AM, 10μM), were incubated for 30 min with NHBEC [2]. Results were expressed as mean ± s.e mean of percent adherent cells over total cells (1.25×10^5) added per well, determined by fluorescence.

ICAM-1 was not detected on resting NHBEC but IFNγ induced expression in a concentration-dependent manner. TNFα (1ng ml^{-1}) did not induce ICAM-1 expression but enhanced IFNγ- (10ng ml^{-1}) induced expression from OD_{405} of 0.99±0.07 to 1.44±0.10. Similar effects were seen with IL-1β but not LPS or IL-4. Basal adhesion of unstimulated eosinophils to untreated NHBEC (10.0±0.7%, n=5) was significantly increased following exposure of eosinophils during the adhesion assay to PMA or C5a but not eotaxin, RANTES or MIP-1α. Activation (24h) of NHBEC with TNFα (1ng ml^{-1})/IFNγ (10ng ml^{-1}) did not increase unstimulated eosinophil adhesion (10.1±1.1%, n=5). Eosinophil adhesion to TNFα/IFNγ-activated NHBEC was increased by C5a (20.2±1.9%, n=4), PMA (25.8±0.6%, n=4) or eotaxin (100, 30ng ml^{-1}; 21.6±1.7 and 19.5±1.9%, respectively, n=4) but not MIP-1α or RANTES. Anti-CD18 monoclonal antibody (6.5E, 20μg/ml) abolished the increased adhesion due to eotaxin.

These results show that eotaxin, PMA or C5a but not RANTES or MIP-1α, induced eosinophil adhesion to TNFα/IFNγ-activated NHBEC. This suggests that eotaxin may play a role in mediating epithelial injury in asthma by increasing eosinophil adhesion to NHBEC via a CD18/ICAM-1 adhesion pathway.

[1] Ponath, P.D., Qin, S., Ringler, D.J., *et al.*, (1996) *J. Clin. Invest.* 97, 604-612.
[2] Burke-Gaffney, A. & Hellewell, P.G. (1996) *Biochem. Biophys. Res. Commun.* (in press)
[3] Burke-Gaffney, A. & Hellewell, P.G. (1996) *Am. J. Physiol.* 270, C552-C561.

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15P IDENTIFICATION OF AN INWARD RECTIFIER K^+ CURRENT IN EOSINOPHILS FROM HUMAN BLOOD

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Eosinophils are effector cells in several human diseases. Although indirect evidence alludes to possible associations between eosinophil activation, mobilisation and changes in membrane potential, little is known about the identity of channels in these cells. This study reveals the existence of an inward rectifier K^+ current in eosinophils from human blood.

Normodense human eosinophils were prepared from blood taken from healthy, non-atopic donors according to the method described previously (Gordienko *et al.*, 1996). Whole cell currents and membrane potential were recorded at room temperature using conventional whole cell patch clamp technique. The cells were bathed in a standard external solution (mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 6; pH 7.0. For solutions of increased external potassium concentration, K^+ replaced Na^+. The standard pipette solution used contained (mM): NaCl 5, KCl 148, MgATP 1, creatine 5, GTP 0.02, HEPES 10 and calcium was clamped to 10^{-8}M with Ca^{2+}/10mM BAPTA buffer; pH 7.0. Mg^{2+}-free pipette solution contained Na_2ATP and EDTA instead of MgATP and BAPTA, respectively. The resting membrane potential of eosinophils measured in current clamp mode was around –60mV (Gordienko *et al.*, 1996). Increasing $[K^+]_o$ produced depolarization of the membrane, and in 100mM K^+ the membrane potential was –9.6 ± 0.8mV (n=5) (mean ± s.e.mean). When the cells were bathed in the standard external solution and the membrane potential was stepped for 0.5-1s from the holding potential of –60mV to more negative voltages, a sustained inward current emerged. The inward current was augmented when $[K^+]_o$ was raised. The reversal potential of the current closely followed the equilibrium potential of K^+ when $[K^+]_o$ was elevated, indicating a potassium current. Exposure to extracellular CsCl (≥ 10^{-6} M) produced a rapid voltage dependent block of the inward current. When $[K^+]_o$ was 100mM, addition of Cs^+ in concentrations in excess of 10^{-4} M revealed this effect at voltages negative to about –30mV (Figure 1A). In the voltage range positive to –30mV Cs^+ was without effect. Ba^{2+} (≥10^{-6} M) also blocked the inward current (Figure 1B). In standard external solution, the outward current recorded was small and of only 1.7 ± 0.2pA (n=7) in amplitude at +50mV. This rectification was partially relieved in cells that were dialysed with a pipette solution free of Mg^{2+}. The outward current was increased 3 fold at –30mV (n=7) and 4 fold at +50mV (n=7). This study reveals the existence of inward rectifier K^+ channels in human eosinophils. These channels are most likely responsible for the maintenance of the resting membrane potential in these cells.

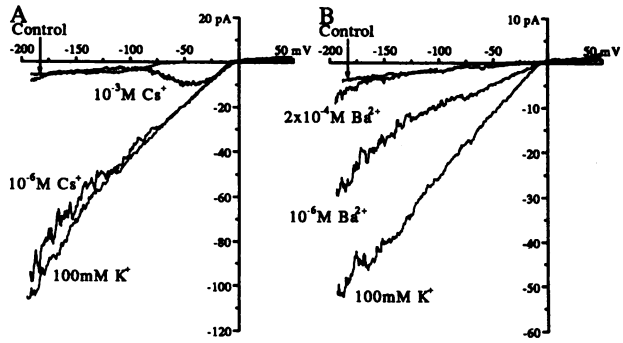

Figure 1. Whole cell voltage clamp recordings from two different eosinophils. The families of averaged current voltage relationships were derived from ramps. The voltage was ramped from –200mV to +50mV at 0.83V/s. Inhibition of the inward rectifier current by caesium (A) and barium (B) is shown in 100mM KCl solution.

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Gordienko D.V., Tare M., Parveen S., Fenech C.J., Robinson, C. & Bolton, T.B. (1996) *J. Physiol.* 496, 299-316.

16P INVESTIGATION OF THE HAEMODYNAMIC EFFECTS OF THE SELECTIVE ADENOSINE A_3 RECEPTOR AGONIST IODOBENZYL-5-N-METHYL CARBOXAMIDOADENOSINE, IB-MECA, IN ANAESTHETIZED CATS

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In rats, activation of adenosine A_3 receptors has been demonstrated to cause hypotension (Carruthers & Fozard, 1993; Patel et al., 1994). Using the selective adenosine A_3 agonist IB-MECA (Gallo-Rodriguez et al., 1994) the present experiments were carried out to investigate if this receptor had a similar function in another species, the cat. In addition, regional haemodynamic variables were also monitored to determine their relative contribution to the hypotensive response observed.

Female cats (2.3- 3.1 kg) were anaesthetized (i.v.) with a mixture of α-chloralose (70 mg kg^{-1}) and pentobarbitone sodium (6 mg kg^{-1}) and artificially ventilated after neuromuscular blockade with vecuronium bromide (200 μg kg^{-1}). Body temperature, blood gases and pH were maintained within the normal physiological range and blood pressure and heart rate were recorded as previously described (Corder et al.,1986). Drugs were given via the jugular vein. Cardiac output (CO) was measured by placing an electromagnetic flow probe (Gould Statham) around the aortic root after performing a thoracotomy. Blood flow in the left renal and mesenteric arteries was also recorded via a retroperitoneal approach using electromagnetic flow probes. From these measurements renal (RAR) and mesenteric (MAR) arterial resistances along with total peripheral resistance (TPR) were derived. A cumulative i.v. dose response curve for IB-MECA (3-300 μg kg^{-1}) was constructed alone (n=4) and in the presence of the adenosine A_1/A_2 receptor antagonists CGS 15943A (3 mg kg^{-1}, i.v., n=4; Ghai et al., 1987) or 8-(p-sulfophenyl)theophylline (8-sPT; 20 mg kg^{-1}, i.v. n=4; Evoniuk et al., 1987). Changes were compared with time-matched saline controls (n=4) in the presence of 20 μl kg^{-1} DMSO by two-way ANOVA and the least significant difference test was used to compare the means.

IB-MECA only caused significant ($P<0.05$) falls in mean blood pressure of 20±6 & 35±6 mmHg at 100 and 300 μg kg^{-1}, respectively. These changes were associated with significant decreases in CO of 14±10 ml min^{-1} kg^{-1} at 100 μg kg^{-1}, TPR of 0.136±0.087 mmHg min ml^{-1} at 300 μg kg^{-1} and RAR of 0.58± 0.21 and 0.93±0.23 at 100 & 300 μg kg^{-1}, respectively. There were no associated changes in HR or MAR. In the presence of CGS 15943A, IB-MECA caused only a significant fall in CO, of 17±6 ml min^{-1} kg^{-1}, at the highest dose of 300 μg kg^{-1}. In the presence of 8-sPT, IB-MECA also caused a decrease in CO, reaching 17±2 ml min^{-1} kg^{-1} at 300 μg kg^{-1}.

The present data indicate that the majority of haemodynamic effects caused by IB-MECA in the cat can be attributed to activation of adenosine A_1 & A_2 receptors except for the decrease in CO. This decrease in CO could, therefore, be attributed to activation of adenosine A_3-like receptors.

Carruthers, A.M. & Fozard, J.R. (1993) *Eur. J. Pharmacol.* 250, 185-188.
Corder, R., Lowry, P.J., Wilkinson, S.J. & Ramage, A.G. (1986) *Eur. J. Pharmacol.* 121, 25-30.
Evoniuk, G. Von Borstel, R.W. & Wurtman, R.J. (1987) *J. Pharmacol. Exp. Ther.* 240, 428-432.
Gallo-Rodriguez, C., Ji, X., Merman, N. et al. (1994) *J. Med. Chem.* 37, 636-646.
Ghai, G., Francise, J.E., Williams, M. et al. (1987) *J. Pharmacol. Exp. Ther.* 242, 784-789.
Patel, M., Sheehan, M.J. & Strong, P. (1994) *Br. J. Pharmacol.* 113, 741-748.

17P ADENOSINE RECEPTORS IN THE BASILAR ARTERY OF THE RAT

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Adenosine causes relaxation of rat cerebral vessels (Haberl *et al*, 1990) but the receptor involved has not been characterised. Here, selective adenosine receptor antagonists and agonists have been used to try and identify the receptors responsible for adenosine-mediated relaxation in this artery.

Segments of basilar artery (2.0 mm long; normalised internal diameter 200-400 μm) from male Wistar rats (250-450 g) were mounted on 40 μm wire in a small vessel myograph (JP Trading, Aarhus, Denmark) for isometric tension recording. The vessel was bathed with physiological salt solution (composition, mM: NaCl 115.3, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5, glucose 11.1) equilibrated with 95% O_2/5% CO_2 at 37°C. Vessels were equilibrated for 60 min before normalisation (Mulvany & Halpern, 1977). After a further 30 min, vessels were precontracted with a submaximal concentration of KCl (40 mM) for determination of cumulative concentration-relaxation curves to adenosine receptor agonists. Responses were measured as changes in tension and expressed as percentage relaxations of KCl-induced tone. Where an agonist did not give clearly defined maximal responses (E_{max}) at the at highest concentration that could be used, the potency is expressed as the mean concentration giving 50% relaxation of the induced tone ($EC_{50\%}$).

5'-N-ethylcarboxamidoadenosine (NECA; Sigma Chemical Co., Poole) was the most potent of the adenosine agonists studied, but its log concentration response curve was biphasic with a maximum relaxation of 97.7±0.1% at 3 mM, the highest concentration used ($EC_{50\%}$ = 141±19.2 μM). R(-)N^6-(2-phenylisopropyl)adenosine (R-PIA; RBI, Natick, MA, USA) and aminophenyl N'-ethyl adenosine (APNEA; RBI) had similar activity ($EC_{50\%}$ values of 79.9±16.8 μM and 388±139 μM, respectively) but did not completely relax the preconstriction (maximal relaxations recorded were 74.7±3.1% and 66.2±8.0%, respectively, both at 300 μM) in the concentration range used (0.1-300 μM). In contrast, 2-[p-(carboxyethyl)phenylethylamine] - 5 - N - ethylcarboxamido-adenosine (CGS 21680, RBI) had an E_{max} of only 8.12±0.45% (EC_{50} = 534±87.1 nM). Adenosine (Sigma) also produced little relaxation with the largest response being 25.5±3.6% at 3 mM, making it the least potent of the agonists tested. At concentrations ≥0.1 mM, adenosine generated biphasic responses; relaxation followed by contraction.

Addition of 8-phenyltheophylline (8-PT; 10 μM; Sigma), an A_1 and A_2 receptor antagonist, antagonised the responses to NECA in the lower concentration range (≤10 μM) The curve was shifted ~30-fold suggesting that NECA mediates relaxation through A_2 receptors at these concentrations. This was confirmed by showing that a selective A_{2a} receptor antagonist, 8-(3-chlorostyryl)caffeine (CSC; 500 nM; RBI), also antagonised responses to these concentrations of NECA. In contrast, responses to higher NECA concentrations were augmented by 8-PT and CSC. Responses to R-PIA were unaffected by 8-PT (10 μM), suggesting the possible presence of A_3 receptors which are considered xanthine-insensitive. However, 10 μM 8-PT shifted the response curve for APNEA, an A_3-selective agonist, rightwards by 30-fold. The A_1-antagonist, 8-cyclopentyl-1,3-dipropylxanthine (10 nM; RBI) did not affect the responses to APNEA.

These results suggest the presence of A_{2a} receptors and another, as yet undefined, site which is resistant to blockade by 8-PT. NECA activates both sites whereas R-PIA apparently activates only the undefined site in a way similar to findings in the rat mesenteric artery (Prentice *et al.*, 1996). It seems unlikely that, under the conditions used, the A_1 receptor mediates any relaxation to these agonists in the rat basilar artery.

HJD is a Medical Research Council Research Student.

Haberl, R.L., Anneser, F., Villringer, A. & Einhäupl, K.M. (1990). *Am. J. Physiol.*, 258, H1840-H1846.
Mulvany, M.J. & Halpern, W. (1977). *Circ. Res.*, 41, 19-26.
Prentice, D.J., Payne, S.L. & Hourani, S.M.O. (1996). *Drug Dev. Res.*, 162.

18P ACTIVATION OF ADENOSINE RECEPTORS INCREASES RAT MESENTERIC AFFERENT NERVE DISCHARGE

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Adenosine is generally considered to be an endogenous inhibitory modulator of central and peripheral neurones (Barajas-Lopez *et al.*, 1991). Nevertheless, there is evidence to suggest that adenosine *stimulates* cutaneous afferent nerve fibres and carotid chemoreceptors (Bleehen *et al.*, 1976; McQueen & Ribeiro, 1986). Furthermore, *in vitro* studies have revealed that stimulation of adenosine receptors produces depolarisation and action potential discharge in enteric neurones (Katayama & Morita, 1989; Barajas-Lopez *et al.*, 1991). The purpose of the present study was to determine the effects of adenosine on intestinal afferent nerve fibre discharge *in vivo*.

Experiments were performed on sodium pentobarbitone-anaesthetised (60mg.kg^{-1}, i.p.) male or female Wistar rats (250-400g). Extracellular recordings were obtained from mesenteric afferent bundles supplying a section of mid-jejunum, which was cannulated to enable intraluminal pressure recording and the local administration of drugs. A jugular vein and carotid artery were cannulated for systemic administration of drugs and for continuous monitoring of haemodynamic parameters, respectively. Data are presented as the mean ±s.e.mean from 3-6 animals, unless otherwise stated.

Intravenous (i.v.) administration of adenosine (30μg-10mg.kg^{-1}) caused a dose-dependent increase in afferent nerve discharge within 5-15s (ED_{50} = 0.85±0.21mg.kg^{-1}), bradycardia (ED_{50} = 0.42±0.14mg.kg^{-1}) and hypotension (ED_{50} = 0.20±0.02mg.kg^{-1}). In addition, adenosine (0.3-10mg.kg^{-1}, i.v.) produced dose-dependent increases in intraluminal pressure, without a maximal response being reached. Luminal distensions with saline (1ml) produced larger increases in intraluminal pressure than observed with an ED_{100} dose of adenosine (1ml distension; 19±2 cmH_2O; adenosine 3mg.kg^{-1}; 6±2 cmH_2O; $P<0.001$ Student's unpaired *t*-test). However, the peak increase in afferent discharge (impulses per 1.25 second) associated with adenosine administration was significantly greater (1ml distension; 154±28 impulses; adenosine 3mg.kg^{-1}; 266±38 impulses; $P<0.05$). The effects of adenosine on blood pressure and heart rate were markedly inhibited by the A_1-receptor-selective antagonist, DPCPX (8-cyclopentyl-1,3-dipropyl-xanthine, 1mg.kg^{-1}, i.v), with mean geometric dose ratios (95% confidence limits) of 9.7(5.7-16.6) and 20.2(10.8-37.9), respectively. However, DPCPX had no antagonistic effect on adenosine-induced responses on luminal distension and afferent discharge. Afferent responses to i.v. bolus doses of CCK-8 (100ng) and 2-methyl-5-hydroxytryptamine (10μg) were also not affected by antagonist or vehicle (5% v/v dimethylsulphoxide, 5% v/v 1M NaOH in saline). Sodium nitroprusside (300ng-3mg.kg^{-1}, i.v.) produced a dose-dependent hypotension (ED_{50} = 6.28±0.83μg.kg^{-1}) but not a rapid activation (>60s) of afferent discharge.

These data indicate that adenosine stimulates mesenteric afferents by a mechanism that does not appear to be related to its haemodynamic effects or solely due to its effects on intraluminal pressure in the jejunum. Moreover, the A_1 receptor is unlikely to mediate this action. Experiments are in progress to characterise further the receptor subtype(s) involved and to determine whether or not adenosine stimulates afferent neuronal discharge in the gut directly.

Anthony J Kirkup is a GlaxoWellcome Research Fellow.

Barajas-Lopez, C., *et al.* (1991). *J. Pharmacol. Exp. Ther.*, **258**, 490-495.
Bleehen, T., Hobbiger, F. & Keele, C.A. (1976). *J. Physiol.*, **262**, 131-149.
Katayama, Y. & Morita, K. (1989). *J. Physiol.*, **408**, 373-390.
McQueen, D.S. & Ribeiro, J.S. (1986). *Br. J. Pharmacol.*, **88**, 615-620.

19P CARDIOPROTECTION FROM ISCHAEMIC INSULT IN ISOLATED WORKING HEARTS BY AN A_3 RECEPTOR AGONIST

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The stimulation of A_3 adenosine receptors is believed to have beneficial effects during ischaemia and reperfusion (Armstrong & Ganote 1994; Lui *et al.*, 1994) The purpose of this study was to investigate the action of adenosine receptor agonist IB-MECA (N^6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide) selective for A_3 receptors upon the myocardial and coronary vascular responses of the guinea-pig working heart preparation, in order to further understand the involvement of adenosine receptor activation in the attenuation of ischaemic/reperfusion injury.

Male Dunkin - Hartley guinea-pigs (300 - 380g) were anaesthetized by sodium thiopental followed by immediate thoracotomy. The heart, still beating, was excised and immersed into heparinized ice cold saline. The endothelium was removed by a 15 second blast of O_2 via the aorta. Both the aorta and the pulmonary artery were cannulated and Langendorff retrograde perfusion commenced with filtered modified Krebs - Henseleit buffer containing (mM): NaCl 118; KCl 4.7; $NaHCO_4$ 25; $MgCl_2$ 1.2; KH_2PO_4 1.2; glucose 11; pyruvate 0.5 and $CaCl_2$ 1.25. The perfusion was maintained at 37°C and gassed with a mixture of 95% O_2 - 5% CO_2 (pH 7.4) for 10 minutes. Perfusion was then switched to anterograde perfusion in the 'working mode', via the left atrium, at a filling pressure (preload) of 10cm of H_2O. The heart ejected against a hydrostatic pressure (after load) of 80cm H_2O. Global mechanical function was continuously recorded by a catheter-tipped manometer placed into the left ventricle. Left ventricular pressure (LVP); dP/dt max; dP/dt min; heart rate; aortic output (AO) and coronary flow (CF) were all determined. Results were expressed as mean percentage change from the pre-drug or pre-ischaemic values ± s.e.m. (n=5). Statistical significance was determined by Students' *t*-test, $P < 0.05$.

In the normoxic experiments, infusions of single concentrations of IB-MECA ($3x10^{-7}$M) were made for 40 minutes. Low flow ischaemia was induced by a reduction in cardiac afterload from 59 mm Hg to 7.8 mm Hg by adjustment of the Starling resistance. During the period of ischaemia, the coronary flow was reduced to approximately 10% of the pre-ischaemic flow rate. Reperfusion was then commenced and the parameters recorded for a further 20 min.

In normoxic conditions IB-MECA ($3x10^{-7}$M) produced no change in CF compared to the control (97.4 ± 3.6 and 97.1 ± 1.9%, respectively), the other parameters also remained close to control values.

When IB-MECA ($3x10^{-7}$M) was perfused commencing at 10 min into the 30 minute period of ischaemia, at reperfusion, there was a significant increase in CF (106.4 ± 8.5%) compared to the control (70.4 ± 3.2%). IB-MECA was also observed to accelerate the recovery of AO at the onset of reperfusion (23.8 ± 6.0 and 4.5 ± 4.5%, respectively). The other parameters remained similar to the control in the presence of IB-MECA under these conditions. When IB-MECA was perfused commencing at reperfusion, there was a significant increase in CF compared to the control (101.3 ± 7.41 and 70.4 ± 3.2%, respectively) at 10 min into reperfusion. IB-MECA also accelerated the recovery of AO at the onset of reperfusion (59.0 ± 8.0 and 4.5 ± 4.5%, respectively). The other parameters remained similar to the control in the presence of IB-MECA under these conditions.

Thus results demonstrate that A_3 receptor mediated responses are not observed under normoxic conditions, but reveal an effect at the onset of reperfusion after an ischaemic insult. It appears that activation of A_3 adenosine receptors enhances recovery after ischaemia and reperfusion, as measured by AO. The myocardial stunning is thereby attenuated, especially when IB-MECA is infused at the onset of reperfusion.

Armstrong S. & Ganote C.E. (1994) Cardiovasc. Res., 28, 1049-1056.
Liu G.S. *et al* (1994) Cardiovasc. Res., 28, 1057-1061.

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20P CARDIOVASCULAR PHENOTYPE OF TRANSGENIC MICE WITH DISTRUPTION OF BRADYKININ B_2 RECEPTOR

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To assess the role of the kallikrein-kinin system in the regulation of blood pressure (BP), we studied the cardiovascular phenotype of a transgenic mouse strain ($Bk2r^{(-/-)}$) in which the bradykinin (BK)-B_2 receptor gene had been knocked out by homologous recombination (Barkowski et al., 1995). Statistical analysis was performed by ANOVA. In conscious male $Bk2r^{(-/-)}$ (25-30 g BW) the hypotensive response to intra-arterial BK (0.1 to 10 µg per mouse) was abolished. Basal systolic BP levels (tail-cuff pletysmography) were higher in conscious $Bk2r^{(-/-)}$ compared to controls c57 Bl/6 and J129 Sv mice (124±1 vs 99±1 and 109±1 mmHg, P<0.01, at least 22 mice in every group). Heart/body weight ratio was greater in $Bk2r^{(-/-)}$ compared to c57 Bl/6 and J129 Sv mice (505±10 vs 458±12 and 458±11 mg/100 g BW, P<0.05). Chronic Hoe 140 (50 µg/mouse per day for 20 days, s.c.), a selective BK-B_2 receptor antagonist, increased the BP of c57 Bl/6 and J129 Sv mice (from 101±1 to 128±3 and from 107±2 to 121±1 mmHg, respectively, P<0.05, n=7 each group), whereas it did not affect the BP of $Bk2r^{(-/-)}$. The administration of a non-peptidic antagonist of angiotensin II AT_1 receptor, 2-(N-propyl-N(2'-[-1H-tetrazol-5-yl] bihenyl 4yl) methyl] amino) pyridine-3 carboxylic acid (A-81988, Abbott Laboratories), at the dose of 200 µg/day for 10 days *per os*, reduced the BP of $Bk2r^{(-/-)}$ (130 ±2 to 109 ±1 mmHg (P<0.01, n=7 each group), while it was ineffective in controls.

The BP response to dietary salt loading (0.84 mmol/g chow) for 15 days was enhanced in $Bk2r^{(-/-)}$ (30 ±3 vs 18 ±3 and 1±1 mmHg, P<0.05, n=8 each group,). At the end of the treatment, direct BP was measured in conscious mice in which, one day in advance, a polyethylene catheter had been inserted into the thoracic aorta through the left carotid artery under the effect of the anaesthetic 2,2,2-tribromoethanol (88 mmol/100g BW, i.p.). Higher direct BP levels were detected in $Bk2r^{(-/-)}$(156±3 vs 113±4 and 119±2 mmHg in c57 Bl/6 and J129 Sv mice respectively).

These data indicate that disruption of BK-B_2 receptor gene results in increased basal BP (possibly due to unbalanced prevalence of vasoconstrictor angiotensin II) and enhanced sensitivity to salt loading. This suggests that the kallikrein-kinin system plays a role in the regulation of BP under basal conditions and during alterations of sodium homeostasis.

Barowski J.A., Ranson R.W., Seabrook G.R., et al. (1995). *J. Biol. Chem.*, 270, 13706-13710.

21P SEXUAL DIMORPHISM OF BLOOD PRESSURE RESPONSE TO BRADYKININ

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Tissue kallikrein is over-expressed in the kidney of female rats, this sexual dimorphism being associated with a greater effect of early blockade of bradykinin (BK) B_2-receptors on female blood pressure (BP) phenotype (Madeddu et al., 1996). We studied the effects of ovariectomy (OX) and oestrogen replacement (oestradiol benzoate, 50 μg kg^{-1}, s.c., every two days, for two weeks) on the mean BP (MBP) responses to intra-arterial injection of graded doses of BK (150-900 ng kg^{-1}) and on the expression of BK B_2-receptors in Wistar rats (250-300 g BW). For MBP measurement, a polyethylene catheter was inserted into the left femoral artery of rats anaesthetised with ether. For drug injection, another catheter was inserted into the thoracic aorta via the left carotid artery. Both catheters were tunnelled under the skin and exteriorised at the back of the neck. The following day, direct MBP was recorded in conscious rats. Statistical analysis was performed by ANOVA. The MBP lowering effect of BK was by 20 to 25% greater in female compared to male rats. The effects of OX and oestrogen replacement on MBP responses to BK are expressed in Table 1.

Table 1

BK (ng kg^{-1})	Controls		OX		OX+Estrogen	
	D	P	D	P	D	P
150	3±1	0±1	2±1*	1±1*	4±1	0±1
450	12±1	0±1	4±1*	5±1*	6±1*	1±1*
900	23±2	0±1	7±2*	5±2*	22±1	2±1*

Absolute changes±s.e.m. represent the area over (Depressor, D) or under (Pressor, P) the MBP curve,*P<0.05 vs Controls. (n=6).

OX reduced the magnitude of the vasodepressor response to BK and unmasked a secondary vasopressor effect. OX also reduced the magnitude of the vasodepressor response to intra-arterial sodium nitroprusside (3-18 μg kg^{-1}), acetylcholine (30-600 ng kg^{-1}), $desArg^9$-BK (150-900 ng kg^{-1}) or prostaglandin E_2 (30-600 ng kg^{-1}) (P<0.05, n=6 per group). Oestrogen replacement restored to normal the vasodepressor response to acetylcholine, $desArg^9$-BK, and prostaglandin E_2, but not that to sodium nitroprusside.
RT-PCR Southern blot analysis (n=3) showed higher levels of mRNA in the kidney, adrenal gland, thoracic aorta, mesenteric artery and left ventricle of female compared to male rats. After OX, the ratio of BK B_2-receptor to β-actin expression was decreased in the aorta and kidney by 24 and 54%, respectively (P<0.05) and it was restored to normal levels by oestradiol. Neither OX nor oestradiol affected the expression of receptors in the heart and uterus. These results indicate that the BP response to BK is influenced by oestrogen, through an effect on BK B_2-receptor gene expression. Since kinins exert a cardiovascular protective action, reduction in their vasodilator activity after menopause could result in an increased risk of pathological events. The beneficial effects of oestrogen replacement might be mediated by an increase in the activity of the kallikrein-kinin system.

Madeddu P., Pinna Parpaglia P., Anania V., et al. (1996), *Hypertension*, 27, 746-51.

22P LOCALLY-ACTING DILTIAZEM, BUT NOT CAPTOPRIL, INCREASES MICROVASCULAR OEDEMA FORMATION

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Heart failure can deteriorate with the administration of calcium channel blockers and improve with angiotensin converting enzyme inhibitor (ACEI) treatment (Packer et al. 1989). We tested the hypothesis that a calcium channel blocker (diltiazem) and a ACEI (captopril) may differ in their effect on local microvascular oedema formation.

The effect of these drugs was examined on the formation of oedema in the skin of male Wistar rats (200-250 g). The animals were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and injected i.v. with ^{125}I-albumin (0.1 MBq/kg). Test agents were injected i.d. in the shaved skin in 0.1 ml volumes according to a balanced site pattern. After 30 min the animals were sacrificed. The injected sites were excised and their ^{125}I-albumin content measured γ-counter. Oedema formation is expressed as μl of per site (mean±SEM).

Diltiazem caused a dose-related increase in plasma albumin leakage (n=6, p<0.05). In rat dorsal skin control (saline) and diltiazem 10^{-9}, 10^{-8}, and $10^{-7.5}$ moles/site caused 14.5±2.0, 15.5±2.6, 19.5±3.2 and 30.0±7.3 μl of albumin leakage per site
respectively. In rat abdominal skin the response to saline, diltiazem 10^{-9}, 10^{-8}, and $10^{-7.5}$ moles/site was 6.9±1.0, 9.0±2.0, 11.0±2.1, and 18.0±2.5 μl respectively (n=6, p<0.05).

In contrast, there was a slight, non-significant, reduction in plasma leakage with captopril. Albumin leakage in rat dorsal skin being 20.0±1.4, 19.5±2.5, 17.0±2.5, and 17.0±1.4 μl respectively (n=9) with saline, captopril 10^{-10}, captopril 10^{-9} and captopril 10^{-8} moles/site. The doses of captopril chosen have been shown previously to cause microvascular vasodilatation (Warren & Loi, 1995).

Although both diltiazem and captopril are vasodilators, they differ in their effect on local oedema formation.

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Packer, M. (1989) Circulation. 80 (suppl IV), 59-67.
Warren, J.B. & Loi, R.K. (1995) FASEB J. 9, 411-418.

23P NIFEDIPINE-INDUCED OEDEMA AND THE ROLE OF MICROVASCULAR VASODILATION

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The calcium channel antagonist nifedipine (nifed) can worsen cardiac oedema and this may be in part via a direct action on the microcirculation. We investigated the effect of locally injected nifed on oedema formation in rat and rabbit skin and assessed the contribution of microvascular vasodilatation to this response.

Male Wistar rats (200-250 g) and New Zealand rabbits (2.5-3.0 kg) were anaesthetized with sodium pentobarbitone (50 mg/kg) and injected i.v. with ^{125}I- albumin. The agents were injected i.d. at 0.1 ml/site according to a balanced site pattern. After 30 min the animals were sacrificed. The injected skin sites were excised and their ^{125}I-albumin content measured by γ-counter. Oedema formation is expressed as µl of plasma per site (mean±SEM).

Nifed caused dose related oedema in rabbit skin from 5.9±0.6 µl in control sites to 6.1±0.9, 7.8±1.0, and 10.0±2.0 µl at 10^{-9}, 10^{-8}, and $10^{-7.2}$ moles/site. PGE_2, a potent microvascular vasodilator, caused similar increases in oedema to 5.9±0.6, 7.0±0.8, and 9.6 ±1.8 µl at 10^{-12}, 10^{-11}, and 10^{-10} moles/site.

At the above concentrations PGE_2 had a greater effect than nifed on rabbit skin microvascular blood flow measured by laser Doppler (Warren et al., 1994). PGE_2 (10^{-12}, 10^{-11}, and 10^{-10} moles/site) increased blood flow by 3±13, 41±13, and 106±5% respectively. Nifed (10^{-9}, 10^{-8}, and$10^{-7.2}$ moles/site) increased blood flow by 17±8, 37±12, and 57±14% respectively suggesting that its effect on oedema cannot be explained by vasodilatation alone (n=6, $p<0.05$ compared to PGE_2).

The effect of nifed was compared with bradykinin (BK) in rat abdominal skin. Nifed ($10^{-7.2}$ moles/site) increased plasma leakage from 3.9±0.6 µl in control sites to 9.8±1.7 µl (n=6, $p<0.05$). The response to BK (10^{-10} moles/site) was higher at 14.5±2.0 µl/site.

The contribution of vasodilatation to the oedemagen effect of nifed was investigated further by coinjecting nifed with BK to assess for potentiation. Potentiation of the BK 10^{-10} moles/site response was greater with PGE_2 10^{-10} moles/site (30.5±5.0 to 76.0±11.5 µl, (n=6, $p<0.05$) than coinjection with nifed (26.0±2.0 to 31.0±5.0 µl). Similarly, the potentiation of the histamine 10^{-8} moles/site response (n=6) although significant, was smaller with nifed $10^{-7.2}$ moles/site (24.5±6.0 to 39.5±10.0 µl) than with PGE_2 10^{-10} (15.0±2.3 to 50.0±11.0 µl).

Nifed acts locally on the microcirculation to increase oedema formation in both rabbit and rat skin. Comparing nifed with locally injected PGE_2 in their effect on oedema, microvasular blood flow, and their ability to potentiate oedemagens suggests nifed has combined oedemagen and vasodilator activity.

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Warren, J.B. et al. (1994) Am. J. Physiol. 266, H1846-1853

24P ATTENUATION OF THE NEGATIVE INOTROPIC EFFECTS OF CHLOROQUINE BY COMBINED ADMINISTRATION OF DIAZEPAM AND ADRENALINE IN ANAESTHETIZED RATS

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Diazepam, combined with early mechanical ventilation and administration of adrenaline, is recommended for the treatment of chloroquine-induced cardiotoxicity (Riou *et al.*, 1988). However, diazepam *per se* failed to reverse the cardiotoxic effects of chloroquine in an anaesthetized rat model (Hughes & Coker, 1996). The present study was designed to investigate the effects of adrenaline and diazepam, alone and in combination, in chloroquine intoxicated, anaesthetized rats.

Male Wistar rats (290 to 375 g) were anaesthetized with sodium pentobarbitone, 60 mg kg^{-1} i.p. A femoral artery and vein were cannulated for blood pressure monitoring and drug administration respectively. Left ventricular pressure (LVP) and a lead II ECG were also monitored. A thoracotomy was performed to facilitate ventilation with air, and arterial blood gases were maintained within normal values. Following a 30 min stablisation period, chloroquine was infused at a rate of 1 mg kg^{-1} min^{-1} i.v. to all rats for 60 min. Treatment intervention was initiated after 30 min of chloroquine infusion. Rats were allocated randomly to one of four groups of n=6: 1) controls - which received diazepam vehicle (DV - ethanol:PEG 300:saline, 1:1:3) 1 ml kg^{-1} and adrenaline vehicle (AV - saline with 1 µg ml^{-1} ascorbic acid) 0.1 ml kg^{-1} min^{-1}; 2) adrenaline - 0.3 µg kg^{-1} min^{-1} + DV; 3) diazepam - 2 mg kg^{-1} + AV; 4) both drugs.

After 30 min of chloroquine infusion, heart rate was reduced from 373 ± 13 to 237 ± 12 beats min^{-1}, mean arterial pressure (BP) from 88 ± 6 to 56 ± 4 mmHg and QRS duration was increased from 29 ± 1 to 33 ± 1 ms in controls. Similar changes were observed in the other three groups. After intervention, there were no differences in these parameters, apart from BP being transiently higher in the adrenaline group than the diazepam group. An index of cardiac contractility, $LV(dP/dt)_{max}$ P^{-1}, was also reduced by chloroquine (Figure 1). Diazepam alone did not alter this negative inotropic response to chloroquine. Similarly, adrenaline failed to reverse the effects of chloroquine when compared to the vehicle group. However, combined administration of adrenaline and diazepam caused a significant improvement of the inotropic status.

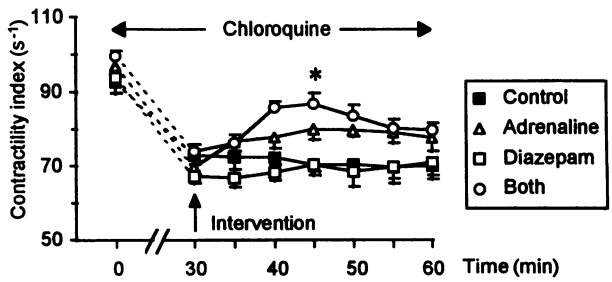

Figure 1. The effects of diazepam, adrenaline or both on the contractility index. Values are mean ± s.e.mean. *P<0.05, Kruskal-Wallis test comparing area under the curve (30 - 60 min) of each intervention with control.

The results suggest that an interaction between diazepam and adrenaline on cardiac contractility may be responsible for reversal of chloroquine-induced toxicity.

DAH received a Wellcome Trust Toxicology studentship.

Hughes, D.A. & Coker, S.J., (1996). *Br. J. Pharmacol.*, 113, 152P.
Riou, B., Barriot, P. *et al.*, (1988). *N. Engl. J. Med.*, 318:1-6.

25P EVIDENCE THAT CENTRAL 5-HT_{1A} RECEPTORS ARE INVOLVED IN THE VAGAL BRADYCARDIA EVOKED BY AORTIC NERVE STIMULATION IN ANAESTHETIZED RABBITS

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Activation of central 5-HT_{1A} receptors potentiates the reflex excitation of cardiac vagal preganglionic neurones (CVPNs) by cardiopulmonary (Bogle et al., 1990) and upper airway afferent stimulation (Futuro-Neto et al., 1993; Dando et al., 1996). CVPNs are also reflexly excited by baroreceptor and chemoreceptor afferents (see Daly 1986). A method of mimicking changes in baroreceptor input is to electrically stimulate the central end of the aortic nerve, which in rabbits contains only baroreceptor afferents (Neil et al., 1949). Experiments were carried out to investigate the effect of the 5-HT_{1A} receptor agonist buspirone, applied i.c. on the cardiovascular effects of electrically activating the aortic nerve and stimulating cardiopulmonary afferents with phenylbiguanide (PBG).

Male rabbits (1.8-3.0 kg) were anaesthetized with urethane (1.5 g kg^{-1}; i.v.) and pretreated with atenolol (1 mg kg^{-1}; i.v.). The animals were allowed to spontaneously breath O_2 enriched air. Simultaneous recordings were made of blood pressure, renal (RNA) and phrenic nerve activity (PNA). ECG was also recorded from which changes in R-R interval were calculated and arterial blood gases and pH were monitored. The left aortic nerve was identified, isolated and placed on a stimulating electrode. The aortic nerve was stimulated (pulse width 1 ms; 5-7.5 V; duration 5 s) at six frequencies (5, 10, 20, 40, 80 and 160 Hz) at 120 s intervals over a 10 min period. 150s after the last aortic nerve stimulation PBG was injected into the right atrium. A dose PBG of between 5-20 μg kg^{-1} was chosen so as to evoke a sub-maximal bradycardia. 150 s later this cycle was repeated and then 150 s after this cycle buspirone (200 μg kg^{-1}) or saline were given i.c. (20 μl over 20s) and the cycle repeated 5 min later starting with PBG. Effect of buspirone (n = 5) on changes caused by these stimulations were compared with that of saline (n = 5) by three (aortic) and two (PBG) -way ANOVA. The least significant difference test was used to compare the means. All values are means ± s.e.mean.

Buspirone caused a significant ($P<0.05$) fall in resting blood pressure (11 ± 2 mmHg) and an increase in R-R interval (41 ± 9 ms). The increase in R-R interval caused by the first set of aortic nerve stimulations was significantly ($P<0.01$) potentiated at the frequencies of 40, 80 and 160 Hz by 60 ± 31, 67 ± 37 and 79 ± 36 ms respectively when compared to that of saline (n=5) -2 ± 3, -3 ± 6 and -2 ± 5 ms. A significant potentiation at these frequencies was still evident for the 2nd aortic stimulation cycle, 22 min 30s after buspirone but was absent after the 3rd (37 min 30s after buspirone). The PBG-evoked increase in R-R interval was significantly potentiated by 113 ± 60 ms compared to -7 ± 5 ms after saline by the second cycle (20 min after buspirone). The hypotension associated with these frequencies of aortic nerve stimulation was reduced as was that evoked by PBG.

These results confirm that central 5-HT_{1A} receptors play a role in the reflex activation of CVPNs by cardiopulmonary receptor stimulation and also show that central 5-HT_{1A} receptors are involved in activation of CVPNs by aortic nerve stimulation.

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Bogle, R.G. et al. (1990) *Br. J. Pharmacol.* 100, 757-760.
Daly, M. de B. (1986) in *Handbook of Physiology* ed. Cherniack, N.S & Widdicombe, J.G. pp 529-594. Bethesda: American Physiological Society.
Dando, S.B. et al. (1996) *Br. J. Pharmacol.* 117, 48P.
Futuro-Neto, H.A. et al. (1993) *Brain Res.* 629, 349-354.
Neil, E. et al. (1949) *J. Physiol.* 109, 392-401.

26P DAMGO [H-Tyr-D-Ala-Gly-MePhe-NH(CH2)2)H] INHIBITS THE BRADYCARDIA ASSOCIATED WITH SEVERE HAEMORRHAGE IN THE ANAESTHETIZED RAT

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The cardiovascular response to a progressive simple haemorrhage is biphasic (e.g. Little *et al.*, 1989). The first phase comprises a tachycardia, increased peripheral vascular resistance while arterial blood pressure is maintained. The second phase includes a reflex bradycardia (vagal activation), fall in vascular resistance (sympatho-inhibition) and hypotension. The sympatho-inhibitory, vasodilator response can be prevented by μ, but not by δ or κ-opioid receptor agonists (Evans *et al.*, 1989). However, these latter studies were conducted in rabbits which did not show a bradycardia even in the absence of the opioid agonists. The aim of the present study was to determine the effect of specific μ (DAMGO), δ (DPDPE) and κ (U-50488H) opioid receptor agonists (see Evans *et al.*, 1989) on the bradycardia associated with severe haemorrhage.

Experiments were performed on 7 groups of male Wistar rats (238-259g). Stainless steel guide cannulae were implanted into the lateral cerebral ventricle (icv) under pentobarbitone anaesthesia (60mg.kg^{-1} ip) 7-15 days before the study. The study was conducted under alphadolone/alphaxalone (16-20 mg.kg^{-1}.h^{-1} iv) anaesthesia. The electrocardiograph was recorded via needle electrodes attached to the skin of the ventrum, blood pressure via the ventral tail artery and body temperature via a rectal probe. Body temperature was maintained constant at 38.0±0.0 °C (mean±s.e.mean) using external heating.

Groups I-IV received a progressive haemorrhage of 40% of total blood volume (BV; measured in separate animals as 6.06 ml.$100g^{-1}$ body weight), at 2% BV.min^{-1}. Group I (n=8) received 0.9% saline, Group II DAMGO (75ng, n=9) Group III DPDPE (3μg, n=8) and Group IV U-50488H (30μg, n=8), 20μl in 0.9% saline icv, administered after the loss of 5% BV. Groups V-VII respectively received DAMGO, DPDPE and U-50488H, but no haemorrhage. Group I displayed an initial tachycardia followed by bradycardia (heart period, HP, decreasing significantly [$P<0.05$, paired t-test] by 7±1ms after 12.2±2.0%BV loss from a control value of 133±3ms, followed by a significant rise of 55±7ms after a 33.0±2.1%BV loss). In Group II the tachycardia was still evident, however the bradycardia was absent since HP did not increase above the baseline of 149±5ms in this group. Furthermore the hypotension was delayed significantly in Group II since MAP did not fall significantly below the baseline of 98±3mmHg until 26.7±0.3%BV was lost (vs 20.2±0.2%BV in Group I, repeated measures analysis of variance, ANOVA). The pattern of response in Groups III and IV were not significantly different to Group I, while there were no significant changes in HP or MAP in Groups V-VII (ANOVA).

These results indicate that the bradycardic response to severe haemorrhage can be abolished, and confirm that the hypotensive response can be delayed by treatment with DAMGO, but not DPDPE or U-50488H.

Evans, R.G., Ludbrook, J. & Van Leeuwen, A.F. (1989) J. Physiol. 419,15-31.
Little, R.A., Marshall, H.W. & Kirkman, E. (1989) Q. J. exp Physiol. 74, 825-833.

27P CENTRAL EFFECTS OF THE 5-HT$_2$ RECEPTOR AGONIST 1-(2,5-DIMETHOXY-4-IODOPHENYL)-2-AMINOPROPANE (DOI) AND NMDA ON LEFT VENTRICULAR dP/dt max IN ANAESTHETIZED CATS

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Activation of central 5-HT$_2$ receptors by 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) at the level of the rostral ventrolateral medulla causes a rise in blood pressure but although heart rate does not change, the force of contraction increases (Mandel et al., 1990). However, in these experiments the increase in the force of contraction may be indirect due to the rise in blood pressure (von Anrep, 1912; Wallace et al., 1963). Therefore the present experiments have been carried out to re-examine the ability of DOI to selectively cause a change in the force of left ventricular contraction. In addition, hindlimb vascular resistance was measured to give an index of centrally induced changes in regional vascular resistance.

Male and female cats (2.5-3.9 kg) were anaesthetized (i.v.) with a mixture of α-chloralose (70 mg kg^{-1}) and pentobarbitone sodium (6 mg kg^{-1}) and artificially ventilated. Blood gases, pH and rectal temperature were monitored and maintained within the normal physiological range. Simultaneous recordings were made of arterial blood pressure, heart rate, left ventricular (LV) pressure, LV dP/dt max, respiratory movements and hindlimb perfusion pressure (limb perfused at a constant flow). Arterial blood pressure was maintained constant and the heart was paced electrically (see Ward et al., 1995). The ventral surface of the brain was exposed as described by Guertzenstein (1973). In two experiments the adrenal glands were tied off. Drugs were given i.v. or applied bilaterally to the rostral ventrolateral medulla (vlm) on 3 mm diameter pledgets of filter paper in a volume of 2.5 µl. For i.v. experiments the cats were pretreated with 2 mg kg^{-1} of the peripheral acting 5-HT$_2$ receptor antagonist BW501C67 (Ramage et al., 1993). For all variables statistical analysis was performed pre-and post-drug for i.v experiments by using a Student's paired t-test while an unpaired t-test was used to compare changes caused by saline with those by drugs for vlm experiments. All values are means ± s.e.mean.

DOI (i.v.; 0.3 mg kg^{-1}; n =6) caused a significant (P<0.05) increase in hindlimb perfusion pressure (P limb) from 126±6 to 165±10 mmHg but no change in LV dP/dt max (5,267±176 to 5,317±419 mmHg s^{-1}). DOI applied to vlm (30 µg per side; n=6) also caused a significant increase in P limb from 107±5 to 157±14 mmHg but no change in LV dP/dt max (5,567±176 to 5,800±286 mmHg s^{-1}). However, application of NMDA (10 µg per side; n=6) resulted in significant increases in both P limb (105±4 to 174±19) and LV dP/dt max (5,783±403 to 7,733± 733 mmHg s^{-1}) but no change in LV end-diastolic pressure. [NMDA had similar effects in two adrenalectomized animals.] NMDA applied after DOI still caused increases in LV dP/dt max and P limb, although the increase in LV dP/dt max was significantly reduced. DOI applied after NMDA resulted in a similar increase in P limb. Glycine (500 µg per side; n=7), after DOI and NMDA, caused significant decreases in LV dP/dt max of 629±195 mmHg s^{-1} and P limb of 35±14 mmHg.

These data indicate that activation of 5-HT$_2$ receptors at the level of the rostral ventrolateral medulla causes differential sympathoexcitation in that hindlimb vascular resistance is increased while left ventricular contractility is unaffected.

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Guertzenstein, P.G. (1973) *J. Physiol.* 229, 395-408.
Mandel, A.K. et al. (1990) *Circ. Res.* 67, 1267-1280.
Ramage, A.G. et al. (1993) *J. Auton. Nerv. Syst.* 42, 53-62.
von Anrep, G. (1912) *J. Physiol.* 45, 307-317.
Wallace, A.G. et al. (1963) *Am. J. Physiol.* 205, 30-36.
Ward, J. et al. (1995) *J. Physiol.* 489, 857-868.

28P THE EFFECT OF ELEVATED GLUCOSE CONCENTRATIONS ON VASCULAR REACTIVITY IN THE STREPTOZOTOCIN-INDUCED DIABETIC RAT

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Diabetes has been linked with alterations in the reactivity of blood vessels to neurotransmitters and circulating hormones (Christlieb *et al.*, 1976) and these factors may be involved in the underlying mechanisms of microvascular disease associated with diabetes. The aims of these experiments were to study the effects of elevated glucose concentrations on the vascular response to constrictors and endothelial-dependent and independent vasodilators in isolated perfused mesenteric arterial preparations from non-diabetic (ND) and streptozotocin-induced diabetic (DIA) rats.

On the day of surgery, age-matched (and initially weight matched) ND and DIA (streptozotocin: 70mg kg^{-1} i.v.; duration of diabetes approximately 4 weeks) Wistar Han rats were subdivided into two further groups in which the isolated mesenteric arterial bed preparations were exposed to either basal (8.3mM) (L) or elevated (32.5mM) (H) glucose perfusion *in vitro*. Concentration dose response curves were performed for noradrenaline (NA, 0.2-20µM); vasopressin (AVP, 0.5-20nM); the endothelial-dependent vasodilator acetylcholine (ACh, 1nM-100µM) and the endothelium-independent vasodilator glyceryl trinitrate (GTN, 1nM-1µM). Vasodilatation studies were performed following pre-constriction with NA (3µM).

At a glucose concentration of 8.3mM, MAX values for both constrictors and both vasodilators were significantly reduced in the DIA group compared to the ND group. At 32.5mM glucose, only the vasoconstrictor response to AVP was significantly lower in the DIA group than the ND group. Within the DIA groups, the higher glucose concentration resulted in significant enhancement of the MAX response for both the vasodilators ACh and GTN.

In conclusion, these results suggest that glucose is an important modulator of vascular responsiveness in both ND and DIA rat mesenteric arterial vessels.

Christlieb, A.R. (1976). *Diabetes*, 25, 820-825.

Table 1. Biochemical and Pharmacological analysis of data. Maximum response (MAX) to vasoconstrictors (mm/Hg) and vasodilators (% max dilatation from NA pre-constriction). values.

		Biochemical Analysis		Pharmacological Analysis			
Group	n	Plasma gl. conc mmol / L	Body Weight (g)	NA MAX	AVP MAX	ACh MAX	GTN MAX
ND (L)	8	8.9±0.4	329±16	157.6±6.0ϕ^{1}	134.1±12.3$\phi^{1,2}$	86.9±10.1ϕ	83.1±6.0ϕ^{1}
ND (H)	10	12.1±1.1	347±17	165.2±6.9ϕ^{2}	121.8±7.3$\phi^{3,4}$	68.3±8.4	98.0±5.2ϕ^{2}
DIA (L)	8	33.1±3.4*	280±16*	122.8±10.5$\phi^{1,2}$	87.5±8.4$\phi^{1,3}$	52.7±4.7ϕ+	64.3±4.0$\phi^{1,2}$+
DIA (H)	10	37.9±3.3*	276±15*	146.5±9.2	97.9±8.6$\phi^{2,4}$	74.1±8.5+	86.8±4.1+

Results for ND and DIA groups are given as mean±s.e.mean. P<0.001(*) for DIA body weights and plasma glucose concentrations vs. ND group. P<0.05 for significant differences in DIA groups (+) and ND groups vs. DIA groups (ϕ).

29P COMPARATIVE EFFECTS OF RILMENIDINE AND AMIODARONE ON NEUROGENIC ARRHYTHMIAS IN ANAESTHETIZED AND CONSCIOUS RABBITS

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Recent studies suggested that stimulation of central imidazoline receptors (IRs) can prevent epinephrine-induced arrhythmias in halothane-anaesthetized animals. It is likely that Rilmenidine (Ril): an antihypertensive agent that acts at IRs, may present antiarrythmic properties.
Therefore, we compared the effects of Ril and Amiodarone (Amio) on neurogenic arrhythmias, mean arterial pressure (MAP) and heart rate (HR) in halothane-anaesthetized and conscious rabbits (n=6) with electrodes chronically implanted into the posterior hypothalamus. Each animal was subjected to 8 randomized experiments and received i.v. injections of saline, Ril (150µg/kg), Amio (5mg/kg) and Tween-80 (Amio-vehicle). Three control hypothalamic electrical stimulations (HES) for 30s every 10 min, before and 4 HES every 20 min after i.v. injection, were performed.
We observed that : 1) in anaesthetized rabbits, control HES induced significant ($p<0.001$) hypertensive effects (59±2 to 76±2 mmHg) and cardiac arrhythmias characterized by an increase in HR (326±2 to 383±4 bpm), premature beats (PB) and ectopic beats (EB) amounts (75±5 and 17±1 respectively), delay (d) and duration (D) of arrhythmias (7±1 and 31±7 s respectively). Halothane-anaesthesia exerts significant ($p<0,01$) hypotensive (-22 %) and tachycardic (+32 %) effects but potentiated arrhythmias (PB= +49 %, d= +75 %).
2) Ril and Amio were equihypotensive ($p<0.05$) and equibradycardic ($p<0.01$) in anaesthetized animals (-15±1 and -12±1 mmHg; -33±4 and -34±3 bpm respectively). They did not antagonise HES-induced hypertension but Amio decreased HR during HES in anaesthetized ($p<0.001$) and conscious ($p<0.05$) rabbits (-68±8 and -40±13 bpm respectively).
3) Maximal anti-arrhythmic effect of drugs was observed 15 to 35 min after injection.
4) Ril produced a significant ($p<0.05$) decrease in PB (-35 %) and EB (-40 %) in anaesthetized but only in EB (-33 %) in conscious animals and significantly ($p<0.05$) increased d (+85 %) in anaesthetized animals. Amio produced a significant ($p<0.05$) decrease in PB (-99 %) and D (-45 %) in anaesthetized animals but, only in PB (-47 %) in conscious rabbits.

In conclusion, we showed that:
i) Ril preferentially prevent HES-induced arrhythmias in anaesthetized rabbits but to a lesser degree than Amio;
ii) in conscious animals, both Ril and Amio were antiarrhythmic but RIL preferentially reduced EB whereas AMIO decreased PB.
iii) the neurogenic component of arrhythmias is enhanced in conscious animals while the cardiac one is predominant in anaesthetized rabbits.

30P ENHANCEMENT OF METABOTROPIC GLUTAMATE RECEPTOR-STIMULATED PHOSPHOINOSITIDE SIGNALLING FOLLOWING PERTUSSIS TOXIN TREATMENT OF BABY HAMSTER KIDNEY CELLS EXPRESSING mGluR1α

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We have previously demonstrated that phosphoinositide signalling in baby hamster kidney cells expressing type 1α metabotropic glutamate receptors (BHK-mGluR1α) is enhanced following pre-treatment with pertussis toxin (PTx) leading to elevations of basal $InsP_1$ and $Ins(1,4,5)P_3$ levels (Carruthers *et al.*, 1996; Mistry *et al.*, 1996). In the present study we have further investigated basal and agonist-stimulated $InsP_1$ responses in control and PTx-treated BHK-mGluR1α in the presence of glutamic-pyruvic transaminase (GPT)/ pyruvate to assess whether L-glutamate release by BHK cells made any contribution to our previous observations.

BHK-mGluR1α cells (passage 3-20) were maintained as described previously (Carruthers *et al.*, 1996) and labelled with 1 µCi ml^{-1} [^{3}H]-inositol for 48 h. Treatment with PTx (100 ng ml^{-1}) was performed by addition 24 h before experimentation. Cells were washed in oxygenated Krebs-Henseleit buffer (KHB) and incubated in the presence of GPT; (3 U ml^{-1})/pyruvate (5 mM) and LiCl (10 mM) for 30 min prior to agonist challenge. Where indicated, antagonist additions were made 15 min before agonist. Cell monolayers were incubated at 37°C for 30 min after agonist addition and experiments were terminated with trichloroacetic acid. [^{3}H]-$InsP_1$ was resolved from neutral cell extracts by ion-exchange chromatography. Data are presented as means ± s.e.mean for 'n' observations.

PTx pre-treatment of BHK-mGluR1α caused an 11.7 fold increase in basal [^{3}H]-$InsP_1$ levels (-PTx, 393 ± 82; +PTx, 5007 ± 640 d.p.m. well^{-1}; n=5). However, incubation of PTx-treated cells in the presence of 3 U ml^{-1} GPT/pyruvate fully reversed the toxin-induced effect (402 ± 12 d.p.m. well^{-1}; n=3). GPT had no effect on basal [^{3}H]-$InsP_1$ accumulation in control cells. The concentration-dependencies of quisqualate and **1S,3R**-ACPD were investigated in control and PTx-treated cells in the presence of GPT (3 U ml^{-1}) + pyruvate. Under these conditions, toxin pre-treatment significantly increased the maximal response to quisqualate by 75 ± 9% and decreased the EC_{50} for this agonist by 65 fold (-log EC_{50} (M): -PTx, 5.45 ± 0.07; +PTx, 7.26 ± 0.23; n=4). Similarly, for **1S,3R**-ACPD the maximal response was increased 3 fold and the EC_{50} decreased ~10 fold (-log EC_{50} (M): -PTx, 3.82 ± 0.42; +PTx, 4.73 ± 0.27; n=4) in PTx-treated cells. The presence of the mGluR antagonist (**S**)-4-carboxy-3-hydroxy-phenylglycine (4C3HPG; 300 µM) elicited similar parallel rightward shifts for quisqualate-stimulated [^{3}H]-$InsP_1$ concentration-response curves in both control and PTx-treated cells (-log EC_{50} (M): control, 5.44 ± 0.07; +4C3HPG, 4.46 ± 0.09; +PTx, 6.81 ± 0.16; +PTx+4C3HPG, 5.73 ± 0.13), yielding K_d values for 4C3HPG of 35 and 26 µM for control and PTx-treated cells respectively.

Thus, PTx pre-treatment results in a dramatic sensitization of the phosphoinositide response evoked by agonists and can account for the apparent basal activation of mGluR1α in PTx-treated cells, via endogenous glutamate release. The changes in the concentration-effect curves for the mGluR agonists are consistent with a model in which mGluR1α associates with PTx-sensitive inhibitory ($G_{i/o}$) and -insensitive stimulatory ($G_{q/11}$) G proteins linked to PLC activity.

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Carruthers, A.M., Mistry, M., Nahorski, S.R. & Challiss, R.A.J. (1996) Br. J. Pharmacol. **118**, 137P
Mistry, R., Carruthers, A.M., Nahorski, S.R. & Challiss, R.A.J. (1996) Br. J. Pharmacol. **119**, 4P

31P EFFECTS OF TYPE I AND II METABOTROPIC GLUTAMATE RECEPTOR AGONISTS AND ANTAGONISTS ON PAIRED PULSE DEPRESSION IN THE RAT DENTATE GYRUS *IN VITRO*

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Field potentials evoked in the medial perforant path of the hippocampus exhibit paired pulse depression (PPD), the extent of which is partially dependent on the interstimulus interval (ISI). A triphasic pattern of a large depression at short intervals (10-50ms), a reduction of this depression at intermediate intervals (50-200ms) and again a large depression at late intervals (>200ms) is observed (Kahle & Cotman, 1993). We have previously demonstrated to the society that metabotropic glutamate receptors (mGluRs) modulate PPD in this pathway although the specific subtypes involved remain unknown (Cassidy & O'Connor, 1995). In the present study we have investigated the effect of group I and II mGluR agonists and antagonists on PPD in the medial perforant pathway of the rat dentate gyrus *in vitro*.

Transverse hippocampal slices were prepared from male Wistar rats (50-150g) by standard methods. Briefly slices were equilibrated for at least 1 hour in a perfusion chamber at 31-33°C, pH 7.3-7.4, in oxygenated artificial cerebrospinal fluid (composition in mM: NaCl, 120; KCl, 2.5; $MgSO_4$, 2.0; $CaCl_2$, 2.0; $NaHCO_3$, 26; NaH_2PO_4, 1.25; D-Glucose, 10). A monopolar glass stimulating electrode was placed in the middle one-third of the molecular layer of the dentate gyrus to evoke field excitatory post-synaptic potentials (EPSPs) in the proximal medial perforant pathway every 20s. Paired pulses were applied at ISIs of 10, 100 and 500ms. Recordings were analysed off-line using the Strathclyde electrophysiology software (Dr J Dempster). All drugs were bath applied onto the slice for at least 30min. All values are given as the mean±sem.

In control slices the second synaptic response was consistently smaller than the first at all ISIs tested (e.g. by 22.1±3.5% and 21.1±1.0% at 10 and 500ms ISI respectively; n=28). All mGluR agonists and antagonists tested were found to have no effect on late phase PPD. The group I mGluR agonist (S)-3,5-dihydrophenyl glycine (DHPG; 20μM) caused an 8.0±1.0% reduction in baseline EPSP amplitude but was without effect on PPD at any ISI (n=6). The group I antagonist (S)-4-carboxyphenylglycine (4-CPG; 200μM), applied on its own reduced baseline EPSP amplitude by 20.1±1.1% (n=5; P<0.05) and attenuated PPD at early phase intervals only (from 38.4±4.4% to 8.9±4.1% PPD at 10ms ISI; n=5; P<0.05). Application of the mGluR group II agonist 2S,3S,4S-α-carboxy-cyclopropyl-glycine (CCG-1; 20μM), had no effect on baseline EPSP amplitude but significantly attenuated PPD at early and intermediate ISIs (e.g. at 10ms ISI from 28.0±4.2% to 7.7±4.9%; n=6; P<0.05). The group II antagonist (RS)-a-methylserine-O-phosphate monophenyl ester (MSOPPE; 200μM) had an inhibitory effect on baseline EPSP amplitude when applied on its own but had no significant effect on PPD (n=5). Interestingly co-application of MSOPPE and CCG-1 reduced baseline EPSP amplitude by 98.2±0.2% (n=5; P<0.001). Increasing test stimulus levels during co-application revealed a paired pulse facilitation (PPF) at both early and intermediate phases (e.g. form 9.1±1.2% PPD to 22.2±5.1% PPF at 100ms ISI; n=5; P<0.01). These results specifically demonstrate the involvement of group II mGluRs in the modulation of PPD in the medial perforant pathway.

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Kahle, J.S. & Cotman, C.W. (1993) J. Pharmacol. Exp. Ther. 266, 207-215.
Cassidy, E.M. & O'Connor, J.J. (1995) Br. J. Pharmacol. 116, 106P.

32P PHARMACOLOGICAL, ELECTROPHYSIOLOGICAL AND IMMUNOHISTOCHEMICAL CHARACTERISTICS OF DISSOCIATED RAT CEREBELLAR PURKINJE CELLS IN CULTURE

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Mature freshly dissociated and *in situ* cerebellar Purkinje cells have two characteristic features. Firstly, the whole cell voltage-dependent calcium current (VDCC) is dominated by the P-type channel which is sensitive to low (<50 nM) concentrations of *Agelenopsis* toxin, ω-AgaIVA (Mintz *et al.*, 1992). Secondly, Purkinje cells are almost unique amongst central neurones, in being insensitive to N-methyl-D-aspartate (NMDA) while retaining sensitivity to α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) (Quinlan & Davies, 1989). In preparation for long term manipulations, we compared the properties of cultured Purkinje cells with those of freshly dissociated cells. Cerebella were dissected out from 16 day old rat embryos, trypsinised, triturated and plated onto coverslips suspended over an astroglial feeder layer (Brorson *et al.*, 1991) in a N2.1-DMEM medium. After 8 days *in vitro* (DIV) the large cerebellar neurones assumed a morphology similar to Purkinje cells *in situ* and also had similar immunofluorescence properties, ie. positive staining for synaptophysin, calbindin, GABA and for α1A (VDCC) subunits, this latter to a greater extent than that for α1B and α1E.

In whole cell patch clamp records from cultured Purkinje neurones, bath perfusion of ω-AgaIVA (50 nM) for 10-20 min reduced the calcium current by 55 ± 4 % (mean ± S.E.M.; n=15) at 8-10 DIV and 66 ± 4 % (n=10) at 13-16 DIV. The remaining current was reduced by ω-conotoxin GVIA (1 μM) and nifedipine (5 μM).

In single cell fura-2 based fluorimetric imaging studies, 50 mM K^+-induced intracellular calcium increases were reduced by ω-AgaIVA (100 μM) by 77 ± 8 % (n=6) at DIV 9. In addition, AMPA 10 μM increased intracellular calcium concentrations by 186 ± 39 nM (n=13) whereas NMDA 40 μM was ineffective.

The above results, from cells 1-2 weeks in culture, are sufficiently close to those from both freshly dissociated and *in situ* Purkinje cells (Mintz, I.H. *et al.*,1992; Quinlan, J.E. & Davies, J. 1989) to conclude that this is a satisfactory system in which to examine effects of chronic treatments on the properties of Purkinje cells.

Brorson, J.R. *et al.*, (1991) J. Neurosci., 11, 4024-4043.
Mintz, I.H. *et al.*, (1992) Nature, 355, 827-829.
Quinlan, J.E. & Davies, J. (1989) Neurosci. Lett., 60, 39-46.

33P EFFECTS OF CALCIUM-SENSING RECEPTOR ACTIVATION IN PRIMARY CULTURED CORTICAL NEURONS

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Recently, an extracellular calcium-sensing receptor has been cloned from bovine parathyroid (Brown et al., 1993). The calcium-sensing receptor is also expressed in various brain regions (Ruat et al., 1995) but its function is hitherto unknown. We have investigated the effects of various calcium-sensing receptor agonists on intracellular calcium mobilization and membrane conductance in cultured rat cortical neurons.

Rat cortical neurons were prepared from embryonic (E18) rats and were cultured in serum free medium. 1-2 weeks after preparation the cells were used for voltage clamp or calcium imaging measurements. The voltage clamp assays were performed using standard techniques and single cell calcium imaging was performed with 340/380 nm excitation ratio-imaging excitation using a Fuji HR-Deltaron 1700 camera attached to an inverted microscope. The data are presented as mean values ± s.e.mean.

Administration of extracellular calcium induced a concentration-dependent increase of intracellular calcium at a concentration-range of 0.5 to 6 mM. This effect was potentiated by co-administration of 10 µM of the calcium-sensing receptor ligand NPS-R-586 ((R)-(+)-[3-(2-Chlorophenyl)-propyl]-[1-(3-methoxyphenyl)-ethyl]-amine). At an extracellular calcium concentration of 2 mM both NPS-R-586 and the polycationic calcium-sensing receptor agonist neomycin induced a concentration-dependent increase of intracellular calcium with pEC_{50}'s of 6.64 ± 0.11 (n = 12) and 3.66 ± 0.23 (n = 5), respectively. The effect of NPS-R-586 was inhibited by 62 ± 7.4 % (n = 9) after depletion of intracellular calcium stores by pretreatment with $5x10^{-6}$ M thapsigargin. In contrast to published results concerning the bovine parathyroid calcium-sensing receptor, in rat cortical neurons the effect of neither calcium nor NPS-R-586 were affected by pretreatment with pertussis toxin (12 h; 200 ng/ml). In addition to the increase in intracellular calcium, NPS-R-586 and neomycin induced a non-selective cationic current. The pEC_{50}-values of NPS-R586 and neomycin for this effect were 6.74 ± 0.15 (n = 5) and 3.79 ± 0.09 (n = 5), respectively. The non-selective cationic current displayed a linear voltage to current relationship and had a reversal potential of -11.3 mV. The cationic current was independent of depletion of the intracellular calcium stores by $5x10^{-6}$ M thapsigarigin and was resistant to pretreatment of cells with pertussis toxin.

We conclude that extracellular calcium, by activating calcium-sensing receptors modulates intracellular calcium and evokes a nonselective cationic current in rat cortical neurons. Activation of the calcium-sensing receptor may explain the modulation of neurotransmission and neuronal excitability by extracellular calcium.

Brown, E. M., Gamba, G., Riccardi, D. et al. (1993) Nature **366**: 712-714.

Ruat, M., Molliver, M. E., Snowman, A. M. et al. (1995) Proc. Natl. Acad. Sci. USA **92**: 3161-3165.

34P EFFECT OF 5-HT ON THE RELEASE OF ENDOGENOUS GLUTAMATE FROM C6 RAT GLIOMA CELLS

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Glial cells express neurotransmitter receptors and may play a role in nervous system function (Kimelberg 1995). For example, bradykinin releases the excitatory amino acid glutamate from astrocyte cultures by mobilising intra-cellular calcium (Parpura et al 1994). 5-hydroxytryptamine (5-HT) mobilises internal calcium in the C6 rat glioma cells, an effect mediated by 5-HT_{2A} receptor (Elliott et al 1996). Here we report the effect of 5-HT on the release of glutamate from C6 cells.

C6 cells were grown in 24 well plates at a density of ~50,000 cells/well for 4 days in DMEM plus 10% FCS and then for 3 days in DMEM plus 1% dialysed FCS. Di-butryl cAMP (1 mM) was used to induce differentiation. Cells were washed 3 times for 10 min with 1 ml Krebs ringer gassed with 95% O_2/5% CO_2 and then incubated at 37 °C with 0.5 ml Krebs (± drugs) in a shaking water bath for 2-12 min. In most experiments the glutamate uptake inhibitor 2,4 trans-pyrollidine dicarboxylic acid (PDC) (50 µM) was present. At appropriate time points supernatant samples (200 µl) were removed, centrifuged and then frozen at -20 °C until assay. Glutamate was detected by the HPLC-EC method previously described by Smith and Sharp (1995). Briefly, 50 µl samples were derivatised with 5 µl fresh OPA/sulphite reagent for 20 min at room temperature. Derivatised glutamate was separated on a 4.6x250 mm C_{18} Microsorb HPLC column with a mobile phase comprised of 0.1 M Na_2HPO_4, 1 mM EDTA, 10 % methanol (v/v) at pH 5.4, and detected using a glassy carbon electrode (+0.85 V vs Ag/AgCl). Data are expressed as mean ± s.e.mean (n) and analysed statistically using Students unpaired t-test.

Glutamate was detectable in supernatant samples collected at 2 min and rose steadily over the following 10 min. Addition of 100 µM 5-HT increased glutamate about 2 fold above control values, at all time points. Using an incubation time of 10 min (when basal glutamate levels were about 300 nM), 5-HT (1, 10 and 100 µM) caused a dose-dependent increase in glutamate (164±11 (5), 189± 6 (6) and 207±14 (6)% of control, respectively). The glutamate response to 100 µM 5-HT was significantly reduced in calcium free Krebs containing 1 mM EGTA and 5 mM $MgCl_2$ (156±6.6 (6) vs. 98.3±9 (5) % of control, $p<0.001$). Removal of calcium also inhibited the 25 fold increase in glutamate levels induced by the calcium ionophore ionomycin (5 µM) (2550±480 (4) vs 375±75 (4) % of control, $p<0.005$). Frusemide (5 mM), which inhibits osmotic swelling, had no effect on the glutamate response to 100 µM 5-HT (227±15 (6) vs. 225 ±10 (6) % of control). 5-HT (100 µM) also released glutamate in the absence of PDC (n=6). Undifferentiated cells were unresponsive to 100 µM 5-HT compared to differentiated cells (103±7 (5) vs. 171±9 (6) % of control, $p<0.001$). The release of glutamate by 100 µM 5-HT was significantly reduced ($p<0.02$) by 1 µM spiperone and not significantly reduced ($p<0.15$) by 1 µM ketanserin (213±17 (6), 154±12 (6) and 174±13 (5) % of control, respectively).

Our data suggest that endogenous glutamate is released from C6 glioma cells and that this is increased by 5-HT in a calcium-dependent manner. The glutamate response to 5-HT is unlikely to be due to osmotic swelling or reversal of glutamate uptake. Preliminary evidence suggests the involvement of the 5-HT_{2A} receptor and experiments are in progress to verify this.

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Elliott, Newberry et al. (1995) Neuroscience 69, p1119-1131.
Kimelberg (1995) Neurochem. Int. 26, p27-40.
Parpura, Basarsky, Liu et al. (1994) Nature 369, p744-747.
Smith and Sharp (1995) B. J. Pharmacol 116, 103p.

35P LONG DURATION NMDA RECEPTOR-MEDIATED SYNAPTIC RESPONSES OF SPINAL MOTONEURONES INDUCED BY LOW THRESHOLD AFFERENTS

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Electrically evoked excitatory morphine-sensitive segmental spinal reflexes tens of seconds in duration are considered to reflect transmission from C fibre primary afferents (Akagi et al, 1985). The present results show that similar reflexes can be evoked by a brief train of stimuli applied to a dorsal root at intensities too low for activation of C fibres.

Hemisected spinal cords from Wistar rats aged between three and six days (unsexed, approximately 10g in weight) were removed following decapitation and prepared as previously described (Otsuka & Konishi, 1974). The bathing solution consisted of (mM) NaCl 118, $NaHCO_3$ 24, KCl 3, $CaCl_2$ 1.5, $MgCl_2$ 1.25 and glucose 12 maintained at 25°C and gassed with 95%/5% O_2/CO_2. Responses in the L4 or L5 ventral root were recorded following stimulation of the corresponding dorsal root. Similar recordings were made from dorsal roots in order to determine the afferent composition of dorsal root volleys. The lowest electrical stimulus intensity (square pulse 0.5ms width) that produced a discernable C wave in the dorsal root was 4 times threshold where threshold was the lowest intensity that produced any discernable reponse.

Single pulses at 2 times threshold produced responses in ventral roots of less than 1.5 s duration. Trains of five pulses (20 Hz) at 2 times threshold produced depolarisations in ventral roots (train EPSP) with a mean time to half decay (from area under the curve) of 5 ±0.6 s (n=6). This compares with a half time of 6.8 ±0.7 s (n=8) for similar responses (supramaximal EPSP) evoked by single supramaximal pulses. Train EPSPs were depressed to 16 ±2% (n=4) and supramaximal EPSPs to 62 ±8% (n=4) of control values by the competitive NMDA antagonist CGP40116 (3 μM, Fagg *et al.*, 1990). Train EPSPs were depressed to 35 ±6% (n=5) in the presence of morphine (1 μM) compared to the value of 14 ±2% (n=8) for the supramaximal EPSP. The results show that it should not be assumed that spinal segmental responses of long duration are necessarily indicative of C fibre primary afferent input. It is possible for A fibres to fire repetitively following single supramaximal shocks (Gasser, 1950)

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Akagi, H., Konishi, S., Otsuka, M. & Yanagisawa, M. (1985) Br. J. Pharmacol., 84, 663-673
Fagg, G.E., Olpe, H.-R., Pozza, M.F. *et al.* (1990) Br. J. Pharmacol., 99, 791-797
Gasser, H.S. (1950) J. Gen. Physiol., 33, 651-690
Otsuka, M. & Konishi, S. (1974) Nature, 252, 733

36P EFFECT OF CYCLOTHIAZIDE ON SYNAPTIC TRANSMISSION IN THE NEONATAL RAT HEMISECTED SPINAL CORD *IN VITRO*

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The α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) class of glutamate receptors mediates fast synaptic transmission in the mammalian CNS. On agonist exposure, AMPA receptors exhibit rapid desensitisation which can be blocked by cyclothiazide (Patneau *et al.*, 1992). There is debate about the role of desensitisation on synaptic transmission (Patneau *et al.*, 1993; Colquhoun *et al.* 1992; Trussell *et al.*, 1993). We therefore investigated the effects of cyclothiazide on reflexes in the neonatal rat hemisected spinal cord preparation superfused 2 ml/min with artificial CSF with 1.4 mM $MgSO_4$ (Evans, 1989). Depolarisation of motoneurones, elicited by superfusion with 4 ml aliquots of AMPA 3 μM, but not by N-methyl-D-aspartate (NMDA) 30 μM, was increased by 55 ± 11 % (n=4; mean ± S.E.M.) by cyclothiazide 10 μM. This effect was concentration-dependent, 1 and 300 μM producing 12 % and 98 % increases, respectively. Cyclothiazide (10 μM) shifted the concentration-response curve for AMPA (0.3-30 μM) to the left (Dose Ratio: 3.2 at control ED_{50} of 1.3 μM) with an 180 ± 14 % increase in the maximum response.

Segmental reflexes in this preparation are characterised by early (10-20 ms) and late (>40 ms) components (as described by Evans, 1989). Cyclothiazide (1-300 μM) increased the integrated area of both components largely due to their slower decay. Thus, 10 and 300 μM (n>4), respectively; increased the early phase by 12 ± 9 and 43 ± 21 % and the late phase by 35 ± 2 and 97 ± 3 %. In 5 out of 7 such cords on which it was measured, the latency to 50% maximal of the early phase was decreased 0.5-1.5 ms by cyclothiazide (10-100 μM).
Only minimal recovery was seen from the effect of cyclothiazide both on AMPA- and afferent-induced responses (<50% at 1 h).
The present data suggest that desensitisation plays an important role in limiting synaptic responses in this preparation where both endogenous levels of glutamate and repetitive firing of interneurones may play a contributory role.

Colquhoun, D. *et al.*, (1992) J. Physiol., 458, 261-287.
Evans, R.H. (1989) Prog. Neurobiol., 33, 255-279.
Patneau, D.M. *et al.*, (1993) J. Neurosci., 13, 3496-3509.
Trussell, L.O. *et al.*, (1993) Neuron, 10, 1185-1193.

37P USE OF THE NOVEL GLYCINE SITE ANTAGONIST, [^{3}H]MDL 105,919, TO STUDY PROPERTIES OF NATIVE AND CLONED NMDA RECEPTOR SUBTYPES

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The N-methyl-D-aspartate (NMDA) receptor is a ligand-gated cation channel that is widely expressed throughout the mammalian CNS. The amino acids, glutamate and glycine, bind to discrete but allosterically connected sites within the receptor and they are both required for full channel activation. Molecular cloning has identified two types of NMDA receptor subunit, NR1 and NR2A-D, with further heterogeneity possible through alternative splicing of the NR1 subunit [McBain and Mayer, 1994]. [^{3}H] MDL 105, 519 ((Z)-2-(phenyl)-3-(4,6 dichloroindol-3-yl-2-carboxylic acid) propenoic acid) has been recently reported to be a novel glycine site antagonist with a high affinity for the NMDA receptor [Siegel et al., 1995]. We report here the first description of its radioligand binding properties to native, cloned and detergent-extracted mammalian NMDA receptors.

Well-washed rat forebrain P2 membranes were used to characterise the radioligand binding properties of [^{3}H] MDL 105, 519 at 4°C using a filtration protocol. Non-specific binding was determined in the presence of 10^{-4}M glycine. All values reported are mean ± S.D (n = 3). Specific [^{3}H] MDL 105,519 reached equilibrium after 90 min and remained stable for at least 3 h. Both the association and dissociation rates were best fitted to a single exponential with $k_{+1} = 3.2 \pm 0.2 \times 10^7 M^{-1} min^{-1}$, $t_{½} = 10 \pm 3$ min and $k_{-1} = 0.080 \pm 0.008$ min^{-1}, respectively ($K_D = 2.50 \pm 0.3$ nM). Competition experiments with a range of known glycine site agonists and antagonists were performed. The resultant curves were best fitted to a single site model with Hill slopes close to unity (analysed using INPLOT; Graphpad). The pharmacological profile was consistent with previous findings using other [^{3}H] glycine site ligands i.e. rank order of potency was L-701, 324 > 5,7 dichlorokynurenic acid > 7-Cl kynurenic acid > glycine > D-serine >> L-serine. HEK 293 cells expressing NR1-1a, NR1-4b or NR2A subunits alone were assayed in parallel for [^{3}H] MDL 105,519 binding activity. Similar levels of specific [^{3}H] MDL 105,519 binding sites were detected for both NR1 splice variants, i.e. at 0.5nM, NR1-1a: 875 ± 32, NR1-4b: 921 ± 73 fmol/mg protein, with no detectable binding found for the NR2A subunit. Therefore, the [^{3}H] MDL 105,519 binding site lies on the NR1 subunit of the NMDA receptor.

Based on immunological studies, optimal NMDA receptor extraction was achieved using 1% Triton X100/1M NaCl (15 ± 3% of the total NR1 immunoreactivity [Chazot and Stephenson, 1996]. Under these extraction conditions, the determination of radioligand binding sites has proved difficult using the channel ligand [^{3}H] MK801, with a recovery of only 5 ± 1% of the initial binding in the membrane fraction. However, using [^{3}H] MDL 105,519, all of the available receptor sites (both assembled and unassembled NR1 subunits) were measured in the detergent extract (with a recovery of 21 ± 3% of the initial binding in the membrane fraction) highlighting the potential usefulness of this ligand in further immunopurification studies.

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Chazot, P.L. and Stephenson, F.A. (1996) *J. Neurochem.* (in press).

McBain, C.J. and Mayer, M.L. (1994) *Physiol. Revs.*74 723-759.

Siegel, B.W. *et al.* (1995) *Neurosci. Abs.* 21 436.10.

38P PRESYNAPTIC 5-HT$_3$ RECEPTORS MEDIATE AN EXCITATORY ACTION OF 5-HT ON DORSAL VAGAL PREGLANGLIONIC NEURONES: AN *IN VIVO* IONOPHORETIC STUDY IN THE RAT

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Ionophoretic application *in vivo* of 5-hydroxytryptamine (5-HT) and the 5-HT$_3$ receptor agonist phenylbiguanide (PBG) excites dorsal vagal preganglionic neurones (DVPNs) of rats and these responses are attenuated by 5-HT$_3$ receptor antagonists (Wang et al., 1996). Since autoradiographic binding studies have demonstrated that 5-HT$_3$ binding sites in dorsal vagal nuclei are located at both postsynaptic and presynaptic sites (Pratt & Bowery, 1989; Leslie et al., 1994), the present study investigated whether presynaptic 5-HT$_3$ receptors mediated excitation of DVPNs.

Male rats (280-370g) were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and artificially ventilated. Activity was recorded from antidromically identified DVPNs using 5- or 7-barrel microelectrodes (Wang et al., 1995, 1996). Ionophoretic application of PBG (0-40nA) excited 27 of 31 DVPNs tested. The excitatory action of PBG was markedly attenuated by co-ionophoresis of Mg^{2+} (1M, pH4, 0-20nA) in 11 out of 12 DVPNs tested. Similarly, Cd^{2+} (100mM, pH4.5, 0-10nA) attenuated the PBG-evoked excitation in the 3 neurones tested. In contrast, ionophoretic application of Mg^{2+}, at the current which attenuated the PBG-evoked excitation, altered neither the spontaneous firing rate nor the excitations evoked by DL-homocysteic acid (n=4), NMDA (n=2), or AMPA (n=2).

The NMDA receptor antagonist 2-amino-5-phosphono-pentanoic acid (AP5) (20mM, pH8.5, 2-10nA) blocked NMDA- but not AMPA-evoked excitations and attenuated PBG-evoked excitation in all 8 dorsal vagal preganglionic neurones tested. Similarly, the non-NMDA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) (2.5mM, pH8.5, 5-20nA), antagonised AMPA- but not NMDA-evoked excitations and also attenuated PBG-evoked excitations in all 3 dorsal vagal preganglionic neurones studied.

These data indicate that excitation of DVPNs by locally applied 5-HT may be mediated by 5-HT$_3$ receptors located presynaptically. Furthermore, activation of such presynaptic 5-HT$_3$ receptors in the DVN appears to increase release of glutamate which acts on both NMDA and non-NMDA receptors to cause excitation.

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Leslie, R.A., Reynolds, D.J.M. & Newberry, N.R. (1994) In: *5-hydroxytryptamine-3 receptor antagonists.* ed King, F.D., Jones, B.J. & Sanger, G.J. pp 79-96. Ann Arbor: CRC Press.

Pratt, G.D. & Bowery, N.G. (1989) *Neuropharmacol.*, 28, 1367-1376.

Wang, Y., Jones, J.F.X., Ramage, A.G. & Jordan, D. (1995) *Br. J. Pharmacol.*, 116, 2291-2297.

Wang, Y., Ramage, A.G. & Jordan, D. (1996) *Br. J.*

39P *IN VITRO* BINDING CHARACTERISTICS OF A SELECTIVE NMDA RECEPTOR 2_B SUBTYPE ANTAGONIST [^{3}H]Ro 25-6981 IN RAT BRAIN

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N-methyl-D-aspartate (NMDA) receptors are ligand-gated cation channels for L-glutamate, the major excitatory transmitter in the mammalian CNS. NMDA receptors are hetero-oligomeric proteins composed of the subunits NR1 (splice variants) and NR2 (A-D). They play key roles in synaptic plasticity, epilepsy and acute neurodegeneration (Kemp & Kew, in press). Ro 25-6981 [R-(R*,S*)]-a-(4-Hydroxyphenyl)-ß-methyl4(phenylmethyl)-1piperidine-propanol] is a potent, state dependent and NR2B-selective NMDA antagonist which is devoid of cardiovascular and behavioural side effects in rats at maximal neuroprotective doses (Fischer et al., 1996; Kemp et al., 1996).

[^{3}H]Ro 25-6981 (spec act 20.7Ci/mmol) was synthesized as a fumarate salt. Male Füllinsdorf albino rats (150-200g) were used. Membranes were prepared by homogenization of the whole brain (minus cerebellum and medulla oblongata) in cold Tris-HCl 50mM, EDTA 10mM, pH 7.1 buffer. They were washed three times before freezing at -80°C. After thawing, the homogenate was washed again three times in cold Tris-HCl 5mM, pH 7.4 (binding buffer). The final membrane concentration was 200µg of protein/ml. For competition experiments, using 5nM [^{3}H]Ro 25-6981, the incubation time was 2 hours at 4°C and the homogenate was filtered onto Whatmann GF/B filters with 5 washes with cold binding buffer. Saturation analyses were performed with a 4 hours incubation in order to reach equilibrium with the lowest concentrations of radioligand used. For film or phosphor imaging radioautography, cryostat sections of fresh-frozen rat brain were pre-washed in buffer then incubated for 90min at 22°C in 5nM [^{3}H]Ro 25-6981, rinsed in buffer (2x 5min + 15 min), dried and exposed to tritium-sensitive Ultrofilm (Amersham) or Fuji plates for 3wks or 3days, respectively. These were subjected to image analysis (MCID, Imaging Res.).

In rat whole brain homogenates, [^{3}H]Ro 25-6981 bound with a high affinity and capacity (K_D= 2±0.4nM; B_{max}= 1.3± 0.05pmoles/mg protein); non-specific binding (in the presence of 100µM spermine) was <10% of total binding. In competition binding experiments, the rank order of affinities (K_i) of reference compounds was: CP-101,606 (9nM), ifenprodil (10nM), haloperidol (270nM), eliprodil (340nM). Dizocilpine was inactive and the sigma site ligands, BMY 14802 and DTG only displaced a moderate fraction of the binding at 100µM (25-30%). Interestingly, both spermine and Mg^{2+} were found to inhibit [^{3}H]Ro 25-6981 binding in an apparently competitive competitive manner (K_i: 4 and 800µM, respectively).

Quantitative receptor radioautography and image analysis revealed a high density of high-affinity specific binding to rat cerebral cortex (layers 1>2,3,4>5,6), hippocampus (CA_1>>CA_4>CA_3), dentate gyrus, striatum and a lower density in thalamus; with some exceptions, e.g. locus coeruleus, binding to midbrain, cerebellum and brainstem was negligible. From saturation studies, K_D values (nM) ranged from 4.36 (caudate putamen) to 14,58 (cerebellum) and B_{max} values (pmoles/mg protein) from 4.47 (hippocampus CA1) to 0.586 (cerebellum).

We conclude that Ro 25-6981 is a high-affinity NMDA receptor ligand with a unique pharmacology and restricted brain distribution. These features probably reflect its selectivity for NR2B subunit-containing receptors as shown electrophysiologically in oocytes (Trube et al., 1996).

Fischer, G., et al., (1996) *Soc Neurosci Abstr* 693.5.
Kemp, J.A. & Kew, J.C.N. (in press) in *Receptor Based Drug Design* ed Leff, P. New York: Marcel Dekker, K.
Kemp, J.A. et al., (1996) *Soc Neurosci Abstr* 693.6.
Trube, G. et al., (1996) *Soc Neurosci Abstr* 693.4.

40P SPIN TRAP AGENT PREVENTS TAU ACCUMULATION IN OLIGODENDROCYTES FOLLOWING FOCAL CEREBRAL ISCHAEMIA IN THE RAT

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Tau-positive oligodendrocyte inclusions are a feature of chronic neurodegenerative conditions such as multiple system atrophy and we have previously reported tau accumulation in oligodendrocytes following stroke in human brain (Irving et. al., 1996). The aim of this study therefore was to determine if pharmacological agents previously shown to reduce ischaemic brain damage could also reduce tau accumulation in oligodendrocytes.

Focal cerebral ischaemia was induced in halothane anesthetised rats by permanent occlusion of one middle cerebral artery (MCA). Following 20, 40 or 80 min of ischaemia the animals were killed and the brains processed for tau immunohistochemistry. Drug treated animals received dizocilpine (0.5mg/kg, i.v) (n=5), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) (30mg/kg,i.v) (n=4) or α-phenyl-tert-butyl-nitrone (PBN) (100mg/kg,i.p) (n=5) 30 min prior to MCA occlusion (MCAO) and NBQX administered again 30 min after MCAO. The density of tau-positive oligodendrocytes mm^{-2} of subcortical white matter was determined and the data expressed as mean ± s.e. mean.

The density of tau-positive oligodendrocytes in the subcortical white matter ipsilateral to the occluded MCA increased 6-8 fold 40 min after MCAO, this time point was therefore selected for the drug intervention study. Pretreatment with the spin trap agent PBN significantly ($p<0.05$) decreased the density of tau-positive oligodendrocytes ipsilateral to the occluded MCA (102 ± 22 mm^{-2}) compared to untreated animals at 40 min after MCAO (191 ± 16 mm^{-2}). In contrast, dizocilpine (278 ± 45 mm^{-2}) and NBQX (233 ± 62 mm^{-2}) pretreatment failed to significantly decrease the density of tau-positive oligodendrocytes following 40 min of ischaemia. Tau accumulation within oligodendrocytes following cerebral ischaemia was therefore mediated through free radical mechanisms. In contrast to PBN, glutamate receptor antagonists failed to reduce the density of tau-positive cells after ischaemia. Free radical mediated mechanisms may be important in the glial pathology of chronic and acute neurodegeneration conditions.

Irving, E.A, Nicoll, J., Graham, D.I., Dewar, D. (1996) Neurosci. Letts. 113, 189-192.

41P MURINE $GABA_A$ RECEPTOR β_3 SUBUNITS PRODUCE A GABA-INSENSITIVE, SPONTANEOUSLY ACTIVE MEMBRANE CONDUCTANCE WHEN EXPRESSED IN *XENOPUS* OOCYTES

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Native $GABA_A$ receptors are hetero-oligomeric assemblies composed of subunits selected from up to 4 families, designated as α, β, γ and δ. Whilst it is unclear if homomeric $GABA_A$ receptors exist *in vivo*, such receptors can be formed in expression systems (Sieghart, 1995). This allows a detailed study of one subunit without the complication of variable subunit stoichiometries and thus receptor heterogeneity. It is of interest to study the β subunit in isolation as this subunit is involved in the partial formation of the GABA binding site, as well as influencing receptor assembly and mediating some effects of protein kinase phosphorylation of $GABA_A$ receptors (Sieghart, 1995; Connolly *et al.*, 1996). Currently homomeric β1 subunits do form functional ion channels (Krishek *et al.*, 1996), whereas β2 receptors do not (Connolly *et al.*, 1996; cf. Cestari *et al.*, 1996). This study now extends the comparison by presenting data obtained from murine β3 $GABA_A$ receptors expressed in *Xenopus* oocytes.
Oocytes were injected with murine $GABA_A$ β3 cDNA (1 μg/μl) and studied using two electrode voltage clamp technique. Oocytes were clamped at -25 mV and membrane conductances determined by applying 10 mV hyperpolarizing steps for 1 sec every 5 sec.

Leak membrane input conductances (g_{leak}) were significantly larger in β3-expressing cells (6.0±1.1 μS; mean±s.e.m., n=18 oocytes) compared to uninjected oocytes (1.1±0.1 μS, n=6, $P<0.05$). The application of GABA (100 μM-1 mM) or muscimol (200 μM) produced negligible increases in g_{leak} (<10%, n=4-9); however, pentobarbitone (PB, 1-2000 μM) evoked an increase in g_{leak} in a concentration-dependent manner, with EC_{50}=169.8±19.1 μM and Hill coefficient, n_H=1.16±0.04 (n=4-21). Current-voltage relationships obtained in the presence of 20 μM PB resulted in a reversal potential of -23.3±0.21 mV (n=11), suggesting that the PB-sensitive current was carried by chloride ions. During the recovery from high PB concentrations (>500 μM), g_{leak} exhibited a transient rebound potentiation, a feature also observed in human embryonic kidney cells transfected with β3 cDNA.

The g_{leak} in β3 subunit expressing oocytes was reduced in a concentration-dependent manner by picrotoxin (PTX, 0.5 nM-10 μM) or zinc (Zn^{2+}, 0.01-100 μM), with IC_{50}'s of 83±11.1 nM (n=3-5) and 328±25 nM (n=5), respectively. The value of g_{leak} in the presence of saturating concentrations of PTX and Zn^{2+} was not significantly different to that of uninjected cells. This observation is compatible with the β3 receptors being spontaneously gated and in an open conformation.
The $GABA_A$ receptor allosteric modulators flurazepam (5 μM) and alphaxalone (10 μM) failed to affect g_{leak}. In contrast, pregnanolone (0.05-5 μM) produced a small, but concentration-dependent enhancement of g_{leak} (13.7±2.4-39.9±3.8%, n=4-5) and propofol (10 μM) produced a significant increase in g_{leak} (102±5.0%, n=5). The antagonists bicuculline (10 μM, 44.7±5.2%, n=4) and strychnine (0.5-100 μM, EC_{50}=3.81±0.88 μM, n_H=0.89±0.14, n=5-9) surprisingly increased g_{leak}, whereas glycine (1 mM) had no effect.

Xenopus oocytes expressing murine β3 subunits possess a GABA-insensitive, spontaneously active membrane conductance which is modulated by PB, PTX and Zn^{2+}. Thus, despite the high level of homology between β subunits (β1-3), there are clear differences in their functional expression and properties.

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Cestari, I.N., Uchida, I., Li, L., Burt D. & Yang, J. (1996) *Neuroreport*, 7, 943-947.
Connolly, C.N., Wooltorton, J.R.A., Smart, T.G. & Moss, S.J. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 9899-9904.
Krishek, B.J., Moss, S.J. & Smart, T.G. (1996) *Molec. Pharmacol.*, **49**, 494-504.
Sieghart, W. (1995) *Pharmacological Rev.*, **47**, 181-234.

42P THE FMRFamide PRECURSOR PROTEIN ENCODES AN AGONIST, A PARTIAL AGONIST AND AN ANTAGONIST OF THE FaNaCh

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cDNA of the first peptide gated ion channel (FaNaCh) has been cloned from *Helix aspersa* (Lingueglia et al., 1995). Previous work showed that this channel occurs in specified *H. aspersa* neurones, is directly gated by the peptide FMRFamide, sodium selective and blocked by amiloride (Green et al., 1994). The FaNaCh cDNA predicts a new type of ligand-gated channel, related to epithelial amiloride-sensitive channels, and also to the degenerins of *Caenorhabditis elegans*. Recently, cDNA (MDeg) of a protein, similar to FaNaCh, was cloned from the mammalian brain; it too may be ligand-gated (Waldmann et al., 1996).

In *H. aspersa*, the precursor for FMRFamide also encodes FLRFamide and pQFYRFamide (See Lutz et al., 1990; Price et al., 1993). All have been detected in extracts of the C3 neurone in approximately the same ratio as in the precursor protein, i.e. 10 FMRFamide: 2 FLRFamide: 2 pQFYRFamide (Price et al., 1996).

A comparison has been made of the effects of these peptides on heterologously expressed FaNaCh. cRNA was prepared from the plasmid pBSK-SP6-globin-FaNaCh, kindly supplied by Drs M. Lazdunski and E. Lingueglia. Amounts (0.5 to 10 ng) cRNA were injected into *Xenopus* oocytes and recordings made 2 to 18 days later. Similar responses, and EC_{50} (~2μM), as reported by Lingueglia et al., (1995) were obtained with FMRFamide. When FLRFamide was compared with FMRFamide on the same oocytes (n = 10), the maximum response was always smaller (ranging from 43 to 59% to that of FMRFamide), indicating FLRFamide is a partial agonist. Surprisingly, pQFYRFamide was not an agonist, but blocked the FMRFamide-response competitively (ID_{50} = 64 ± 16μM); it also antagonised FLRFamide. This may be the first report of an endogenous antagonist for a natural receptor.

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Green, K.A., Falconer, S.W.P. & Cottrell, G.A. (1994) *Pflugers Arch.* 428, 232-240.
Lingueglia, E., Champigny, G., Lazdunski, M. *et al.*, (1995) *Nature* 378, 730-733.
Lutz, E.M., MacDonald, M., Hettle, S. *et al.* (1992) *Mol. & Cell. Neurosci.* 3, 373-382.
Price, D.A., Doble, K.E., Lesser, W. *et al.* (1993) *J. Soc. Neurosci. Abstract* 383.
Price, D.A., Denslow, N.D., Reynolds, S. *et al.* (1996) *J. Physiol.* 494, 82P.
Waldmann, R., Champigny, G., Voilley, N. *et al.* (1996) *J. Biol. Chem.* 271, 10433-10436.

43P ANTAGONIST EFFECTS OF THE PEPTIDES BIM-23055 AND BIM-23056, AT SOMATOSTATIN RECEPTORS IN GUINEA-PIG ISOLATED VAS DEFERENS AND RIGHT ATRIAL PREPARATIONS

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The genes for at least five distinct somatostatin (SRIF) receptors have been cloned (see Hoyer *et al.*, 1994) but determination of the operational characteristics of these receptors when either heterologously expressed in cell lines or naturally expressed in tissues has been hampered by the lack of specific SRIF receptor blocking drugs. In the present study we describe the antagonistic effects of the linear peptides BIM-23055 and BIM-23056 (Raynor *et al.*, 1993) on SRIF-induced inhibition of neurotransmission in the guinea-pig vas deferens and SRIF-induced negative inotropy in the guinea-pig right atrium.

Guinea-pig isolated vas deferens were prepared for measurement of isometric tension changes to electrical field stimulation as previously described in detail (Feniuk *et al.*, 1993). Spontaneous contractions of guinea-pig isolated right atrial preparations were also recorded (see Feniuk *et al.*, 1993). Concentration-effect curves to SRIF-28 were obtained before and after a 30min exposure to either BIM-23055 or BIM-23056. The pK_B values of the antagonists were determined from the Gaddum-Schild equation (Jenkinson *et al.*, 1995) by measuring the concentration ratio (CR) from the respective agonist EC_{50} values in the presence and absence of different concentrations of antagonist. All values quoted are the mean ± se mean or geometric mean (95% confidence limits) from n experiments.

SRIF-28 (1nM-1μM) caused a concentration dependent inhibition of neurogenically mediated contractile responses in the vas deferens [EC_{50}: 4.7(4.0-5.6)nM, n=20] and a negative inotropic effect in right atria [EC_{50}: 16.8(13.0-21.9)nM, n=17]. At the highest concentration tested SRIF-28 abolished neurogenic contractions in the vas deferens and maximally inhibited developed tension in the atria by 73±2%. Consecutive concentration-effect curves to SRIF-28 in the absence of antagonist were highly reproducible [CR: 1.4(1.28-1.74) and 0.89(0.62-1.27)] in vas deferens and atria, respectively. BIM-23055 and BIM-23056 (0.3,1 and 3μM) caused a concentration-dependent blockade of SRIF-28-induced inhibition of neurogenic contractions in the vas deferens with no effect on the maximum response (pK_B values of 6.43±0.07 and 6.40±0.28 respectively, n=12 and 8). Neither BIM-23055 nor BIM-23056 (both 3μM) had any direct effect in the vas deferens and did not modify the inhibitory effect of clonidine (1-30nM) [CR:0.85(0.44-1.64) and 0.83(0.60-1.16) respectively, n=4]. BIM-23055 and BIM-23056 (both 3μM) also had no direct effects in guinea-pig atria and antagonised SRIF-28 induced negative inotropic effects [CR:5.36(2.42-8.83) and 5.01(1.67-8.36), respectively, n=4 and 5]. The mean pK_B estimates were 6.16±0.09 and 6.02±0.15, respectively. At these concentrations, negative inotropic effects induced by carbachol (1nM-1μM) were not modified [CR:1.13(0.63-2.02) and 0.94(0.48-1.34)].

These findings demonstrate the specific SRIF receptor blocking actions of the linear peptides, BIM-23055 and BIM-23056. Importantly both BIM-23055 and BIM-23056 were devoid of agonist activity. Based on comparisons of relative agonist potencies, we have previously suggested that the SRIF receptor mediating inhibition of neurotransmission in the guinea-pig vas deferens and that mediating negative inotropy in the atria are similar (Feniuk *et al.*, 1993). The results from the present study with specific SRIF-receptor blocking drugs are consistent with this conclusion.

Feniuk, W. et al., (1993). *Br. J. Pharmacol.*, **110**, 1156-1164.
Hoyer, D. et al., (1994). *Naunyn Schmied. Arch. Pharmacol.*, **350**, 441-453.
Jenkinson, D.H. et al., (1995). *Pharmac. Rev.*, **47**, 255-266.
Raynor, K et al., (1993). *Mol. Pharmacol.*, **43**, 838-844.

44P PEPTIDE YY(PYY)/NEUROPEPTIDE Y RECEPTOR-MEDIATED RESPONSES IN THE EPIDIDYMIS AND VAS DEFERENS OF THE GUINEA-PIG: EVIDENCE FOR A PYY SELECTIVE RESPONSE

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Neuropeptide Y (NPY) and peptide YY (PYY) are members of a family of regulatory peptides which act presynaptically to inhibit electrically-evoked contractions in the vas deferens of rabbit, rat and guinea-pig (Doods & Krause, 1991; Ellis & Burnstock, 1990; Grundemar & Hakanson, 1990). At least 5 distinct receptor subtypes are thought to respond to NPY and PYY (as reviewed in Gerald, *et al.*, 1995). However, there has been no clear demonstration of the presynaptic NPY/PYY receptors subtypes mediating contractile reponses in the cauda epididymis and vas deferens of the guinea-pig.

Peptide effects on electrically-evoked contractions in the prostatic vas deferens and cauda epididymis of the guinea pig were assessed. Male Duncan-Hartley guinea-pigs (600-1000g) were killed by cervical dislocation. Preparations of epididymis and vas deferens were placed in an organ bath containing modified Krebs buffer (mM: $NaCl_2$ 118, KCl 4.7, Mg_2SO_4 0.45, K_2HPO_4 25, $NaHCO_3$ 25, $CaCl_2$ 1.9, glucose 11) gassed with O_2(95%):CO_2(5%) at 35-36°C and suspended under 0.35g and 1g resting force, respectively. Following 40 min equilibration preps were field stimulated (9Hz, 0.1ms duration; supramaximal voltage) with 10s trains of pulses every 14 min. Peptides (Calbiochem-Novabiochem, UK Ltd. and Peninsula, USA) were added to organ baths at least 2 min prior to field stimulation, after which tissues were washed with fresh Krebs buffer.

Both PYY and NPY inhibited electrically-evoked contractions in the vas deferens (Table 1). Time control tissues showed no change in response over the duration of each experiment. The Y2-receptor selective NPY13-36 and the Y1-receptor selective [Leu^{31},Pro^{34}]NPY also inhibited electrically-evoked contractions in the vas deferens. In the cauda epididymis, PYY inhibited electrically-evoked contractions, but NPY (100nM), NPY13-36 (1 μM), [Leu^{31},Pro^{34}]NPY (1 μM) and pancreatic polypeptide (PP) (1 μM)) did not. These results indicate that inhibition of electrically-evoked contractions is PYY-selective in the guinea pig cauda epididymis, while responses to NPY are restricted to the vas deferens.

Table 1 $^1EC_{50}$ nM (95% confidence limits) (maximal % inhibition)

Peptide Agonist	Cauda Epididymis	Vas Deferens
PYY	0.17 (0.01, 4.55) (42 .0 ± 7.0%)	0.86 (0.32, 2.29) (76.4 ± 4.7%)
NPY	NI	1.82 (0.83, 3.98) (66.8 ± 4.4%)
NPY 13-36	NI	40.6 (14.7, 112.1) (48.6 ± 4.8%)
[Leu^{31},Pro^{34}]NPY	NI	$>10^{-6}$M (35.3 ± 9.9%)
PP	NI	NI

^{1}The effects on electrically-evoked contractions of increasing concentrations of peptides were determined and are expressed as the percent of the response to the first stimulation. EC_{50} values (95% confidence limits), and maximal % inhibition are shown for n=5-8 separate determinations. Peptides were tested in the range of 10pM to 1mM, except NPY at 10pM to 100nM. NI = no inhibition.

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Doods, H.N. & Krause, J. (1991) *Eur. J. Pharmacol.* **204**, 101-103.
Ellis, L. & Burnstock, G. (1990) *Br. J. Pharmacol.* **100**,457-462.
Gerald, C. *et al.* (1995) *Nature*, **382**, 168-171.
Grundemar, L. & Hakanson, R. (1990) *Br. J. Pharmacol.* **100**, 190-192.

45P CONSTANT LIGHTING DOES NOT AFFECT THE FUNCTIONAL RESPONSE OF ML_1-LIKE RECEPTOR IN THE TAIL ARTERY OF JUVENILE WISTAR RATS

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Melatonin enhances neurogenic contractions of ring segments of isolated tail artery (from rats housed in a 12-hour light/dark cycle) (Ting *et al.*, 1996). Several other studies have shown that prolonged exposure to melatonin down-regulates its own receptor (eg. Gauer *et al.*, 1993). If vascular melatonin receptors are also subject to regulation by plasma melatonin, it is postulated that the density of the receptors might be increased by exposing the rats to constant illumination - which decreases plasma melatonin. We have compared the effect of melatonin against neurogenic responses of the caudal artery from rats housed in normal 12-hour light/dark cycle (LD) and 72-hour constant illumination (LL), and pharmacologically characterised the receptor involved.

Male Wistar rats (55-80 g wt; LD (control) lights on 8.00am or LL) were killed by decapitation (8-9am) and 2-3mm proximal segments of the tail artery mounted in a wire myograph containing Krebs-Henseleit saline (34°C; 95% O_2/5% CO_2). All vessels were placed under 0.1-0.2 g wt resting tension, exposed to 60 mM KCl, and then stimulated with a 5 sec train of electrical pulses (2-3 Hz, 15-30 volts, 0.3 ms pulse width) at 4-5 min intervals. Once stable contractions were established, melatonin or melatonin analogues were added non-cumulatively to each bath. Responses to melatonin have been calculated as a percentage of neurogenic contractions prior exposure to drug and are shown as the mean ± s.e.mean. The maximum potentiation (E_{max}) of each analogue has been expressed as a ratio of that produced by melatonin and the negative logarithm of the concentration producing 50% of the maximum response (pD_2) has also been determined. Differences between mean values have been compared using an unpaired Student's t-test ($p<0.05$).

The electrically-evoked contractions were sensitive to tetrodotoxin (0.5μM) and prazosin (0.1μM) indicating the neurogenic responses are mediated by α_1-adrenoceptors. Melatonin produced concentration-dependent enhancement of neurogenic contractions in caudal arteries from LD and LL rats. Unpaired Student's t-test ($p>0.05$) indicates that there was no difference in either the E_{max} (LD-282.5±39.2%; LL-191.7±2.5%) nor pD_2 (LD-8.31±0.12; LL-8.47±0.16) values of the two groups.

The pharmacological rank for (LD) the melatonin analogues (in decreasing potency; pD_2) investigated is as follows: 2-iodomelatonin (9.70±0.12;n=6) > (-) AMMTC [N-acetyl-4-aminomethyl-6-methoxy-9-methyl-1,2,3,4-tetrahydrocarbazole] (9.23±0.15; n=6) ≥ **melatonin** (lower and upper limit: 8.73±0.13; n=8 and 8.99±0.12; n=11 respectively) > 6-hydroxymelatonin (7.59±0.16; n=5) > (+) AMMTC (6.64±0.16; n=6) > N-acetyl-serotonin (5.72±0.12; n=4). All drugs possessed full agonist characteristics (E_{max} ratio between 1.02 and 0.91). None of these agents produced a consistent contraction.

Our data suggests that modulation of lighting conditions does not affect the potency and efficacy of melatonin at its receptor in the tail artery. From this pharmacological profile, it appears that melatonin is acting through the putative ML_1-like receptor. In particular, the enantiomeric ratio of 400 for (+) and (-) AMMTC in this preparation is entirely consistent with that described for melatonin ML_1 receptors. (Sugden *et al.*,1995).

Gauer, F., Masson-Pevet, M. and Pevet, P. (1993). *Brain Res.*, **602**, 153-156.
Sugden, D., Davies, D.J., Garratt, P.J., *et al.* (1995). *Eur. J. Pharmacol.*, **287**, 239-243.
Ting, K.N., Scalbert, E., Delagrange, P. and Wilson, V.G. (1996). *Br. J. Pharmacol.*, **119**, 113P.

46P CALPAIN INHIBITOR I ATTENUATES CIRCULATORY FAILURE, ORGAN INJURY AND THE EXPRESSION OF NITRIC OXIDE SYNTHASE AND CYCLOOXYGENASE II IN ENDOTOXIC SHOCK

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Local or systemic inflammatory responses are associated with the induction by cytokines (or endotoxin) of the inducible isoform of nitric oxide (NO) synthase (iNOS) and cyclooxygenase (COX-2) (Vane et al., 1993). There is limited evidence that the expression of the genes for iNOS or COX-2 involve the activation of the nuclear transcription factor NFκB. This study investigates the effects of calpain inhibitor I (Cal-I; inhibits the activation of NFκB by preventing IκB proteolysis), or chymostatin (Chym) on *(i)* systemic haemodynamics, *(ii)* renal and liver dysfunction, and *(iii)* the induction of iNOS and COX-2 protein in rats with endotoxic shock.

Male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.). The carotid artery was cannulated for the measurement of mean arterial pressure (MAP) and the femoral vein for the administration of compounds. After stabilisation of haemodynamic parameters (20 min), animals received either vehicle (saline, 1 ml kg^{-1}, i.v., n=6) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v., n=25) as a slow injection over 10 min. Different groups of animals received 2 h prior to LPS a bolus injection of vehicle (50% ethanol/PBS, 1 ml kg^{-1}, i.p., n=12), Cal-I (10 mg kg^{-1}, i.p., n=7) or Chym (10 mg kg^{-1}, i.p., n=6). At 6 h, blood samples were taken and analysed for glutamate-pyruvate-transaminase (GPT), bilirubin (liver integrity) and urea (renal function) by a contract laboratory for veterinary chemestry and 6-keto-$PGF_{1\alpha}$ by a specific RIA. In addition, lungs were removed to determine the expression of iNOS and COX-2 protein by Western blot analysis.

LPS caused hypotension, renal and liver dysfunction, an increase in 6-keto-$PGF_{1\alpha}$, as well as in iNOS and COX-2 protein expression in the lung ($p<0.05$, Table 1). Pretreatment of LPS-rats with Cal-1, but not with Chym, ameliorated the delayed hypotension and attenuated the rise in the serum levels of GPT, bilirubin and 6-keto-$PGF_{1\alpha}$ ($p<0.05$), but had no effect on urea (Table 1). In addition, Cal-I, but not Chym, significantly inhibited the expression of iNOS and COX-2 protein elicited by LPS in the lung ($p<0.05$, Table 1).

Thus, inhibition of the activation of the nuclear transcription factor NFκB by Calpain inhibitor I, reduces the delayed circulatory failure, liver dysfunction and the induction of iNOS and COX-2 protein in rats with endotoxic shock. We propose that prevention of the activation of NFκB *in vivo* may be useful in the therapy of disorders associated with local or systemic inflammation.

HR is a fellow of the DFG (Ru595/1-1). CT is a Senior Research Fellow of the BHF (FS/96018).

Vane, R. et al. (1993). *Proc. Natl. Acad. Sci. USA*, **91**, 2046-2050.

Table 1. Treatment	MAP (at 6 h) (mmHg)	bilirubin (iu l^{-1})	GPT (iu l^{-1})	6-keto-$PGF_{1\alpha}$ (pmol ml^{-1})	Urea (μM)	iNOS protein (OD*mm^2)	COX-2 protein (OD*mm^2)
Sham	109±2	2±0.3	107±14	1.0±0.2	7±1	2±1	2.2±0.2
LPS + vehicle	71±5	6±1	422±71	8.1±1.7	23±1	21±2	3.8±0.9
LPS + Cal-1	100±5*	3±1*	169±59*	1.6±0.2*	18±2	7±2*	1.3±0.3*
LPS + Chym	77±2	8±1	481±87	8.9±1.5	20±1	26±3	3.1±0.6

mean ± s.e.mean. *$p<0.05$ vs. LPS + vehicle, unpaired Student's *t* test.

47P EFFECTS OF INHIBITORS OF TYROSINE KINASE ON HAEMODYNAMICS, ORGAN FAILURE AND EXPRESSION OF NITRIC OXIDE SYNTHASE IN RATS WITH ENDOTOXIC SHOCK

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There is some evidence that activation of protein tyrosine kinase is a key event in the signal transduction pathway which leads to the induction of the inducible isoform of nitric oxide synthase (iNOS) by endotoxin in macrophages (Glaser et al., 1993). This study investigates the effects of the tyrosine kinase inhibitors tyrphostin AG126, tyrphostin A1 or genistein (Gen) on *(i)* systemic haemodynamics, *(ii)* renal and liver dysfunction, *(iii)* serum concentration of tumour necrosis factor alpha (TNFα), and *(iv)* the induction of iNOS protein in rats with endotoxic shock.

Male Wistar rats were anaesthetised with thiopentone sodium (120 mg kg^{-1}, i.p.). The carotid artery was cannulated for the measurement of mean arterial pressure (MAP) and the femoral vein for the administration of compounds. After stabilisation of haemodynamic parameters (20 min), animals received either vehicle (saline, 1 ml kg^{-1}, i.v., n=4) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v., n=29) as a slow injection over 10 min. Different groups of animals received 2 h prior to LPS a bolus injection of vehicle (50% DMSO/PBS, 1 ml kg^{-1}, i.p., n=10), AG126 (5 mg kg-1, i.p., n=7), A1 (5 mg kg^{-1}, i.p., n=6) or Gen (10 mg kg^{-1}, i.p., n=6). At 90 min after LPS, blood samples were collected to measure TNFα by ELISA. At 6 h, blood samples were taken and analysed for glutamate-pyruvate-transaminase (GPT) and bilirubin (liver integrity), and urea (renal function). In addition, lungs were removed to measure iNOS protein expression by Western blot analysis.

LPS caused hypotension, renal and liver dysfunction, an increase in iNOS activity in the lung and (at 90 min after LPS) a rise in the serum levels of TNFα ($p<0.05$, Table 1). Pretreatment of LPS-rats with AG126, A1 or to a lesser extent by genistein ameliorated the delayed hypotension and attenuated the rise in the serum levels of GPT and bilirubin, but not of urea (Table 1). In addition, AG126, A1 or genistein significantly inhibited the expression of iNOS protein in lung homogenates and the increase in the serum concentrations of TNFα elicited by LPS ($p<0.05$, Table 1).

Thus, inhibition of the activation of protein tyrosine kinase by the tyrphostins AG126 and A1, or genistein reduces the delayed circulatory failure, liver dysfunction and the expression of iNOS protein in rats with endotoxic shock.

HR is a fellow of the DFG (Ru595/1-1). CT is a Senior Research Fellow of the BHF (FS/96018).

Glaser, K.B. et al. (1993). *Biochem. Pharmacol.*, **45**, 711-715.

Table 1. Treatment	MAP (at 6 h) (mmHg)	bilirubin (iu l^{-1})	GPT (iu l^{-1})	Urea (μM)	iNOS protein (OD*mm^2)	TNFα (ng ml^{-1})
Sham	108±2	1.1±0.3	87±11	6.4±0.5	4.7±1	0.1±0.04
LPS + vehicle	76±6	6±1	478±85	20±2	28.9±4	6.5±0.4
LPS + AG126	100±4*	1.2±0.5*	246±45*	22±1	12±2*	2.7±0.2*
LPS + A1	104±2*	2.4±0.2*	116±8*	20±1	9±2*	3.2±0.5*
LPS + Gen	90±6	5±1	252±48*	23±2	16±3*	3.6±0.8*

mean ± s.e.mean. *$p<0.05$ vs. LPS + vehicle, unpaired Student's *t* test.

48P N$^{\omega}$-OH-D.L-INDOSPICINE, A POTENT AND SELECTIVE INHIBITOR OF ARGINASE IN RAT AND RABBIT ALVEOLAR MACROPHAGES (AMΦ) CAN PROMOTE UTILIZATION OF L-ARGININE BY NITRIC OXIDE SYNTHASE (NOS)

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L-Arginine serves as substrate of NOS and arginase, enzymes which play a particular role in AMΦ. Whereas NOS has to be induced by bacterial toxins or cytokines, arginase appears to be expressed constitutively. N$^{\omega}$-OH-D,L-Indospicine has recently been described as potent and selective inhibitor of liver arginase without being a substrate of NOS (Custot *et al.*, 1996). The present study should test whether arginase in AMΦ is also inhibited by N$^{\omega}$-OH-D,L-indospicine and whether such an effect would affect L-arginine utilization by NOS.

Rat or rabbit AMΦ (2.5 or $3*10^6$ cells per well, respectively) were cultured for 20 h in DMEM-F12 medium containing 5 % FCS in the absence or presence of LPS. Thereafter, the activity of NOS and arginase was determined by measuring the accumulation of ^{3}H-L-citrulline and ^{3}H-L-ornithine in incubation media during 1 h incubation with ^{3}H-L-arginine (37 kBq, 100 nM, Hey *et al.*, 1995). In addition, L-arginine uptake was studied by measuring the cellular radioactivity after 2 min of incubation with ^{3}H-L-arginine. Absolute values are expressed per 2.5 or $3*10^6$ cells, respectively, given are means±s.e.m. of n experiments.

Rabbit AMΦ which are known to express arginase, but no NOS (Hey *et al.*, 1995) did not show any significant formation of ^{3}H-L-citrulline, but produced substantial amounts of ^{3}H-L-ornithine (149±16 d.p.m.*1000, n=38). N$^{\omega}$-OH-D,L-indospicine, present during incubation with ^{3}H-L-arginine, concentration-dependently inhibited the formation of ^{3}H-L-ornithine by more than 80 % at 10 μM (IC_{50}: 2 μM). N$^{\omega}$-OH-D,L-Indospicine up to 100 μM had no significant effect on uptake of ^{3}H-L-arginine. In rat AMΦ, in which different levels of NOS had been induced by culture in the presence of 0.1 or 1 μg/ml LPS, N$^{\omega}$-OH-D,L-indospicine also reduced ^{3}H-L-ornithine formation, but this was accompanied by an increase in ^{3}H-L-citrulline accumulation. ^{3}H-L-citrulline and ^{3}H-L-ornithine formation by AMΦ, cultured with 0.1 μg/ml LPS amounted to 183±11 and 168±18 d.p.m.* *1000, respectively. These values were shifted to 327±52 and 22±6 d.p.m.*1000 by 10 μM N$^{\omega}$-OH-D,L-indospicine and to 390±53 and 8±1 d.p.m.*1000 by 30 μM N$^{\omega}$-OH-D,L-indospicine, respectively. ^{3}H-L-citrulline and ^{3}H-L-ornithine formation by AMΦ, cultured with 1 μg/ml LPS amounted to 265 ±16 and 64±4 d.p.m.*1000, respectively. These values were shifted by 10 μM N$^{\omega}$-OH-D,L-indospicine to 339±5 and 4±1 d.p.m.* *1000, respectively (n=3-5).

In conclusion, N$^{\omega}$-OH-D,L-indospicine is a potent and selective inhibitor of arginase in rat and rabbit AMΦ. When NOS is induced, this enzyme appears to compete with arginase for the substrate L-arginine. Under these conditions, inhibition of arginase can promote NO synthesis.

Custot, J., Boucher, J.-L., Vadon, S., et al., (1996) *J. Biol. Inorg. Chem.*,.1, 73-82
Hey, C., Wessler, I. & Racké, K. (1995) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 351, 651-659

49P THE ANTI-INFLAMMATORY DRUG LEFLUNOMIDE INHIBITS *IN VITRO* AND *IN VIVO* THE ACTIVITY OF COX-2 MORE POTENTLY THAN THE INDUCTION OF COX-2 OR iNOS

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Cyclooxygenase (COX) and nitric oxide synthase (NOS) exist in both constitutive and inducible isoforms (Mitchell *et al.*, 1995). The anti-inflammatory drug leflunomide has been proposed to be an inhibitor of the induction of COX-2 *in vitro* (Weithmann *et al.*, 1994). This action may possibly be exerted via inhibition of the activity of tyrosine kinases of the Src family (Xu *et al.*, 1995), which can be involved in the process of induction of COX-2 and iNOS (Akarasereenont *et al.*, 1994). Here we have examined the effects of leflunomide, and its active metabolite A771726, on the induction of COX-2 and iNOS *in vitro* and *in vivo*.

For *in vitro* studies, confluent J774.2 cells were exposed to LPS (10 μg/ml) for 24 h in the presence or absence of leflunomide (0.003-30 μg/ml), A771726 (0.3-1000 μg/ml), or vehicle (0.1 % DMSO for leflunomide, saline for A771726). The concentrations within the medium of NO_2 (as a measure of the activity of iNOS) and PGE_2 (as a measure of the activity of COX-2) were then determined by Griess reaction and radioimmunoassay, respectively (Akarasereenont et al., 1994). For *in vivo* studies, anaesthetised rats (male Wistar, 270±5 g) were infused with LPS (0.2 mg/kg/h) for 6 h following pre-treatment (t -1 h) with leflunomide (20 mg/kg, i.p.) or vehicle (DMSO). Blood samples (450 μl) were taken at 0, 2, 4 and 6 h for the determination of the plasma concentrations of NO_2/NO_3 (following treatment with NO_3 reductase) and 6-keto-$PGF_{1\alpha}$ (as a measure of PGI_2 formation). The expressions of iNOS and COX-2 proteins in homogenates of J774.2 cells or rat lung were determined by Western blot analysis.

In vitro, A771726 inhibited in concentration-dependent manners the accumulations of PGE_2 and NO_2, with respective IC_{50} values of 3.5 μg/ml and 380 μg/ml (n=9-12). Leflunomide similarly inhibited the accumulation of PGE_2 (IC_{50}, 2.2 μg/ml; n=9-12) but due to its poor solubility could not be given at sufficient concentrations to inhibit strongly the accumulation of NO_2. Western blot analyses indicated that the inhibitions of PGE_2 and NO_2 accumulation caused by the highest concentrations of A771726 and leflunomide were associated with reductions in the expressions of both iNOS and COX-2. Lower concentrations of A771726, which caused reductions in the accumulation of PGE_2, did not effect the expression of COX-2 protein. *In vivo*, LPS caused a significant increase in plasma NO_2/NO_3 (t=0, 9±3 μM; t=6 h, 292±83 μM; n=5) and 6-keto-$PGF_{1\alpha}$ (t=0, 0.2±0.2 ng/ml; t=6 h, 14.7±1.8 ng/ml; n=3). Leflunomide significantly reduced the elevation in plasma 6-keto-$PGF_{1\alpha}$ caused by LPS (t=6 h, 0.6±0.2 ng/ml; n=4; $p<0.05$, Mann-Whitney U test) but did not affect the increase in plasma NO_2/NO_3 (t=6 h, 363±29 μM; n=7). Western blot analysis indicated that LPS markedly increased the expression of COX-2 within lung homogenates and that this expression was unaffected by leflunomide.

Thus, leflunomide either directly, or through its active metabolite A771726, inhibits the inductions of both COX-2 and iNOS. However, the anti-inflammatory activity of leflunomide may be due to direct inhibition of COX-2 activity, for it inhibits the accumulation of COX metabolites at concentrations below those that inhibit the induction of COX-2.

Supported by a grant from Servier, France. TDW holds a British Heart Foundation Lectureship (BS/95003). Leflunomide and A771726 were a generous gift of Dr. R.R. Bartlett (HMR).

Akarasereenont, P. et al. (1994). Br. J. Pharmacol., 113, 1522-1528.
Mitchell, J.A. et al. (1995). Biochem. Pharmacol., 50, 1535-1542.
Weithmann, K.U. et al. (1994). Agents Action, 41, 164-170.
Xu, X. et al. (1995). J. Biol. Chem., 270, 12398-12403.

50P INHIBITION OF NO SYNTHASE ACTIVITY REDUCES THE SKELETAL MUSCLE NECROSIS CAUSED BY ISCHAEMIA-REPERFUSION OF THE HINDLIMB

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Skeletal muscle, unlike cardiac muscle, is rich in neuronal nitric oxide (NO) synthase (nNOS or NOS type I), the exact function of which remains unclear (Kobzik *et al.*, 1994). Ischaemia-reperfusion (I/R) injury in skeletal muscle results in muscle necrosis, with subsequent multiple organ failure and limb loss. There is an enhanced formation of NO and superoxide anions, which may result in the formation of peroxynitrite, which is a potent oxidant, and, hence, cytotoxic. Here, we investigate the effects of various inhibitors of NOS activity in a model of I/R in the hindlimb of the anaesthetised rabbit.

Male New Zealand white rabbits (2.0-2.8 kg) were premedicated with Hypnorm (0.15 $ml \cdot kg^{-1}$, s.c.). General anaesthesia was induced and maintained with sodium pentobarbitone (60 $mg \cdot ml^{-1}$) via a marginal ear vein. The rabbits were mechanically ventilated on room air and their temperature maintained at 37.5-38.5°C. Hindlimb ischaemia was induced, at laparotomy, by placement of a vascular clamp over the distal abdominal aorta. Following 4 h of ischaemia, reperfusion was allowed for 3 h. The right and left gracilis muscles of each animal were stained with nitro-blue tetrazolium (0.5 $mg \cdot ml^{-1}$ for 20 min at 37°C) to assess viability. I/R caused a substantial skeletal muscle necrosis (~50%), which was significantly reduced by the injection (prior to reperfusion) of the NOS inhibitors 7-nitroindazole (7-NI) ($30 mg \cdot kg^{-1}$, i.p., 30 min. prior to reperfusion), aminoethylisothiourea (AE-ITU) ($10 mg \cdot kg^{-1}$, i.v., 1 min. prior to reperfusion) and aminoguanidine (AG) ($30 mg \cdot kg^{-1}$, i.v., 1 min. prior to reperfusion). Results are means ± s.e.mean ($p<0.05$, compared to controls by unpaired Student's t-test) (Fig.1).

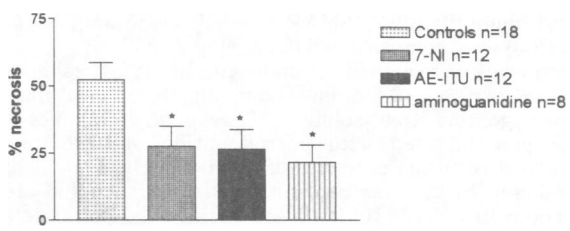

Fig.1

Thus, inhibitors of NOS activity reduce the degree of skeletal muscle necrosis caused by I/R of the hindlimb. As 7-NI is a potent and highly selective inhibitor of nNOS activity (Bland-Ward & Moore, 1995), we speculate that an enhanced formation of NO by nNOS contributes to the development of tissue necrosis.

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Bland-Ward PA & Moore PK, (1995) *Life Sciences* 57:PL131-135.
Kobzik L *et al.*, (1994) *Nature* 372: 504-505

51P PARS INHIBITION REDUCES THE MUSCLE NECROSIS CAUSED BY ISCHAEMIA-REPERFUSION INJURY OF THE HINDLIMB

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Ischaemia-reperfusion (I/R) injury of the lower limb may result in muscle necrosis and limb loss. A number of mechanisms are thought responsible for this injury, including the production of oxygen free radicals, which cause DNA strand breaks.

Poly (adenosine 5' -diphosphate ribose) synthetase (PARS) is a nuclear enzyme responsible for the repair of DNA strand breaks. PARS transfers ADP-ribose units from NAD to nuclear acceptor proteins (Banasik & Ueda, 1994). This leads to a fall in ATP, and ultimately, cell death. Here, we show that a range of inhibitors of PARS activity reduce the degree of skeletal muscle necrosis, in a model of I/R injury in the anaesthetised rabbit.

Male New Zealand white rabbits (2.0-2.8 kg) were premedicated with Hypnorm (0.15 $ml \cdot kg^{-1}$, s.c.). General anaesthesia was induced and maintained with sodium pentobarbitone ($60mg \cdot ml^{-1}$) via a marginal ear vein. The rabbits were mechanically ventilated on room air and their temperature maintained at 37.5-38.5°C. Lower limb ischaemia was induced, by placement of a vascular clamp at the distal abdominal aorta. Following 4 h of ischaemia, reperfusion was allowed for 3 h. In the treated animals, the PARS inhibitors were given as an intravenous bolus injection, in 2 ml of vehicle (saline or 10% DMSO), 1 min prior to reperfusion. The PARS inhibitors used were 3-aminobenzamide (3-AB), benzamide (BZ), nicotinamide (NIC), 4-amino-,1,8-naphthalimide (AN) and 1,5-dihydroxyisoquinoline (IQ). The right and left gracilis muscles of each animal were stained with nitro-blue tetrazolium (0.5mg $ml^{-1}$for 20 min at 37°C) to assess viability. Ischaemia and reperfusion of the hindlimb caused a substantial necrosis of the gracilis muscle, which was attenuated by 50-70% by the PARS inhibitors used (Fig. 1). Results are means ± s.e.mean ($p<0.05$, unpaired Student's t-test).

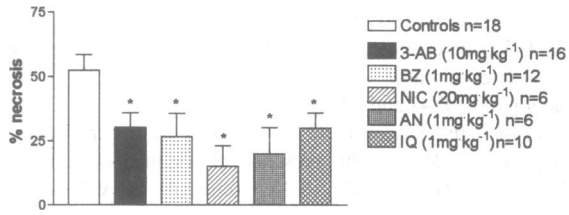

Fig1.

In contrast, 3-aminobenzoic acid (which is similar in structure to 3-AB, but does not inhibit PARS activity) had no effect on the degree of necrosis caused by I/R.

Thus, PARS inhibition reduces the muscle necrosis induced by I/R injury of the hind limb, suggesting that the activation of PARS leads to the extension of tissue injury during reperfusion of ischaemic skeletal muscle.

CT is a Senior Fellow of the BHF (FS/96018)

Banasik M & Ueda K (1994) *Mol Cell Biochem* 138:185-197

52P VASODILATORY PROPERTIES OF A NOVEL NITROSATED GLYCO-AMINO ACID IN RAT ISOLATED FEMORAL ARTERIES: POTENTIAL AS A SLOW RELEASE NITRIC OXIDE DONOR DRUG

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S-nitrosothiols decompose readily to release nitric oxide (NO) but the unpredictable nature of the decay of existing nitrosothiols limits their suitability as therapeutic NO donor drugs. Here, the vasodilator properties of a novel S-nitroso-N-acetyl penicillamine (SNAP)-related glyco-amino acid (RIG200) were compared to those of SNAP.

Experiments were carried out on lengths of rat femoral artery (7.5mm) perfused (0.6ml min^{-1}) and superfused (1ml min^{-1}) with oxygenated Krebs solution (Flitney*et al*, 1992). Vessels were precontracted with phenylephrine (4-10μM) and developed perfusion pressures of 66-104mmHg. Endothelial denudation by air was confirmed using carbachol. Bolus injections of SNAP (10μl; 10^{-8}-10^{-3}M) into the perfusate of vessels caused dose-dependent, transient vasodilatations which recovered to pre-injection pressure within 5min. Similar bolus injections of RIG200 produced more protracted responses which recovered incompletely at intermediate and high concentrations, resulting in a stepwise and persistent loss of tone. Pre-injection pressure was fully restored by perfusing with ferrohaemoglobin (Hb; 10μM), a NO scavenger (fig 1).

Superfusion with SNAP (10^{-7}-10^{-5}M) caused rapid, dose-dependent vasodilatations which tended to recover spontaneously over a period of 1-2hr. Responses to equivalent doses of RIG200 were slower to develop than those to SNAP. Vasodilatation in response to 10^{-5}M RIG200 was maximal and sustained throughout the period of superfusion but could be fully reversed by co-superfusion with 10μM Hb.

Our results are consistent with the hypothesis that SNAP-induced vasodilatation is caused predominantly by spontaneous release of NO in the perfusate or superfusate ($T^1/_2$ for SNAP decomposition =45 min at 24°C in Krebs), resulting in rapid, transient responses. In contrast, RIG200 appears to be retained in the tissue and slowly decomposes ($T^1/_2$ =300 min), releasing sufficient NO to maintain a "vasodilator tone" which persists for >60 min after bolus washout.

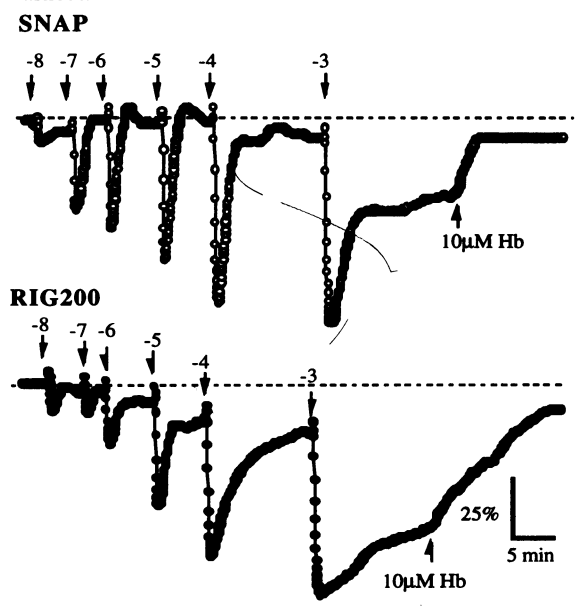

Fig1. Representative traces showing responses to bolus injections of SNAP or RIG200 (% pre-injection pressure; log molar concentrations as indicated). Persistent loss of tone following 10^{-3}M injections is reversed by perfusing with 10μM Hb.

Supported by the British Heart Foundation.

Flitney *et al* (1992) *Br. J. Pharmacol.*, **107**: 842-848.

53P ASCORBATE: CONTENT, RELEASE AND PROTECTION OF NO-INDUCED RELAXATIONS IN THE ANOCOCCYGEUS MUSCLE

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Nitrergic relaxations of smooth muscle are resistant to inhibition by superoxide anions and certain other NO-scavengers which do, however, block relaxations to exogenous NO. One explanation is that such tissues contain (and release) substances which protect neurotransmitter NO from attack. In the anococcygeus, superoxide dismutase appears to fulfill such a role, but the protection is only partial and is only manifest against superoxide anions (Lilley & Gibson, 1995). A further candidate 'minder-molecule' is ascorbate, which protects exogenous NO from inhibition by superoxide anion generators (duroquinone, DQ) and NO-scavengers (hydroquinone, HQ; carboxy-PTIO; Lilley & Gibson, 1996). Here, we have compared the pharmacology of ascorbate with its oxidised form dehydroascorbate (DHA) in the mouse anococcygeus, and measured ascorbate content and release in the rat anococcygeus.

Mouse anococcygeus muscles were set up for the recording of isometric tension responses as described (Lilley & Gibson, 1995). Ascorbate content of rat anococcygeus muscles was determined by HPLC, using perchloric acid (50mM) as the extraction/stabilising agent (Pachla & Kissinger, 1979). Release of ascorbate was determined by incubating individual anococcygeus muscles (rat; 20-30mg) in 100µl Krebs solution for 30min at 37°C and then assaying the ascorbate content of the bathing medium using HPLC. The Krebs solution used was either normal (Lilley & Gibson, 1995) or high-K (70mM KCl with appropriate reduction in NaCl). Results are expressed as mean±s.e.. Statistical analysis was by Student's t-test.

In mouse anococcygeus muscles, authentic NO (15µM) relaxed carbachol (50µM)-induced tone by 48±5% (n=11). DQ (100µM), HQ (100µM) and carboxy-PTIO (50µM) inhibited these NO-induced relaxations by 42±8%, 83±2% and 69±9% respectively (all n=6). Ascorbate (400µM) reversed the inhibition of NO-induced relaxations by DQ, HQ and carboxy-PTIO by 37±9%, 81±9% and 93±6% respectively (all n=4). DHA (400µM) produced no such protection against any of the drugs. HPLC measurement of ascorbate levels in rat brain and anococcygeus produced values of 790±70 nmol g^{-1} and 49±6 nmol g^{-1} respectively (both n=6). HPLC signals were attributed to ascorbate, since ascorbate oxidase (10 U ml^{-1}) abolished them. DHA produced no signal. A detectable amount of anococcygeus ascorbate was released into normal Krebs solution (4.91±0.85 nmol g^{-1}; n=6), and this was increased in high-K Krebs (to 12.35 ±1.35 nmol g^{-1}; n=6; $P<0.05$).

The results confirm that ascorbate, but not DHA, can protect exogenous NO from attack. In addition, the anococcygeus contains significant amounts of ascorbate. Release of this ascorbate occurs at rest, and is increased under depolarising conditions. It will be important to determine the source of the released ascorbate, and whether ascorbate depletion renders nitrergic relaxations sensitive to NO-scavengers.

E.L has an M.R.C. studentship.

Lilley, E. & Gibson, A. (1995) *Br. J. Pharmacol.* **116**, 3231-3236.
Lilley, E. & Gibson, A. (1996) *Br. J. Pharmacol.* **119**, 432-438.
Pachla, L.A. & Kissinger, P.T. (1979) *Meth.Enzymol.* **62**, 15-24.

54P DIFFERENTIAL ACTIONS OE CHARYBDOTOXIN ON RABBIT CENTRAL EAR AND DAUGHTER BRANCH ARTERIES

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K_{Ca} channels modulate vascular tone through direct and indirect mechanisms. In vascular smooth muscle, their opening leads to vasodilatation, whilst in endothelium they may modulate NO release stimulated by both agonists and flow. They may also modulate the relaxant action of NO. Using an X-ray microangiographic technique (Griffith *et al.*, 1987), we have investigated the actions of charybdotoxin (ChTX; 1nM), a K_{Ca} channel blocker, and L-NAME (100 µM) on diameter-flow and pressure-flow relationships in the isolated rabbit ear preconstricted with 5-HT (100 nM).

In the intact ear, ChTX induced an upwards shift in the pressure-flow curve relative to control levels (rising by 32.2±20.9 mm Hg at 5 ml min^{-1}; $p<0.001$; n=9) whilst subsequent addition of L-NAME induced a further shift (rising by 38.7±29.0 mm Hg at 5 ml min^{-1}; $p<0.001$; n=9). L-NAME itself caused an upwards shift in the pressure-flow curve relative to control (rising by 99.6±25.1 mm Hg at 5 ml min^{-1}; $p<0.001$; n=7), but subsequent addition of ChTX had no effect. Direct visualisation with X-ray microangiography revealed that ChTX predominantly constricted the central ear artery (G_0) rather than the first (G_1) or second (G_2) generation daughter branch arteries. In comparison, L-NAME constricted G_0, G_1 and G_2 vessels. Pressure-flow curves were also constructed using isolated G_0 preparations. ChTX was again found to cause an upwards shift in the pressure-flow curve relative to control (rising by 20.0±15.9 mm Hg at 5 ml min^{-1}; $p<0.01$; n=9) with subsequent addition of L-NAME causing a further shift (rising by 28.7±19.8 mm Hg at 3 ml min^{-1}; $p<0.05$; n=9). L-NAME itself caused an upwards shift in the pressure flow curve relative to control (rising by 24.8±17.7 mm Hg at 5 ml min^{-1}; $p<0.01$; n=6), with no effect on subsequent addition of ChTX. In isolated G_1 preparations, pressure-flow curves were obtained over the range 0.25–1 ml min^{-1}. ChTX had no effect on the pressure-flow curve relative to control but subsequent addition of L-NAME resulted in an upwards shift (rising by 61.3±18.8 mm Hg at 1 ml min^{-1}; $p<0.001$; n=6). L-NAME itself caused an upwards shift relative to control (rising by 60.0±32.9 mm Hg at 1 ml min^{-1}; $p<0.01$; n=6), but subsequent addition of ChTX had no effect.

The data indicate that ChTX exhibited a differential action on central and daughter branch arteries of the isolated rabbit ear causing constriction in G_0 but not in G_1 or G_2 vessels. This differential action was also seen between isolated G_0 and G_1 vessels indicating it is due to a difference in the sensitivity of these vessels to ChTX, and not merely the result of integrated network behaviour. This may reflect a heterogeneous distribution of ChTX-sensitive channels within the rabbit ear vasculature. The action of ChTX in intact ears and G_0 preparations was not seen in the presence of L-NAME and may therefore be due to an inhibition of the release or action of NO.

Griffith, T.M., Edwards, D.H., Davies, R.L., Harrison, T.J. & Evans, K.T. (1987). *Nature*, **329**, 442-445.

55P EFFECTS OF PEROXYNITRITE ON CONTRACTION AND RELAXATION RESPONSES IN THE SMALL MESENTERIC, CORONARY AND BASILAR ARTERIES OF THE RAT

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Peroxynitrite ($ONOO^-$) is a reactive species formed by the reaction of superoxide and nitric oxide (Pryor & Squadrito, 1995). It has diverse biological effects, possibly including damage to blood vessels. This study compares the effects of $ONOO^-$ on the contraction and relaxation of the left anterior descending coronary (LAD), the third generation mesenteric and the basilar arteries.

Artery segments (2.0 mm long; internal diameter 200-400 μm), from male Wistar rats (250-500g) were mounted in a wire myograph (JP Trading, Aarhus) and bathed in physiological solution (mM: NaCl 115.3, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5, glucose 11.1 or 5 for the mesenteric artery) equilibrated with 95% O_2/5% CO_2 at 37°C. Vessels were incubated for 45 min before normalisation (Mulvany & Halpern, 1977). After another 30 min, control responses were obtained before vessels were exposed for 10 min to $ONOO^-$ (Alexis Corp., Nottingham) or its vehicle (0.3 M NaOH). Concentration-response curves were again determined 30 min later. $ONOO^-$ concentration was determined by spectrophotometry (Beckman *et al.*, 1990). Statistical significance was determined by analysis of variance.

In the mesenteric artery, concentrations up to 1 mM $ONOO^-$ did not change contractions to methoxamine. In contrast, 10 and 100 μM $ONOO^-$ increased the potency of carbachol (CCh) at relaxing methoxamine- (10 μM) precontracted vessels. Effects on the maximal relaxation (E_{max}) varied; it was slightly enhanced by 100 μM $ONOO^-$ but reduced by 2 x 100 μM (i.e. two exposures to 100 μM in 10 min) or one exposure to 1mM $ONOO^-$ (Table 1).

Table 1 Relaxation to carbachol in rat small mesenteric artery

	E_{max} (% induced tone)	EC_{50} (nM)	*n*
Control	71.2 ± 2.2	948 ±107	8
10 μM $ONOO^-$	75.1 ± 1.2	615 ± 48*	6
100 μM $ONOO^-$	80.7 ± 1.2**	502 ± 28**	5
2 x 100 μM $ONOO^-$	45.0 ± 1.6***	1077 ±100	9
1 mM $ONOO^-$	29.1 ± 2.2***	1588 ±337	12

*, **, ***: $P < 0.05$, 0.01 & 0.001 respectively relative to control

In the LAD, neither 2 x 100 μM $ONOO^-$, nor a single application of 1 mM affected the contraction to 5-hydroxytryptamine (5-HT). However, 2 x 100 μM $ONOO^-$ potentiated relaxation to CCh and CCh also relaxed vessels at resting tension. Exposure to 1 mM $ONOO^-$ decreased the E_{max} to CCh (Table 2).

Table 2. Relaxation to carbachol in LAD precontracted with 5-HT

	E_{max} (% induced tone)	EC_{50} (nM)	*n*
Control	64.7 ± 1.7	773 ± 132	12
2 x 100 μM $ONOO^-$	147.9 ± 6.3***	941 ± 222	5
1 mM $ONOO^-$	38.1 ± 1.3***	2291 ±378**	5

**, ***: $P < 0.01$ & 0.001 respectively relative to control.

In the basilar artery, 2 x 100 μM $ONOO^-$ did not affect contractions to KCl (10 mM-100 mM), but exposure to 1mM $ONOO^-$ abolished this response. Endothelin-1 contractions were reduced by 2 x 100 μM $ONOO^-$ (E_{max}: control, 11.9±0.6 mN; after $ONOO^-$ 7.6±0.5 mN, $n = 4$) and by 1 mM (E_{max}: 4.8±0.5 mN, $n = 4$). CCh does not induce a large relaxation of the basilar artery; but these responses were reduced by exposure to $ONOO^-$.

Thus there was variation in the sensitivity of arteries to $ONOO^-$ but contractile responses were relatively resistant. Changes in CCh responses were more complex; exposure to lower concentrations of $ONOO^-$ enhanced relaxation in the coronary and mesenteric arteries. Exposure to 1 mM $ONOO^-$ consistently reduced the E_{max} for CCh-mediated relaxation but EC_{50} values were unchanged.

CEO, SPC and HJD are Medical Research Council Students.

Beckman, J.S., Beckman, T.W., Chen, J. *et al.* (1990). *Proc. Natl. Acad. Sci. (USA)*, 87, 1620-1624.
Mulvany, M.J. & Halpern, W. (1977). *Circ. Res.*, 41, 19-26.
Pryor, W.A. & Squadrito, G.L. (1995). *Am. J. Physiol.*, 268, L699-L722.

56P INTERACTION OF PEROXYNITRITE WITH MEMBRANE PERMEANT AND IMPERMEANT SUGARS

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We recently reported that peroxynitrite anion ($ONOO^-$, PN) reacts with glucose to form a novel vasorelaxant whose action is augmented by the thiol, L-cysteine (L-cys, Dowell & Martin, 1996). In this study we wished to investigate whether the formation of a novel relaxant occurred following the reaction of PN with both membrane permeant and impermeant sugars.

PN was synthesised and stabilised at pH 12.4 (Beckman *et al.* 1990). To investigate the formation of stable vasodilator substances, PN (1 mM) was mixed (30 s) with the membrane permeant sugars, D-glucose (11 mM) or glycerol (22 mM), or the impermeant sugars, L-glucose, mannitol or sorbitol (each 11 mM). These solutions were then neutralised to remove unreacted PN and their relaxant actions assayed on endothelium-denuded rings of rat aorta contracted with phenylephrine (PE, 20 nM, Dowell & Martin, 1996).

L-cys (1 mM) had no effect by itself on the tone of PE-contracted rings. As previously reported (Dowell & Martin, 1996), the reaction of D-glucose with PN led to formation of a new stable relaxant, and the additional presence of L-cys (1 mM) in the tissue bath significantly potentiated its relaxant action (Table 1). When neutralised PN (NEU) was mixed with D-glucose no new relaxant was formed, but the solution did have weak dilator activity, consistent with contaminating nitrite; this relaxant action was unaffected by L-cys. New stable relaxant activity was also seen following reaction of PN with D-glucose or glycerol and their ability to produce relaxation too was potentiated by L-cys. If, however, mannitol and sorbitol, were mixed with PN no new relaxant activity was evident. Reactions had occurred however, since with each sugar a more potent dilator action was seen in the presence of L-cys.

Table 1. Log EC_{50} values for relaxant activity generated following mixing of NEU or PN with sugars.

	NEU	PN	PN + L-cys (1 mM)
D-Glucose	-5.19±0.04	-5.78±0.02$^{\$\$\$}$	-6.18±0.03 ***
L-Glucose	-5.26±0.04	-5.84±0.03$^{\$\$\$}$	-6.18±0.03 ***
Glycerol	-5.34±0.05	-5.62±0.06$^{\$\$\$}$	-5.88±0.05 *
Mannitol	-5.31±0.07	-5.39±0.03	-5.74±0.03 ***
Sorbitol	-5.44±0.08	-5.53±0.05	-5.90±0.06 ***

(mean ± s.e. mean, n≥5) $\$\$\$$ $p<0.05$ c.f. NEU, * $p<0.05$, *** $p<0.001$ c.f. PN (ANOVA, followed by Bonferroni analysis).

Thus, the membrane impermeant sugars, mannitol and sorbitol, do react with PN, but expression of the vasorelaxant properties of their derivatives requires the presence of a thiol (L-cys). Although L-cys also potentiates the relaxant actions of the derivatives formed from the reaction of PN with the membrane permeant sugars, D-glucose and glycerol, these derivatives have intrinsic vasodilator activity. Membrane permeability cannot explain the difference in activity, however, as the impermeant sugar, L-glucose, also has intrinsic activity.

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Beckman, J.S., Beckman, T.W., Chen, J. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 86,1620-1624.
Dowell, F.J. & Martin, W. (1996) *Br. J. Pharmacol.*, Dundee BPS meeting.

57P SYNERGISM BETWEEN INTERLEUKIN-4 AND LIPOPOLYSACCHARIDE IN THE INDUCTION OF VCAM-1 ON HUMAN LUNG MICROVASCULAR ENDOTHELIAL CELLS

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Endothelial vascular cell adhesion molecule-1 (VCAM-1) is thought to play a key role in eosinophil recruitment into the lung in asthma. Interleukin (IL)-4 has been shown to induce VCAM-1 but not intercellular adhesion molecule-1 (ICAM-1) or E-selectin expression, on human umbilical vein endothelial cells [1]. In the present study, we investigated effects of IL-4 alone, or with lipopolysaccharide (LPS: *Escherichia coli* 055:B5) on VCAM-1 expression, mRNA induction & eosinophil or neutrophil adhesion to human lung microvascular endothelial cells (HLMVEC).

HLMVEC (Clonetics, San Diego, USA) were maintained in endothelial cell growth medium with foetal calf serum (5%), epidermal growth factor (10ng ml^{-1}) & antibiotics. Confluent HLMVEC monolayers were incubated for 6, 24, 48 or 72h with IL-4 (100ng ml^{-1}) and/or LPS (0.01 or 1μg ml^{-1}). A specific enzyme-linked immunosorbant assay was used to measure VCAM-1, ICAM-1 or E-selectin expression [2]. Results were expressed as mean optical density (OD_{405}) ± s.e. mean of 4 experiments. VCAM-1 mRNA was quantified by Northern blot analysis [2] following extraction of total HLMVEC mRNA. Leukocytes, isolated from peripheral blood of adult donors, labelled with a fluorescent dye (Calcein-AM, 10μM), were incubated (30 min) with HLMVEC [3]. Results were expressed as mean ± s.e. mean of percent adherent over total cells (1.25×10^5) added per well, as determined by fluorescence.

VCAM-1 was not detected on resting HLMVEC but was induced by IL-4 only at 72h (OD_{405}: 0.12±0.01). LPS (0.01 or 1μg ml^{-1}) alone did not induce VCAM-1 (24h) but synergised with IL-4 to induce expression (0.16±0.01 and 0.40±0.02). Similar effects were seen at 48 or 72h. LPS (1μg ml^{-1}) and IL-4 also synergised to induce VCAM-1 mRNA at 4h. IL-4 did not induce ICAM-1 or E-selectin, or alter LPS-induced expression. Pretreatment of HLMVEC with IL-4 and LPS (1μg ml^{-1}) together, but neither stimuli alone, significantly ($P<0.05$) increased eosinophil adhesion from 8.2±0.1 to 28.4±3.3% (n=4). Adhesion was significantly ($P<0.05$) reduced by an anti-VLA_4 monoclonal antibody (mAb: 2B4, 30μg ml^{-1}) to 24.2±4.9% (n=3) of LPS/IL-4 induced-adhesion; anti-CD18 mAb (6.5E; 30μg ml^{-1}) had no effect. Neutrophil adhesion to LPS-treated HLMVEC (16.7±2.0%, n=3) was significantly ($P<0.01$) increased compared with basal adhesion (4.40±0.50%, n=4) but was not altered by co-stimulation with IL-4 (19.4±1.7%, n=4).

These results show that IL-4 and LPS synergise to induce VCAM-1 expression and mRNA in HLMVEC resulting in a VLA_4-dependent increase in eosinophil adhesion. This suggests that IL-4, in the presence of LPS, may trigger recruitment of eosinophils into the lung.

[1] Schleimer, R.P., Sterbinsky, S.A., Kaiser, J. *et al.*, (1992) *J. Immunol.* 184, 1086-1092.

[2] Burke-Gaffney, A. & Hellewell, P.G. (1996) *Am. J. Physiol.* 270, C552-C561.

[3] Burke-Gaffney, A. & Hellewell, P.G. (1996) *Biochem. Biophys. Res. Commun.* (in press)

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58P CHOLINESTERASE ACTIVITY IN HUMAN PULMONARY VESSELS

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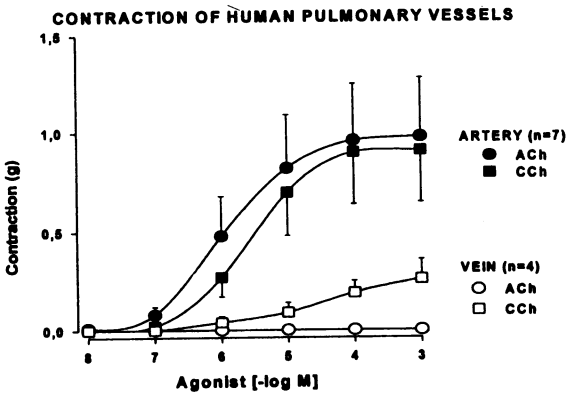
Acetylcholine (ACh) has been shown to induce either vasorelaxation or vasoconstriction (Norel *et al.*, 1996). These effects may depend on ACh degradation by cholinesterases (Nandiwada *et al.*, 1983). The aim of this study was to determine the role of cholinesterases in human pulmonary vessels.

In human pulmonary arteries and veins (endothelium removed), contractions induced by ACh or carbachol (CCh) were measured. The vascular preparations were cut as rings (3-6 mm internal diameter), set up in organ baths containing Tyrode's solution and placed under an initial load (1.5 g). Changes in force were recorded using an isometric force displacement transducer and physiographs. Dose response curves are shown in the figure below as means ± SEM expressed in grams.



A biochemical study was also performed to determine the enzymatic activities of acetylcholinesterase (AChE, EC 3.1.1.7.) and butyrylcholinesterase (BChE, EC 3.1.1.8.) in both types of preparations. Kinetic parameters of cholinesterases were measured (Ellman's colorimetric method) after osmotic shock on arterial and venous homogenates. Results are shown below (means ± SEM; n=6 lung samples; *indicates $P<0.05$ vs artery).

	AChE		BChE	
	Vmax	Km	Vss	Kss
Artery	1.73 ± 0.24	97 ± 17	1.83 ± 0.22	362 ± 72
Vein	3.36 ± 0.26*	106 ± 19	4.71 ± 0 17*	491 ± 87

Vmax and Vss are expressed in mIU/mg protein; Km and Kss are expressed in µM. AChE and BChE activities were measured in the presence of iso-OMPA (10 µM) and BW284C51 (1 µM), respectively.

A greater cholinesterase activity (two fold) was observed in veins when compared with results obtained in arteries. This observation may partly explain why arteries are more sensitive to ACh than veins.

Norel *et al.*, (1996) Br. J. Pharmacol. 119, 149-157.

Nandiwada *et al.*, (1983) Circ. Res. 53, 86-95.

59P PHARMACOLOGICAL EVIDENCE THAT NITRIC OXIDE AND CHOLINESTERASE IN THE EPITHELIAL LAYER SUPPRESSES THE ACETYLCHOLINE-INDUCED CONTRACTIONS IN GUINEA-PIG AIRWAYS

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Nitric oxide (NO) released by the epithelial layer upon stimulation with pharmacological agents suppresses the contraction of guinea pig tracheal tubes (Nijkamp *et al.*, 1993, Folkerts *et al.*, 1995a,b). In the present study it was investigated whether the acetylcholine-induced contractions were modulated by endogenous NO and/or cholinesterase in the epithelial layer.

Male Dunkin Hartley guinea pigs (400-500 g) were killed with Euthesate© (1 g/kg, i.p.). A method was used in which only the mucosal (in-)side of the guinea pig tracheal tube was stimulated with pharmacological agents and isometric contractions measured. Cumulative concentration-response (C/R) curves with acetylcholine were made in intact or epithelium-denuded trachea in organ baths containing Krebs-solution (37°C). Intact tissues were incubated with the NO synthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 120 μM, 20 min) to investigate the role of endogenous NO in the acetylcholine-induced contractions. In a separate set of experiments the cholinesterase inhibitor physostigmine (10 μM) was added to the preparations to investigate the role of cholinesterase in the acetylcholine-induced contractions.
Data are expressed as the mean ± SEM. Significant differences between experimental groups were determined by two-way ANOVA and/or a Students unpaired *t*-test.

The maximal contraction in response to acetylcholine (Emax) was 674±81 mg and the pD_2 value was 4.22±0.12 (n=6). Emax values were enhanced ($p<0.05$) after incubation with L-NAME (Emax=1374±174 mg, pD_2=4.80±0.15, n=6). Epithelium removal had a far more pronounced effect (Emax=2486±120 mg, pD_2=6.37±0.06, n=5). This could be due the high activity of cholinesterase. Indeed, addition of physostigmine induced a contraction of itself (see later) and the total contraction and pD_2-value after the acetylcholine C/R curve (n=5) was increased to the levels observed in epithelium -denuded preparations. In epithelium-denuded tissues with physostigmine only the pD2 value was increased (7.48±0.04, n=4). Interestingly, as mentioned above, in intact tissues, physostigmine itself induced a potent contraction 1841±223 mg (n=6) which was completely inhibited by atropine (10 μM, 10 min, n=5). However, in epithelium-denuded tissues the physostigmine-induced contractions were decreased by 45% ($p<0.05$, 1028±260 mg, n=4). These findings suggest the presence of acetylcholine in the epithelial layer.

In conclusion, a decreased cholinesterase activity and/or a decreased production of NO contributes to an enhanced tracheal contraction in response to acetylcholine. The suggestion for the presence of acetylcholine in the epithelial layer may signify the physiological relevance of these observations.

Folkerts, G., Linde, van der H.J. & Nijkamp, F.P. (1995a) *J. Clin. Invest.* 95, 26-30.
Folkerts, G., Linde, van der H.J. *et al.* (1995b) *Br. J. Pharmacol.* 115, 1194-1198.
Nijkamp, F.P., Linde, van der H.J. Folkerts, G. (1993) *Am. Rev. Resp. Dis.* 148, 727-734.

60P ACETYLCHOLINE INHIBITS HISTAMINE RELEASE FROM ISOLATED HUMAN BRONCHI VIA STIMULATION OF MUSCARINIC RECEPTORS

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Human lungs are a considerably rich source of histamine which is mainly stored in secretory granules of mast cells and partly in basophils. Mast cells are widely distributed throughout the respiratory tract and are even found between airway epithelial cells rather close to the airway lumen. These mast cells are among the first cells to interact with inhaled antigens and other agents and in consequence may frequently be activated to release histamine which is involved in human asthma.

In the present experiments we have investigated whether a regulatory link exists between the cholinergic system and human pulmonary mast cells. Human bronchi obtained at thoracotomy from patients with lung tumor were incubated in organ baths to measure histamine release. The medium was exchanged in 5 min intervals. Histamine content was determined after derivatisation with *o*-phthaldialdehyde by reverse phase HPLC combined with fluorometric detection.

Human epithelium-intact segmental bronchi contained 260±30 nmol/g histamine (means±s.e.m.), removal of the surface epithelium reduced the tissue content to 140±30 nmol/g (n=4). Incubated human bronchi released spontaneously about 2-3 nmol/g*5min histamine. Exposure (1 min) to the calcium-ionophore A23187 (10 μM) increased histamine release by 146 ±29 % (n=11) above the pre-stimulation level. Likewise, human anti-IgE ab (1:3000) stimulated histamine release by 127±32 % (n=6) above the pre-stimulation level, whereas bradykinin (1 μM) or compound 48/80 (10 μg/ml) were ineffective. In epithelium-denuded bronchi A23187-induced histamine release was reduced by 80% indicating a preferentially activation of mucosally localized mast cells and basophils.

Acetylcholine reduced anti-IgE ab- and A23187-induced histamine release maximally by 70±7% (n=6) and 89±9% (n=4), respectively, in an atropine-sensitive manner. Surprisingly low concentrations (0.1-100 nM) of acetylcholine were already effective in reducing evoked histamine release, but acetylcholine lost its inhibitory potency at a concentration of 10 μM. Oxotremorine (1 nM), a stable agonist at muscarinic receptors, completely suppressed A23187-induced histamine release, whereas concentrations higher than 1 nM were less effective. Also physostigmine (0.1 μM), a cholinesterase inhibitor, reduced A23187-induced histamine release by 58±10 % (n=12).

In conclusion, the present experiments demonstrate an inhibitory link between acetylcholine and histamine release from human airways, an effect mediated by muscarinic receptors. The inhibitory action of physostigmine indicates the involvement of endogenous, probably non-neuronal acetylcholine which is expressed in human airway epithelium (Münch *et al.*, 1996; Reinheimer *et al.*, 1996). Epithelial damage may cause desinhibition of pulmonary mast cell function.

Münch, M., Reinheimer, T., Klapproth, H., *et al.*, (1996) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **354**, R6.
Reinheimer, T., Bernedo, P., Klapproth, H., *et al.*, (1996) *Am. J. Physiol.* **270**, L722-L728.

61P NON-NEURONAL ACETYLCHOLINE, A WIDESPREAD SIGNALLING MOLECULE IN MAN

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Acetylcholine is known as a prominent neurotransmitter in the central and peripheral nervous system. In addition, non-neuronal cells of mammals have already been shown to synthesize acetylcholine (placenta, corneal epithelium, spleen, skin; see Sastry & Sadavongvivad, 1979). In the experiments described here we have investigated the expression of acetylcholine in non-neuronal surface cells of man.

Epithelial cells of the airways and alimentary tract were analysed i) by measuring acetylcholine content in extracts of surface cells, ii) by measuring the activity of the synthesizing enzyme choline acetyltransferase (ChAT), iii) by immunohistochemistry for ChAT-like proteins, and iv) by Western blot analysis. Human tissue was obtained at surgery from patients with tumors. Epithelial cells were removed by rubbing the luminal surfaces without penetrating the underlying basal membrane. Acetylcholine was measured by a sensitive and highly specific HPLC-method using substrat-specific enzyme reactor columns. A polyclonal anti-ChAT ab was used for immunohistochemistry and Western blot (Schemann *et al.*, 1993).

Acetylcholine was detected in the surface epithelium of human bronchi (33±10 pmol/g; n=14; means±s.e.m.), oral mucosa of males (8±2 pmol/sample (4)) and females (0.7±0.3 (5)), small and large intestine (800-16 pmol/g), gall-bladder (12±5 pmol/g (5)) and vagina (6±2 pmol/sample (5)). Acetylcholine was also detected in human skin (1000±300 pmol/g (7)), hairs (1500± 200 pmol/g), saliva and surface cells covering ureter and urinary bladder. ChAT-activity was found in isolated epithelial cells of human bronchi and small intestine (3.5±1.3 (5) and 28±11 (5) nmol/mg protein/h, respectively). Airway and intestinal epithelial cells expressed strong ChAT-like immunoreactivity. ChAT-like proteins extracted from isolated bronchial epithelial cells were also demonstrated by Western blot (54 and 41 kd).

In functional experiments it was tested whether acetylcholine affects the poliferation of human bronchial epithelial cells in primary culture. Proliferation was investigated by measuring the conversion of MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolin) to coloured formazan. Acetylcholine (1, 100, 1000 nM) mediated a significant increase in the proliferation, whereas bromoacetylcholine, the specific ChAT-inhibitor, caused the opposite effect. The stimulatory effect of acetylcholine was prevented by atropine (1 μM) and tubocurarine (30 μM), but only when added together.

In conclusion, the present experiments demonstrate for the first time a rather widespread expression of non-neuronal acetylcholine in surface cells of man. We propose that non-neuronal acetylcholine acts as an autocrine/paracrine signalling molecule controlling important cell functions.

Sastry, B.V.R. & Sadavongvivad C. (1979) *Pharmacol. Rev.* **30**, 65-132.
Schemann, M., Sann, H., Schaaf, C. *et al.* (1993) *Am. J. Physiol.* **265**, G1005-G1009.

62P ATYPICAL MUSCARINIC CHOLINOCEPTOR (mAChR) MEDIATING CARBACHOL-INDUCED CONTRACTION OF THE GUINEA-PIG UTERUS

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In many smooth muscle tissues, a minor M_3 mAChR population mediates contraction, despite the presence of a larger M_2 mAChR population. However, it has been suggested that this may not be the case for guinea pig uterus, where radioligand binding and contractile studies support a dominant role for M_2 mAChRs (for review see Eglen *et al.*, 1996). In tissues where a heterogeneous mAChR population exists, selective alkylation procedures have been used to isolate single subtype populations (Thomas *et al.*, 1993). In the current study, estimates of antagonist affinity have been made before and after selective alkylation procedures, together with estimates of agonist affinity to characterise more fully the mAChR population mediating carbachol-induced contractions in the guinea pig isolated uterus.

Adult, female oestrogen-dominant (diethyl stilboestrol 0.1mgkg^{-1} i.p.) Hartley guinea pigs were killed by CO_2 asphyxiation. Uterine horns were each cut into four longitudinal strips and suspended in 10ml organ baths (initial tension 1g) in a modified Sund's solution (mM;NaCl 154, KCl 5.63, $MgCl_2$ 0.98, $NaHCO_3$ 5.95, $CaCl_2$ 0.48, glucose 2.78, indomethacin 3μM, tetrodotoxin 1μM, cocaine 30μM, and corticosterone 30μM) at 32°C, aerated with 95% O_2/5% CO_2.

Antagonist affinity estimates were obtained by Schild regression analysis of carbachol-induced responses (Cumulative additions; Control pEC_{50}=5.79±0.05; max tension 2-4g) using at least five concentrations of antagonist. Schild slopes were not significantly different from 1 (P>0.05), and were constrained to 1 to estimate pK_B values (Table 1). Contractions to carbachol were abolished by phenoxybenzamine (1-3μM; incubated for 20min) and could be protected by equilibration with methoctramine (0.1μM; 1hr), followed by extensive washing (90min, 5min intervals). At this concentration, methoctramine occupies ~85% of M_2 and <10% M_3 mAChRs in a heterogeneous population. However, when antagonist affinity estimates were made at these 'protected' receptors, using single antagonist concentrations, the affinity profile was not significantly different from that seen for untreated tissues (P>0.05) (Table 1).

Table 1. Affinity estimates at mAChR mediating contraction

Antagonist	Untreated	'Protected'	M_2 Atria	M_3 Ileum
Atropine	9.3 ± 0.1	ND	[a]9.1	[a]9.3
Methoctramine	7.1 ± 0.1	7.1 ± 0.1	[a]7.9	[a]6.0
Zamifenacin	8.3 ± 0.1	8.6 ± 0.1	[a]6.6	[a]9.3
Tripitramine	8.1 ± 0.1	8.3 ± 0.1	[b]9.7	[b]6.5

Values shown (pK_B/pA_2) are means ± s.e. mean, n≥7; ND not determined; [a] Caulfield (1993) [b] Chiarini et al (1995)

Furthermore, after partial receptor inactivation with phenoxybenzamine and using simultaneous operational model curve fitting methods, an agonist affinity value (pK_A) was calculated for L-660,863 (Harris *et al.*, 1991) of 5.44 ± 0.30 (n=6). This result does not support a role for M_2 mAChRs in contraction of this preparation (pK_A at M_2 receptors 7.6 ± 0.05: Harris *et al.*, 1991). In contrast, the pK_A value for carbachol (4.22 ± 0.17; n=8) agrees with that reported for guinea pig ileum (4.7 ± 0.5; Ford *et al.*, 1991).

These data suggest that contractions to carbachol in guinea pig isolated uterus are mediated not by M_2 mAChRs, as previously reported, but by a single homogeneous population of mAChRs with an atypical operational profile.

Caulfield M.D. (1993) *Pharmacol. Ther.* 58, 319-379
Chiarini *et al.* (1995) *Br. J. Pharmacol.* 114, 1507-1517
Eglen R.M. *et al.* (1996) *Pharmacol. Rev.* (in press)
Ford A.P.D.W. *et al.* (1991) *Mol. Neuropharmacol.* 1, 117-127
Harris G.C. *et al.* (1991) *Br. J. Pharmacol.* 102, 197P
Thomas E.A. *et al.* (1993) *Mol. Pharmacol.* 44, 102-110

63P CHARACTERIZATION OF THE INTERACTION OF TOLTERODINE AT MUSCARINIC RECEPTOR SUBTYPES *IN VITRO* AND *IN VIVO*

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The aim of the present study was to characterize the interaction of tolterodine, (a novel compound under evaluation for the treatment of urge incontinence; Nilvebrant *et al.*, 1996), at muscarinic receptors *in vitro* and *in vivo*.

Methods. The inhibitory potency of the compound at volume-induced bladder contractions (VIBCs) and oxotremorine induced salivation (OIS) in rat (Sprague-Dawley, 200-250g) was measured according to the method of Hegde *et al.*, (1996). Competition radioligand binding studies were conducted at human recombinant muscarinic receptors, stably expressed in CHO-K1 cells using [^{3}H]*N*-methyl scopolamine as the radioligand. Functional estimates of affinity were obtained according to Watson *et al.*, (1995), using guinea-pig (male Dunkin Hartley, 200-250g) atria (left, paced at 2 Hz, supramaximal voltage), oesophageal muscularis mucosae, ileum, trachea and rat isolated bladder. In all studies, tolterodine was equilibrated with the tissues for 60 min., prior to constructing agonist concentration-effect curves.

Results. In rat isolated bladder, tolterodine exhibited an apparent pK_B value of 8.9 ± 0.2. *In vivo*, the compound inhibited VIBCs with a mean (95% confidence intervals) ID_{50} value of 0.036 (0.014 - 0.097) mg kg^{-1}, iv. The ID_{50} at inhibiting OIS was 0.176 (0.033 - 0.32) mg kg^{-1}, iv. These data indicated an *in vivo* selectivity of 4.9 fold for the urinary bladder. Binding studies at human recombinant muscarinic receptors (Table 1; conducted in a Tris-Krebs buffer and therefore with a physiologically appropriate ionic strength) showed little or no subtype selectivity of the compound, even though this was was observed with other antagonists. The functional studies (Table 2) suggested a limited selectivity for the M_2 over the M_3 receptor (6 fold) but no differentiation between the three M_3 receptors studied.

Table 1. pK_i values for ligands at human recombinant muscarinic receptors. (values are mean, s.e. mean < 5%, n=3).

Antagonist	m_1	m_2	m_3	m_4	m_5
atropine	9.1	8.9	9.5	9.2	9.1
pirenzepine	8.0	6.3	6.8	7.0	6.9
methoctramine	6.6	7.6	6.1	6.9	6.4
4-DAMP	9.2	8.1	9.3	8.4	8.9
p-F-HHSiD	7.3	6.6	7.5	7.2	6.7
himbacine	6.6	7.9	6.9	7.4	6.1
tripitramine	8.4	9.4	7.1	7.8	7.3
darifenacin	7.8	7.0	8.9	7.7	8.1
zamifenacin	7.5	7.1	7.9	6.7	7.4
tolterodine	8.5	8.4	8.5	8.1	8.6

(All Hill slopes were not significantly different from unity).

Table 2. pA_2 values for tolterodine at guinea-pig muscarinic receptor subtypes (values are mean ± s.e. mean, n = 9-16).

Tissue		pA_2	Schild slope
atria	(M_2)	8.3 ± 0.2	1.1 (0.88 - 1.26)
oesophagus	(M_3)	9.1 ± 0.1	0.9 (0.76 - 1.06)
ileum	(M_3)	9.1 ± 0.1	1.1 (0.99 - 1.21)
trachea	(M_3)	8.7 ± 0.1	1.1 (0.93 - 1.33)

Summary. Tolterodine was found to be a muscarinic antagonist with little discrimination between muscarinic receptor subtypes, *in vitro*, supporting previous data by Newgreen & Naylor (1996). and Nilvebrant *et al.*, (1996). The *in vivo* bladder selectivity of tolterodine was similar to that reported for oxybutynin (3 fold; Hegde *et al.*, 1996).

Hegde, S.S. *et al.*, (1996) *Proceedings of the 26th Annual Meeting of the International Continence Society, abstract 126.*
Newgreen, D.T. & Naylor, A.M. (1996) *Br. J. Pharmacol.* **117**, 107P
Nilvebrant, L. *et al.* (1996) *Neurol. Urodyn.* **15**, 310-311.
Watson, N. *et al.*, (1995) *Eur. J. Pharmacol.* **285**, 135-142.

64P INHIBITION OF HYPOXIC PULMONARY VASOCONSTRICTION IN ISOLATED RAT PULMONARY ARTERIES BY IODONIUM DIPHENYL

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The NADPH oxidase inhibitor diphenyleneiodonium (DPI) has previously been shown to selectively inhibit hypoxic pulmonary vasoconstriction (HPV) in the isolated rat lung (Thomas *et al.*, 1991). We have therefore investigated whether the analogue of DPI, iodonium diphenyl (ID) has a similar effect on HPV in isolated rat pulmonary arteries *in vitro*. Vessels (n=31) were mounted as rings in an automated small vessel myograph (Cambustion Ltd, UK) and precontracted twice with potassium chloride (KCl, 80mM) to ensure that each vessel was reacting consistently to the same concentration of agonist. Subsequent contractions were standardised by expressing as a percentage of these KCl contractions. Initially, vessels were primed with a submaximal concentration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 0.5μM), prior to an hypoxic challenge. This was necessary for the production of HPV in this preparation. Hypoxic contractions were then repeated following the addition of either ID (0.1, 10 or 50μM) or the vehicle, distilled water (250μl) so that the effect of ID on the first contractile phase of the hypoxic response and on the priming contraction due to $PGF_{2\alpha}$, could be studied.

ID (10 and 50 μM) significantly inhibited the first contractile phase of the hypoxic response. In the presence of 10μM ID the hypoxic contraction was 3.36% compared to a control value of 15.97%, ($P<0.01$), and 2.82% in the presence of 50μM ID compared to a control value of 15.01% ($P<0.01$). ID (10 and 50μM) also increased the size of the priming contraction following the addition of $PGF_{2\alpha}$ (0.5μM). In the presence of 10μM ID the $PGF_{2\alpha}$ contraction was 25.63% compared to a control value of 4.02% ($P<0.01$), and 22.73% in the presence of 50μM compared to a control value of 5.99% ($P<0.01$). No significant effect was seen with either 0.1μM ID or distilled water (250μl) on either the first contractile phase of the hypoxic response, or the contraction due to the addition of the priming concentration of $PGF_{2\alpha}$ (0.5μM).

The fact that an inhibitor of NADPH oxidase, the enzyme responsible for the production of reactive oxygen species from molecular oxygen, reduced the pulmonary vascular response to hypoxia, may indicate that this or a similar enzyme is involved in oxygen sensing in the pulmonary artery. This action may be non specific, however, since $PGF_{2\alpha}$ contractions are also affected.

Thomas, III, H.M., Carson, R.C., Fried, E.D. *et al.*(1991) *Biochem.Pharm.* 42, R9-R13.

65P INHIBITION OF HYPOXIA-, PROSTAGLANDIN $F_{2\alpha}$- AND POTASSIUM CHLORIDE-INDUCED CONTRACTIONS IN RAT ISOLATED PULMONARY ARTERIES BY HYDROGEN PEROXIDE

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It is well documented that hydrogen peroxide (H_2O_2) is a toxic agent, being involved in many types of tissue injury (Grisham & McCord, 1986). Since oxygen radicals are produced within the lung, we have studied the effect of H_2O_2, on the first contractile phase of the hypoxic response, on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced contractions and on potassium chloride (KCl)-induced contractions, to establish whether pulmonary reactivity is altered. Isolated rat pulmonary arteries (n=20) were mounted in a small vessel myograph (Cambustion Ltd, UK) and initially preconstricted twice with a maximal concentration of KCl (80mM) to ensure reproducibility of the contractile response. Subsequent contractions were standardised by expressing as a percentage of these KCl contractions. One of three methods was then followed; 1) precontraction with a priming concentration of $PGF_{2\alpha}$ (5μM) followed by production of a hypoxic contraction (n=7), 2) production of a $PGF_{2\alpha}$ (1-100μM) concentration-response curve (n=6), or 3) production of a KCl (1-100mM) concentration-response curve (n=7). These responses were then repeated in the presence and then absence of H_2O_2 (0.03 or 0.5mM).

H_2O_2 (0.03mM) had no significant effect upon either the hypoxic contraction (29.9% compared to the control value of 30.3%, $P>0.1$), or the priming concentration of $PGF_{2\alpha}$ (17.2% compared to a control value of 22.4%, $P>0.1$). H_2O_2 (0.5mM) significantly inhibited both the hypoxic contraction (1.6% compared to the control value of 30.3%, $P<0.01$), and the contraction to the priming concentration of $PGF_{2\alpha}$ (0%, $P<0.01$).Both the hypoxic and the $PGF_{2\alpha}$ contraction obtained following washout of H_2O_2 were identical to control values (30.3% and 19.5% respectively, both $P>0.1$). H_2O_2 (0.5mM) also significantly reduced the efficacy (V_{max}) of the $PGF_{2\alpha}$ concentration-response curve (6.4% compared to the control value of 56.6%, $P<0.01$), and the efficacy of the KCl concentration-response curve (23.2% compared to the control value of 65.9%, $P<0.01$). Following washout of H_2O_2 the V_{max} of both concentration-response curves was significantly higher than the test curves (42.8% and 43.9% respectively, both $P<0.01$).

Since the inhibitory effect of H_2O_2 appears to be partially reversible, H_2O_2 may inhibit the release of Ca^{2+} ions from intracellular stores, as this is the common mechanism of each of these contractile responses, rather than cause toxicity.

Grisham,M.B. & McCord,J.M. (1986) in *Physiology of Oxygen Radicals* ed Taylor,A.E. *et al.* pp1-18. American Physiological Society.

66P INHIBITION OF DIABETIC HYPERFILTRATION BY JB1, A PEPTIDE ANTAGONIST OF THE TYPE 1 IGF RECEPTOR

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Insulin-like growth factor 1 (IGF-I) increases the glomerular filtration rate (GFR) (Hirschberg *et al.*, 1991) and recent evidence from isolated glomerulii, shows an increase in IGF-I and its receptor following the induction of diabetes at both the mRNA and protein level (Sugimoto *et al.*, 1995). Antagonists of the IGF receptor have been described, which are 12-amino acid cyclic peptides, designed to mimic the D-domain of the human IGF protein. In the present study, diabetic hyperfiltration has been measured in the presence and absence of JB1, an antagonist of the type I IGF receptor (Pietrzkowski *et al.*, 1992), using an isolated kidney preparation *ex vivo* (McKillop *et al.*, 1995).

Diabetes was induced in male Wistar rats (350-500g) by a single injection of streptozotocin (45 mg/kg, i.p.). After 7 days, rats were anaesthetised using thiopentone (100mg/kg, i.p.). The right kidney perfused by a non-ischaemic technique at a constant pressure of 100mmHg using a physiological solution based on the plasma volume expander Haemaccel. GFR was assessed from the renal clearance of [^{14}C] inulin. The glucose concentration of the perfusate was increased from 5 to 20mM when kidneys from diabetic rats were perfused compared to non-diabetic controls.

Diabetic rats achieved blood glucose concentrations of 20.2 ± 1.4 vs 5.6 ± 0.4 mmol/l and showed an increase in kidney growth, left kidney wet weight being significantly higher than non-diabetic controls 1.46 ± 0.05 vs 1.26 ± 0.04 ($P<0.01$, $n=12$ per group). In kidneys perfused from diabetic rats, [^{14}C] inulin clearance was significantly higher than in non-diabetic controls (1.07 ± 0.09 vs 0.64 ± 0.11 ml/min $P<0.01$, $n=12$ per group). In kidneys perfused from non-diabetic rats, [^{14}C] inulin clearance was stable with time and remained unaffected by the presence of JB1 1μg/ml in the perfusate (0.67 ± 0.11 vs 0.70 ± 0.13 ml/min NS, $n=6$ per group). In kidneys obtained from diabetic rats, the elevated [^{14}C] inulin clearance was markedly reduced from 1.08 ± 0.16 to 0.64 ± 0.17 ml/min ($P<0.05$ $n=6$) following the addition of JB1 1μg/ml (perfusate concentration). In contrast to the changes in [^{14}C] inulin clearance, the addition of JB1 to the perfusion circuit was not associated with any change in renal perfusate flow to kidneys obtained from either diabetic or non-diabetic animals.

The decrease in GFR following the administration of JB1, an antagonist of the type 1 IGF receptor, to kidneys obtained from the diabetic rat suggest that renal IGF-I may help to mediate diabetic hyperfiltration.

Hirschberg, R., Kopple, J.D. Blantz, R.C. *et al.* (1991) *J. Clin. Invest.* 87, 1200-1206.
McKillop, I., Haylor, J. & El Nahas, A.M. (1995) *Exp. Nephrol.* 3, 49-57.
Pietrzkowski, Z., Wernicke, D., Porcu, P. *et al.* (1992) *Cancer Res.* 52, 6447-6451.
Sugimoto, H., Shikata, K., Makino, H. *et al.* (1995) *J. Diabet. Complic.* 9, 262-264.

67P INTERACTIONS OF GR127935, A 5-HT$_{1B/D}$ RECEPTOR LIGAND, WITH FUNCTIONAL 5-HT RECEPTORS

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In binding studies GR127935 exhibits high and selective affinity for 5-HT$_{1B/D}$ receptors (pK$_i$: 9.9 and 8.9, respectively) and it antagonizes a number of 5-HT$_{1B/D}$ receptor-mediated responses (Skingle et al., 1996). The present experiments were performed to investigate the selectivity of GR127935 against functional responses mediated by 5-HT$_1$-like, 5-HT$_{2A}$, 5-HT$_3$ and 5-HT$_4$ receptors in several *in vivo* preparations. In vagotomized, pentobarbital anaesthetized rabbits (n=7), the 5-HT$_1$-like receptor agonist sumatriptan (1-100 μg kg^{-1}, i.v.) decreased carotid blood flow and arterial blood pressure (baseline 53.9±7.8 ml min^{-1} and 74.7±2.4 mmHg, respectively). Both responses were potently antagonized by GR127935 (0.3 mg kg^{-1}, i.v.; Table 1). As described by Saxena & Lawang (1985), 5-HT (3-30 μg kg^{-1}, i.v.) produced a triphasic response in pentobarbital anaesthetized rats with intact vagus (n=6), consisting of an early depressor (5-HT$_3$), a pressor (5-HT$_{2A}$) and a late depressor ('orphan' 5-HT$_1$-like; 5-HT$_7$?) phase from baseline (111±2 mmHg and 265±13 beats min^{-1}, respectively). Treatment with GR127935 (0.5 mg kg^{-1}, i.v.) did not affect the initial depressor response, but attenuated the pressor response and slightly enhanced the late depressor response (Table 1). The 5-HT$_2$ receptor agonist DOI (1-100 μg kg^{-1}, i.v.) produced a pressor response without altering heart rate in 6 vagotomized, pentobarbital anaesthetized rats (baseline 86±2 mmHg and 216±29 beats min^{-1}). This pressor response was attenuated by GR127935 (0.5 mg kg^{-1}, i.v.; Table 1). In anaesthetized, vagosympathectomized pigs (n=5) pretreated with methiothepin (0.5 mg kg^{-1}), 5-HT (10 and 30 μg kg^{-1}, i.v.) increased (via 5-HT$_4$ receptors; Villalón *et al.*, 1991) heart rate (baseline 105±7 beats min^{-1}) by 45±8 and 70±9 beats min^{-1}, respectively, without altering baseline blood pressure (87±3.1 mmHg). These tachycardiac responses to 5-HT were not blocked after i.v. administration of 0.5 mg kg^{-1} of GR127935 (59±7 and 73±9 beats min^{-1}, respectively). In conclusion, the present study demonstrates that GR127935 is a selective 5-HT$_{1D}$ receptor antagonist devoid of interactions at 'orphan' 5-HT$_1$-like (5-ht$_7$?), 5-HT$_3$ and 5-HT$_4$ receptors. However, GR127935 possesses a moderate 5-HT$_{2A}$ receptor blocking property, which is consistent with its binding profile (pK$_i$: 7.4; Skingle *et al.*, 1996). Lastly, in view of potent antagonist action of GR127935, the sumatriptan-induced hypotension in rabbits seems to be mediated by 5-HT$_{1B/D}$ receptors.

Skingle, M. *et al.* (1996). *Behav. Brain Res.*, 73, 157-161.
Saxena, P.R. & Lawang, A. (1985). *Arch. Int. Pharmacodyn.*, 277, 235-252.
Villalón, C.M. *et al.* (1991). *Br. J. Pharmacol.*, 102, 107-112.

Table 1. Changes in carotid blood flow (CBF; ml min^{-1}) and blood pressure (mmHg) by various compounds before and after GR127935.

	Sumatriptan-induced responses in rabbits				5-HT-induced triphasic blood pressure response in rats						DOI in rats	
Agonist	CBF		Depressor		Early depressor		Pressor		Late depressor		Pressor	
(μg kg^{-1})	*Before*	*After*[a]	*Before*	*After*[a]	*Before*	*After*[b]	*Before*	*After*[b]	*Before*	*After*[b]	*Before*	*After*[b]
1	-1.4±0.4	0.1±0.1*	-1.7±0.3	0.7±0.3							10.7±1.3	3.0±0.7*
3	-4.1±1.0	-0.0±0.3*	-3.3±1.1	-0.5±0.3	0.0±0.0	0.0±0.0	3.0±2.1	2.8±1.5	-21.7±2.6	-31.4±2.9*	19.2±1.9	5.0±0.7*
10	-11.4±2.2	0.1±0.4*	-8.0±2.1	-0.4±0.3*	-32.2±7.3	-26.0±12.7	15.2±5.8	5.4±3.5	-38.7±3.3	-39.6±4.9	30.8±2.2	9.8±1.2*
30	-17.0±3.4	0.5±0.4*	-18.0±2.2	0.6±0.3*	-55.5±6.7	-57.8±10.3	39.3±6.1	9.8±4.1*	-46.8±2.6	-48.0±4.4	33.3±3.1	15.5±1.8*
100	-20.0±5.3	-0.4±0.8*	-19.4±3.5	-1.5±1.4*								21.5±1.7

*[a], GR127935 0.3 mg kg^{-1}; [b], GR127935 0.5 mg kg^{-1}. *, P<0.05 vs corresponding value before GR127935.*

68P CURRENT AND FUTURE ANTI-MIGRAINE DRUGS IN THE HUMAN ISOLATED CORONARY ARTERY

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The anti-migraine drugs ergotamine and sumatriptan contract the human isolated coronary artery (HCA) (e.g. Bax & Saxena, 1993). We have now compared HCA contraction to a number of anti-migraine drugs - ergotamine, dihydroergotamine, methysergide and its metabolite methylergometrine, the 5-HT$_1$ receptor agonists sumatriptan, naratriptan, zolmitriptan, rizatriptan and avitriptan as well as 5-HT - in a paired parallel design, which is important in view of the variability of contractile responses to sumatriptan (Kaumann *et al.*, 1994; MaassenVanDenBrink *et al.*, 1996). Also, we investigated whether the pD$_2$ values correlated with the pK$_i$ values for the 5-HT$_{1B}$ (former 5-HT$_{1D\beta}$), 5-HT$_{1D}$ (former 5-HT$_{1D\alpha}$), and 5-HT$_{1F}$ receptor (P.J. Pauwels, personal communication). Ring segments of right epicardial coronary artery (9 heart valve donors; 5 M, 4 F; 7-50 yr), with intact endothelium, were suspended in organ baths. Tension was recorded isometrically and expressed as percentage of contraction to 100 mM K$^+$. All anti-migraine drugs investigated induced dose-dependent contractions of HCA (Table). Ergotamine, dihydroergotamine and methylergometrine as well as, to a lesser degree, methysergide, avitriptan, naratriptan and zolmitriptan, were more potent than sumatriptan. The maximal effect (E$_{max}$) of 5-HT and ergotamine was higher than that of sumatriptan, whereas the E$_{max}$ of methysergide, avitriptan and naratriptan tended to be somewhat smaller. The pD$_2$ values of sumatriptan, naratriptan, rizatriptan, zolmitriptan, and dihydroergotamine did not significantly correlate to the pK$_i$ values for either the 5-HT$_{1B}$, 5-HT$_{1D}$, or 5-HT$_{1F}$ receptor. In conclusion, our results show that HCA contraction to ergotamine, dihydroergotamine and methylergometrine is more potent than that to sumatriptan, and that the E$_{max}$ of ergotamine is higher. This difference in potency is more than the difference in the clinical dose of the compounds (Bax & Saxena, 1993). Although some of the newer 5-HT$_1$ receptor agonists were slightly more potent and/or less efficacious than sumatriptan on HCA, it remains to be seen whether these differences are clinically relevant.

Bax, W.A. & Saxena, P.R. (1993). *Lancet* 341, 1420.
Kaumann, A.J., Frenken, M., Posival, H. *et al.* (1994). *Circulation* 90, 1141-1153.
MaassenVanDenBrink, A., Bax, W.A., Ferrari, M.D., *et al.* (1996). *Br. J. Pharmacol.* 119, 855-862.

(mean±s.e.mean)	pD$_2$	E$_{max}$ (%K$^+$)
Sumatriptan	6.2±0.2	14.0±2.9
5-HT	6.6±0.1	58.3±7.9‡
Naratriptan	6.9±0.1‡	10.1±2.1†
Rizatriptan	6.5±0.2	10.1±2.7
Zolmitriptan	6.4±0.1†	11.8±2.5
Avitriptan	7.1±0.1‡	7.7±1.8†
Ergotamine	8.0±0.1‡	20.5±3.7†
Dihydroergotamine	8.0±0.2‡	13.0±2.5
Methysergide	7.0±0.2†	7.8±1.9†
Methylergometrine	7.6±0.2‡	13.4±3.5

†) Significantly different from sumatriptan (0.05≥P≥0.01)
‡) Significantly different from sumatriptan (P<0.01; paired *t*-test)

69P DYNORPHIN A IS THE ONLY ENDOGENOUS OPIOID PEPTIDE WITH HIGH AFFINITY FOR THE ORL_1-BINDING SITE

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Although classical opioids have little or no affinity for ORL_1 receptors (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), dynorphin A and, to a lesser extent, dynorphin B have been shown to interact with the ORL_1 receptor in a number of assay systems (Mollereau *et al*, 1994; Reinscheid *et al.*1995; Zhang & Yu,1995). Furthermore, dynorphin A was found to inhibit the binding of [^{125}I]-nociceptin with a K_i value of 449nM (Reinscheid *et al.*, 1996).

We have demonstrated the presence of high affinity binding sites for [^{3}H]-nociceptin in homogenates of guinea-pig brain (Paterson & McKnight, 1996). We have investigated the interaction of the endogenous opioid peptides with the ORL_1-binding site and compared it with their activity at μ-, δ - and κ - opioid sites.

Membranes prepared from the brains of male Dunkin-Hartley guinea-pigs were incubated with tritiated ligands for 90 min at 0°C to minimise metabolism of the peptides. The ORL_1-binding sites were labelled with 0.1nM [^{3}H]-nociceptin, the μ-sites with 1nM [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, the δ-sites with 1.5nM [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin and the κ-sites with 0.3nM [^{3}H]-bremazocine in the presence of 100nM unlabelled [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin and [D-Ala^2, D-Leu^5] enkephalin. Non-specific binding was determined with 100nM unlabelled nociceptin or 1μM diprenorphine for opioid ligands.

Nociceptin displaced the binding of [^{3}H]-nociceptin with a pK_i of 9.43±0.18 (n=3). Although dynorphin A and dynorphin B inhibited the binding of [^{3}H]-nociceptin with pK_i values of 6.99±0.20 (n=3) and 5.79±0.21 (n=4), respectively, none of the other endogenous fragments derived from prodynorphin, proenkephalin or pro-opiomelanocortin caused >20% inhibition at a concentration of 1μM (n=3). Dynorphin A(2-17), which is inactive at opioid binding sites, was inactive at the ORL_1-sites. The ability of the endogenous opioid peptides to inhibit [^{3}H]-nociceptin binding did not correlate with their activity at any of the three opioid sites. The fact that only dynorphin A retains significant activity at ORL_1-sites may explain the abnormal locomotor effects reported with high doses of dynorphin A *in vivo*.

Meunier, J.-C., Mollereau, C., Toll, L. *et al.* (1995). *Nature* 377, 532-535.
Mollereau, C., Parmentier, M., Mailleux, P. *et al.* (1994). *FEBS Lett.* 341, 33-38.
Paterson, S.J. & McKnight, A.T. (1996). *Br. J. Pharmacol.* 119, 35P.
Reinscheid, R.K., Nothacker, H.-P., Bourson, A. *et al.* (1995). *Science* 270, 792-794.
Reinscheid, R.K., Ardati, A., Monsma, F.J. *et al.* (1996). *J. Biol. Chem.* 271, 14163-14168.
Zhang, S. & Yu, L. (1995). *J. Biol. Chem.* 270, 22772-22776.

70P EFFECT OF NEUROTROPHIN 3 ON THE RELEASE OF SUBSTANCE P FROM THE RAT SPINAL CORD

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Neurotrophin 3 (NT-3) belongs to the superfamily of neurotrophic factors, the neurotrophins. In developing rats large myelinated proprioceptive fibres depend on NT-3 for survival whilst umyelinated fibres (nociceptors) depend on nerve growth factor (NGF). In the adult rat the effects of NT-3 are not yet defined whereas NGF is emerging as a nociceptive substance which can increase substance P (SP) release (Malcangio et al.,1996).

In this study we have investigated the effect of 2 week treatment of male Wistar rats with NT-3 (1 mg/kg s.c. 3 times a week) on the release of the nociceptive peptide SP from the central terminals of primary afferent fibres. The effect of acute superfusion with NT-3 of naive rat spinal cord slices on the peptide release was also evaluated. Horizontal lumbar spinal cord slices with dorsal roots (L_4 and L_5) attached were mounted in a 3-compartment bath and superfused with Krebs' solution at room temperature (Malcangio & Bowery, 1993). Three 8 ml-fractions were collected to measure SP basal outflow. The release of SP was induced by electrical stimulation of the dorsal roots (12.3±1.2 mA for 8 min) or by capsaicin 10^{-6}M superfused for 2 min. At the end of each experiment, slices were blotted, weighed and SP extracted in acetic acid. SP-like immunoreactivity (SP-LI) content in concentrated perfusates was determined by specific radioimmunoassay (sensitivity 1 fml/tube). In the acute experiment, NT-3 (1-100 ng/ml) was present 8 min prior to and during 8 min-stimulation of the dorsal roots.

Basal outflow of SP-LI from control spinal cord slices was 17.0 ± 1.1 fmol/8 ml fraction (mean± s.e. mean n=3) and increased by 18.2 ± 1.3 fmol/8 ml fraction over basal during stimulation of the dorsal roots. Treatment with NT-3 did not modify SP basal outflow (16.3 ±1.0 fmol/8 ml fraction, n=5) but significantly inhibited electrically evoked release which was 10.7 ± 1.9 fmol/ 8 ml fraction over basal ($P<0.05$ v controls). However, capsaicin-induced release of SP-LI (20.9 ± 4.7 and 15.8 ± 6.2 fmol/ml control and NT-3, respectively) and total SP-LI content of the cords (94.3 ± 26.8 and 77.3 ± 18.5 fml/mg tissue control and NT-3, respectively) were not significantly decreased by NT-3. Acute superfusion of rat spinal cord slices with NT-3 dose-dependently (1-100 ng/ml) inhibited electrically-evoked SP-LI release over basal outflow (control: 15.5 ±1.8 fmol/8ml fraction, n=6; NT-3 100ng/ml: 1.1 ±0.8 fmol/8ml fraction , n=6).

Thus, both 2 week treatment of rats and acute superfusion of the spinal cord with NT-3 reduced evoked release of SP-LI. We suggest that NT-3 might act as endogenous inhibitor of NGF-pro-nociceptive effect through its effect on peptide release at the spinal cord level.

We thank Regeneron for the gift of NT-3.
Malcangio M. *et al.* (1996) *Br. J. Pharmacol.* **118**, 48P
Malcangio M. & Bowery N.G. (1993) *J. Pharmacol. Exp. Ther.* **266**, 1490-1496.

71P CYCLOOXYGENASES IN RAT SPINAL CORD: SELECTIVE INDUCTION OF COX-2 DURING PERIPHERAL INFLAMMATION

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In a previous study (Willingale & Grubb, 1996) we showed that intrathecally administered NSAIDs have a spinal site of action since they reduce wind-up in a nociceptive flexor withdrawal reflex. In the present study we have examined whether cyclooxygenase (cox) isoforms are present in spinal cord and whether their expression can be regulated following the development of peripheral inflammation.

Rats were killed by barbiturate overdose (sodium thiopentone, 500 mg/kg, i.p.) and perfused transcardially with 0.9% saline. Spinal cords were removed for Western blotting and immunocytochemistry. In order to measure changes in cox levels in spinal cord twenty-eight rats received multi-site injections of Freund's complete adjuvant (total volume 0.15 ml, 1 mg/kg, mycobacterium tuberculosis) at the ankle joint 6 h to 14 days prior to tissue collection (Grubb et al, 1991). Thin frozen sections (10-15 µm) were cut from the lumbar segments L3-L6, fixed in 2% paraformaldehyde and incubated in 1° antibody overnight at 4°C. Following several washes sections were incubated in an FITC-conjugated 2° antibody. For Western blotting spinal cords were finely chopped on dry ice, homogenised in a standard buffer containing Triton X-100 (1%), and centrifuged at 20,000 rpm at 4°C. Protein samples were separated by SDS-PAGE (10%) and transferred to a nitrocellulose filter prior to probing with 1° antibody overnight at room temperature. This was followed by a 2-hour incubation in 2° antibody and exposure using a standard chemiluminescent system. Primary antisera were a commercial cox-1 monoclonal and a cox-2 polyclonal (Cayman Chemical Co). Six rats were anaesthetised with sodium thiobutabarbital (125 mg/kg, i.p.) and subdural catheters placed over the lumbar spinal cord. Artificial csf (pH 7.4) was superfused over the spinal cord (200 µl/min) and collected over ice. Indomethacin (200µM) and EDTA (5mM) were added to each sample to prevent further prostaglandin formation. The concentration of prostaglandins in the spinal cord samples was measured by radioimmunoassay.

Western blot analysis of protein from normal animals showed the presence of both cox-1 and cox-2 isoforms. Tissue sections incubated with cox-1 antisera showed no localised cox-1-like immunoreactivity (li) whereas those incubated with cox-2 antisera showed cox-2-li in laminae I, II, V,VII, VIII and X. This labelling was observed in a halo around neuronal cell bodies indicating that it may be localised in the plasma membrane. More diffuse staining was also observed throughout the grey matter indicating that non-neuronal structures may also be labelled. RIA revealed measurable levels of PGE_2 (173±7 pg/ml/min) and PGD_2 (294±22 pg/ml/min) but not $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ both of which were below detectable limits. A quantitative densitometric analysis of the films revealed a 3.5 fold increase in the amount of cox-2 protein in the spinal cord samples 6 ($p<0.05$, Anova and Duncan's test), 12 and 24 ($p<0.01$) hours after induction of ankle inflammation. At the 3 day time point cox-2 levels were not significantly different from control ($p>0.05$). Cox-1 protein levels did not change during inflammation. These data indicate that both isoforms of cox are present and active in spinal cord and in a location consistent with their involvement in spinal pain processing.

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Grubb, B.D., Birrell, G.J., McQueen, D.S. et al (1991) *Exp. Brain Res.* 84, 383-392.
Willingale, H.L. & Grubb, B.D. (1996) *Br.J. Pharmacol.* 118, 49P

72P INDUCTION OF CYCLO-OXYGENASE-2 IN HUMAN INTERNAL MAMMARY ARTERY AND SAPHENOUS VEIN IN ORGAN CULTURE

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The release of prostacyclin (PGI_2) by vessels is regulated by cyclo-oxygenase (COX; COX-1). Certain cytokines or bacterial lipopolysaccharide (LPS) can induce prostanoid release through the expression of a novel isoform of COX (COX-2), (see Mitchell et al., 1995). We have previously demonstrated that LPS stimulates the release of COX metabolites by saphenous vein (SV) and internal mammary artery (IMA) in organ culture (Bishop-Bailey et al., 1996). We have extended these studies to compare the ability of human SV and IMA to express COX-2 mRNA and release metabolites in organ culture. Furthermore we have assessed the ability of interleukin-1β (IL-1β) to modulate COX-2 expression in these two vessels.

SV and IMA were obtained from the same patients (n=4; 3 male; age range 59-68), undergoing coronary artery bypass graft surgery. Vessels were cleaned and dissected under sterile conditions. Tissue rings of approximately equal weight and size (2-5 mm wide; approximately 10mg) were cut, and placed in separate wells of a 48 well culture plate containing 500 µl of DMEM supplemented with 2 mM glutamine, penicillin (1000 IU.ml^{-1}) and streptomycin (1 mg.ml^{-1}) (37°C; 5% CO_2; 95% air). After 1h and 24h the medium was replaced and IL-1β (10ng.ml^{-1}) and/or the selective COX-2 inhibitor NS-398 (N- (2- cyclohexyloxy -4 -nitrophenyl) methanesulfonamide; 30µM; Futaki et al., 1994) added. After the second 24h incubation period medium was removed and stored at -20°C. COX-metabolites accumulated in the medium were measured by radioimmunoassay (Mitchell et al., 1993). The tissue was removed, blotted dry and weighed, and stored at -80°C, until northern blot analysis of COX-2 using a 520 bp probe corresponding to bases 1297-1816 of the COX-2 sequence (Hla and Neilson, 1992), could be performed. COX-2 mRNA was not detected in fresh SV or IMA. In contrast after 48h in organ culture significant levels of COX-2 mRNA (4.5 kb) were detected in both SV and IMA. Furthermore, the level of COX-2 mRNA was elevated when IL-1β was included in the culture medium. Table 1 shows the release of COX-metabolites by IMA and SV after the second 24h incubation period.

		6-keto $PGF_{1\alpha}$	PGE_2	TXB_2
IMA	control	0.8±0.1	2.4±0.6	0.0±0.0
	IL-1β	7.2±5.9	13.2±10.3	0.2±0.1
SV	control	1.8±0.5	4.9±0.6	0.1±0.0
	IL-1β	11.8±2.5*	29.0±3.5*	0.4±0.1*

*Table 1: Release (mean ± s.e.mean) and prostanoids (ng.ml^{-1}.mg^{-1}; 24h) from human IMA and SV (n=4). *=$p<0.05$ between control and IL-1β (paired t-Test).*

COX-2 metabolites released by SV were significantly increased in the presence of IL-1β. Segments of IMA released less prostanoids than segments of SV. In addition, IL-1β did not significantly increase prostanoid release by IMA. NS-398, significantly attenuated 6-keto $PGF_{1\alpha}$ (the hydrolysis product of PGI_2) release from SV by 96±4%* under control and by 98±1%* when stimulated with IL-1β, and from incubations of IMA stimulated with IL-1β by 56±14%*; (* $p<0.05$; one sample test, n=4).

These observations show that COX-2 is induced in human SV and IMA, and suggests that induction may occur in man following coronary artery bypass graft surgery. The induction of COX-2 and subsequent release of PGI_2 may represent an endogenous defence mechanism against endothelial damage incurred during surgical preparation of the these vessels for bypass.

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Bishop-Bailey, D. Larkin, S.W., Williams, T.J., et al. (1996) *Br. J. Pharmacol.* **117**, 79P.
Mitchell, J.A., Akarasereenont, P., Thiemermann, C. et al., (1993). *Proc. Natl. Acad. Sci. (USA).* **90**, 11693-11697.
Mitchell, J.A., Larkin, S.W. and Williams, T.J. (1995). *Biochem. Pharmacol.* **50**, 1535-1542.
Futaki, N., Takahashi, S., Yokoyama, M., et al., (1994). *Prostaglandins.* **47**, 55-59.
Hla and Nielson (1992). *Proc. Natl. Acad. Sci. (USA)* **89**, 7384-7388

73P CYCLO-OXYGENASE-2 ACCOUNTS FOR THE SUSTAINED RELEASE OF PROSTAGLANDIN E_2 FROM CYTOKINE STIMULATED HUMAN VASCULAR SMOOTH MUSCLE CELLS

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The release of prostacyclin (PGI_2) and prostaglandin (PG)E_2 by vessels is regulated by cyclo-oxygenase (COX; COX-1) localised primarily in the endothelium. A cytokine-inducible isoform, COX-2, has been extensively studied in macrophages, and demonstrated in human endothelial cells after stimulation. However, the possibility that human vascular smooth muscle cells can express COX-2 has not been comprehensively addressed. In this study, we have investigated the induction of COX-2 in cultures of human vascular smooth muscle cells derived from internal mammary artery (IMA).

IMA were obtained from patients undergoing coronary artery bypass graft surgery. Explants of vascular smooth muscle cells were grown in DMEM supplemented with 2 mM glutamine, penicillin (1000 IU.ml^{-1}), streptomycin (1 mg.ml^{-1}), and 20% foetal calf serum (37°C; 5% CO_2; 95% air). Smooth muscle cells (passages 2-6) were identified by morphological hill and valley growth pattern, and by smooth muscle α-actin immunostaining. Prostaglandin formation from endogenous or exogenous stores of arachidonic acid (AA) was measured in cells cultured on 96-well plates, for Western blot analysis cells were grown in 6-well plates with or without cytokines for 24h. Cells were extracted and blotted according to Mitchell et al., (1993) using a specific COX-2 antibody (Merck Frosst, Montreal; Chen et al., 1995). For measurement of release from endogenous stores of AA, cells were treated with a combination of IL-1β (500U.ml^{-1}), tumour necrosis factor-α (10ng.ml^{-1}), interferon-γ (1000U.ml^{-1}) and lipopolysaccharide (10μg.ml^{-1}) for 6, 12, 24 and 48h, medium was removed and the accumulated PGE_2 measured by radioimmunoassay. For COX activity supported by exogenous AA, fresh medium containing 30μM AA was added for 10min and PGE_2 measured. In separate experiments, indomethacin or its COX-2 selective derivative L-745,337 (5- methanesulfonamido- 6- (2,4- difluorothiophenyl) -1-indanone; Chan et al., 1995), (10 pM to 100 μM) were added to cells prior to the addition of cytokines for 24 h.

COX-2 protein (approximately 70 kDa) was detected in cells cultured with, but not without, cytokines. Furthermore, cells stimulated with cytokines released prostanoids from both endogenous (closed squares) and exogenous (open squares) stores of AA (figure 1). Indomethacin or L-745,337 inhibited cytokine-induced PGE_2 release with pIC_{50} (95% confidence interval) values (-log M) of 8.9 (8.3- 9.6) and 8.9 (8.5- 9.3) respectively, indicative of COX-2 being the active isoform.

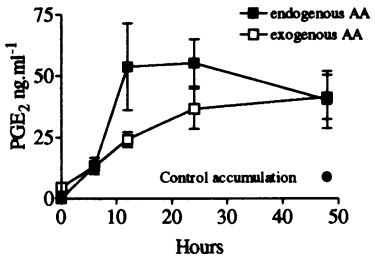

Figure 1. Formation of PGE_2 by IMA smooth muscle cells treated with cytokines. Filled squares, release form endogenous arachidonic acid; open squares release from exogenous arachidonic acid. Results are the mean ± s.e.mean of n=9 determinations from 3 patients.

Thus, as has been demonstrated in other human cell types, COX-2 is induced in human vascular smooth muscle cells. In larger vessels, such as IMA, an induction of COX-2 in the smooth muscle layer may compensate for a damage or loss of the endothelium. These observations suggest that during inflammation, the vascular smooth muscle may become an important source of prostanoids.

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Mitchell, J.A., Akarasereenont, P., Thiemermann, C. et al., (1993). *Proc. Natl. Acad. Sci. (USA).* **90**, 11693-11697.
Chan, C.C., Boyce, S., Brideau,C. et al., (1995). *J. Pharmacol. Exp. Ther.* **274**, 1531-1537.

74P desArg^{9}BK-INDUCED MECHANICAL HYPERALGESIA AND ANALGESIA IN THE RAT: INVOLVEMENT OF IL-1, PROSTAGLANDINS AND PERIPHERAL OPIOIDS

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In naïve Sprague-Dawley rat knee joints, injection of the B_1 selective agonist desArg^{9}BK has no effect whereas the B_2 kinin receptor agonist bradykinin induces mechanical hyperalgesia (Davis & Perkins 1994a). However in animals with joints pretreated with IL-1ß desArg^{9}BK can cause either hyperalgesia or analgesia depending on the dose administered (Davis & Perkins 1994b).

In this study we have characterised further the mechanism of hyperalgesia and analgesia induced by desArg^{9}BK in IL-1ß pretreated joints. The involvement of prostaglandins and IL-1 in the desArg^{9}BK-induced hyperalgesia and the involvement of opioids in the desArg^{9}BK-induced analgesia have been investigated.

The method used for assessment of mechanical hyperalgesia was as described in the previous studies (Davis & Perkins 1994a,b). Animals were injected with 1unit IL-1ß into a single knee joint 24 h prior to testing, a dose used in our previous study (Davis & Perkins 1994b). On the test day the load tolerated by the IL-1 pretreated joints was assessed before or after administration of desArg^{9}BK.

DesArg^{9}BK (0.5nmol)-induced hyperalgesia was blocked when co-administered with indomethacin (1-25μg). This dose of indomethacin had no effect when administered alone.

The response to desArg^{9}BK (0.5nmol) was also antagonised when co-administered with IL-1ra (0.1-1μg) or Lys-D-Pro-Thr (0.2-2μg). IL-1ra also partially reversed the basal level of hyperalgesia in IL-1 pretreated joints.

DesArg^{9}BK (50pmol)-induced analgesia was not antagonised when coadministered with the opioid receptor antagonists CTOP or naltrindole. However the analgesia was dose dependently antagonised by norBinaltorphimine and naloxone (see table 1). Norbinaltorphimine caused a slight hyperalgesia when injected alone into IL-1 pretreated joints, but was without effect in naïve joints. The doses of antagonists used here have been shown to be selective for their respective receptors in other inflammatory models (Schafer et al., 1994).

In summary we have shown that in IL-1ß pretreated joints desArg^{9}BK can have both pro and antinociceptive effects. Furthermore the analgesic actions of desArg^{9}BK seem to be via release of peripheral opioids, possibly acting via the k-receptor.

Davis A.J. & Perkins M.N. (1994a). Neuropharmacol., 33, 127-133.
Davis A.J. & Perkins M.N. (1994b). Br. J. Pharmacol., 113, 63-68.
Schafer M. et al., (1994). Proc.Natl.Acad.Sci. USA, 91, 4219-4223.

Table 1. Summary of effects of specific antagonists on desArg^{9}BK-induced hyperalgesia and analgesia in IL-1ß pretreated rats.

	Indomethacin		**IL-1ra**		**KDPT**			
	Control	25μg	Control	0.1μg	Control	0.2μg		
desArg^{9}BK 0.5nmol	63±2g	88±3g*	64±1g	96±3g*	64±1g	90±2g*		
	CTOP		**naltrindole**		**norbinaltorphimine**		**naloxone**	
	Control	1μg	Control	50μg	Control	5μg	Control	5μg
desArg^{9}BK 50pmol	103±3g	103±5g	92±2g	94±1g	94±1g	81±2g*	98±3g	83±3g*

*P<0.05 compared to the relevant control group (the control represents the effect of desArg^{9}BK administered alone). All results are expressed as load tolerated ± s.e. mean (g) by the treated joint, n=8 animals per treatment group.

75P BRADYKININ B_2 RECEPTOR ANTAGONIST CP-0597 REDUCES INFARCT VOLUME AFTER FOCAL CEREBRAL ISCHAEMIA IN THE RAT: COMPARISON WITH HOE 140, NPC 17731 AND MK801

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Bradykinin has been characterized as an initial mediator of inflammation and is thought to be involved in the pathogenesis of various forms of neuronal injury (Francel, 1992).

We examined the effect of a selective B_2 receptor antagonist CP-0597 (Goodfellow *et al.*, 1995) on the extent of brain infarction after focal cerebral ischemia in the rat. The effects of CP-0597 were compared with those of other B_2 receptor antagonists HOE 140 (Wirth *et al.*, 1991) and NPC 17731 (Kyle *et al.*, 1991) and with the NMDA receptor antagonist MK801.

Focal cerebral ischaemia was induced in male Sprague Dawley rats (250-300 g) by permanent occlusion of the left middle cerebral artery (MCAO) under halothane anaesthesia. 30-45 min prior to arterial occlusion, primed miniosmotic pumps were implanted subcutaneously to release 300 ng/kg/min CP-0597, HOE 140 or NPC 17731, or isotonic saline over the ensuing 24h period. MK801 was administered as a single bolus dose (4 mg/kg, ip) 30 min prior to surgery.

24 h after MCAO, rats were sacrificed and infarct size measured by tetrazolium staining of 500 um coronal brain sections of fresh brain tissue. Infarct area on each section was sketched onto stereotaxic maps and quantified using image analysis. 10-14 rats were included in each treatment group. Statistical analysis was performed using one way ANOVA with Dunnett's test. Results are expressed as mean % control values ± s.e. mean.

Treatment with CP-0597 significantly reduced total infarct volume to 42.8±7.4% ($P<0.01$) and striatal infarct volume to 5.6±2.7% ($P<0.01$) of vehicle treated control values.

Treatment with HOE 140 and NPC 17731 did not significantly reduce total infarct volume (62.3±12.5% and 75.7±13.9% of control values respectively) but did significantly reduce striatal infarct volume to 16.6±11.4% ($P<0.01$) and 47.9±13% ($P<0.01$) of control values respectively.

MK801 treatment significantly reduced total infarct volume to 44±11.5% of control values ($P<0.01$) but did not significantly reduce striatal infarct volume (72.9±31% control).

These data indicate that bradykinin is involved in the pathogenesis of ischemic brain injury in the rat and that activation of the B_2 receptor results in neurodegeneration after MCAO. Treatment with CP-0597 showed comparable efficacy to MK801 in reducing total infarct volume as well as causing a dramatic reduction in striatal infarction. Marked selective striatal protection by the B_2 antagonists CP-0597, HOE 140 and NPC 17731 suggest an important role for the B_2 receptor in neurodegenerative processes in this region of the brain.

Francel P.C. (1992) J.Neurotrauma. 9, S27-S45.

Goodfellow V.S., Marathe M.V., Kuhlman K.G. *et al.* (1995) J. Med. Chem. 39, 1472-1484.

Kyle D.J., Martin J.A., Burch R.M. *et al.* (1991) J.Med.Chem. 34, 2649-2653.

Wirth K., Hock F.J., Albus U. *et al.* (1991) Br. J. Pharmacol. 102, 774-777

76P BLOCKADE OF THE BRADYKININ B_1 RECEPTOR REVERSES THE NEUROPROTECTIVE EFFECT OF B_2 RECEPTOR ANTAGONISM AFTER FOCAL CEREBRAL ISCHAEMIA IN THE RAT

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Two types of bradykinin receptor exist, termed B_1 and B_2 (Regoli & Barabe, 1980) and both have been implicated in inflammatory processes (Marceau,1995).

We examined the effects of bradykinin B_1 and B_2 receptor antagonists used both alone and in combination to evaluate the respective roles of B_1 and B_2 receptors in the pathogenesis of ischemic brain injury. The compounds used were the selective B_1 antagonists; B9858 (Gera *et al*, 1996) and Lys0-des-Arg9-Leu8-BK (Regoli & Barabe, 1980), the B_2 antagonist, CP-0597 (Goodfellow *et al.*, 1995) and the combined B_1 and B_2 antagonist B9430 (Stewart *et al.*, 1996).

Focal cerebral ischaemia was induced in male Sprague Dawley rats (250-300g) by permanent occlusion of the left middle cerebral artery (MCAO) under halothane anaesthesia as described previously (Relton *et al.*, 1996). 30-45min prior to arterial occlusion, primed mini-osmotic pumps were implanted subcutaneously to release 300 ng/kg/min of drug solution or isotonic saline over the ensuing 24 h period.

24h after MCAO, rats were sacrificed and infarct size measured by tetrazolium staining of 500 um coronal sections of fresh brain tissue. Infarct area on each section was sketched onto stereo-taxic maps and quantified using image analysis. 10-14 animals were included in each treatment group. Statistical analysis was performed using one-way ANOVA with Dunnett's test. Results are expressed as mean % control values ± s.e. mean.

Treatment with the B_2 receptor antagonist CP-0597 significantly reduced total infarct volume to 42.8±7.4% of vehicle-treated control values ($P<0.01$). Treatment with the selective B_1 antagonist, B9858 did not affect infarct size compared to control animals (110.2±17.6% control infarct size). Combined treatment with CP-0597 plus either B9858 or Lys0-des-Arg9-Leu8-BK reversed the protective effect of CP-0597 treatment and tended to exacerbate infarction, although this effect did not reach statistical significance (130.4±15% and 111.8± 10.7% control infarct size respectively). Similarly, infarct size was slightly, but not significantly, exacerbated in rats treated with B9430, a combined B_1/B_2 receptor antagonist (128.5±19% control infarct size).

In summary, selective B_2 receptor blockade with CP-0597 resulted in markedly reduced brain infarction whereas selective B_1 blockade did not significantly effect neurological outcome after MCAO. The protective effect of B_2 receptor blockade with CP-0597 was reversed by co-administration of B_1 receptor antagonists.

Together these data suggest that bradykinin B_1 and B_2 receptors may have differential actions in the neurodegenerative processes triggered after an ischemic insult to the brain in the rat.

Gera L., Stewart J.M., Whalley E.T. *et al.* (1996) Immuno-pharmacology. 33,183-185.

Goodfellow V.S., Marathe M.V., Kuhlman K.G. *et al.* (1995) J. Med. Chem. 39, 1472-1484.

Marceau F. (1995). Immunopharmacology. 30, 1-26.

Regoli D. & Barabe J. (1980) Pharm. Rev. 32, 1-46.

Relton J., Martin D., Thomson R.C. *et al.* (1996) Exp. Neurol. 138, 206-213.

Stewart J., Gera L., Hanson W. *et al.* (1996). Immunopharmacology. 33, 51-60

77P BRADYKININ RELEASE DURING CEREBRAL ISCHAEMIA IN ANAESTHETIZED RATS

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Cerebral ischaemia, produced by intra-carotid infusion of starch microspheres in anaesthetized rats, resulted in a permeability increase of pial venular capillaries consistent with the formation of cerebral oedema (Kurokawa & Fraser, 1994). The cerebral vascular permeability increase was totally prevented and even reversed by the bradykinin B_2 receptor antagonist HOE-140 (Kurokawa & Fraser, 1995).

In the present study cerebral ischaemia was produced in a similar manner and the release of immunoreactive bradykinin (IRBK) was measured in rat brain superfusates before and during microsphere administration, and during reperfusion. The samples were obtained from a 0.5 ml cup placed over the exposed cerebral surface from which the meninges had been removed. Results are reported as median; Q1, Q3 and analysed using the Mann-Whitney U-test (significance at $P \leq 0.05$).

There was no significant change in superfusate concentration of IRBK (pg ml^{-1}) in the 30 min preceding microsphere infusion (52; 34, 68; n=7) compared with sham operated animals (38; 34, 764; n=7). During the first 30 min following infusion there was a median increase in IRBK from 52 pg ml^{-1} to 92 pg ml^{-1}, but this did not quite reach significance (P=0.064). There was, however, a significant (P=0.0487) increase in IRBK, compared with normally perfused time-matched controls, in the second 30 min sampling period (92; 52, 894). It had been shown previously that reperfusion was likely to have occurred during this period.

The increased BK release supports the idea that kinins have a significant role in the vascular sequelae of cerebral ischaemia.

Kurokawa, T. & Fraser, P. A. (1994) J. Physiol. **480**, 10P.
Kurokawa, T. & Fraser, P. A. (1995) J. Physiol. **483**, 140P.

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78P THE ROLE OF PROSTAGLANDINS IN THE BRADYKININ-INDUCED ACTIVATION OF SEROSAL AFFERENTS OF THE RAT JEJUNUM *IN VITRO*

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Considerable research documents the role of bradykinin (BK) and prostaglandins (PGs) in inflammatory nociception. Studies performed *in vivo* have suggested that the activation of serosal afferent neurones of the rat jejunum by BK may be dependent on the presence of PGs (Stebbins *et al.*, 1985). In the present study we have utilised a novel *in vitro* preparation of the rat jeunum to study serosal afferents in isolation. We have investigated the role of PGs in the activation of these serosal afferents by BK.

Hooded Lister rats (either sex, 350-400g) were anaesthetised with Urethane ($1.5 gkg^{-1}$ i.p.) and a 3cm long segment of jejunum excised and placed in a recording chamber superfused with bicarbonate buffer (composition in mM: NaCl 117, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 11, $CaCl_2$ 2.5; equilibrated with 95% O_2 and 5% CO_2, maintained at 34°C, at a flow rate of $10 ml min^{-1}$). The serosa was carefully dissected free and the remainder of the jejunum was discarded. The mesenteric arcade was drawn into a separate chamber containing paraffin, from which nerve bundles were teased out for extracellular recording of afferent activity. All afferents recorded were both capsaicin-sensitive and mechanosensitive. Drugs were applied by bath perfusion. The Student's t-test was used for statistical analysis and values of $P<0.05$ were considered to be significant.

BK (1 μM, 2 min) elicited an increase in multi-unit afferent activity from 11.8 ± 3.5 impulses per second (mean ± s.e.m., $imps^{-1}$) to 38.5 ± 11.5 $imps^{-1}$. The time from application to the peak response was 36.6 ± 7.9 s and the overall increase in nerve discharge was 4773 ± 1530 impulses (n=47). The effect of BK was found to be concentration-dependent (EC_{50} = 0.62 ± 0.12 μM, n=5). Repeatable increases in afferent activity were observed to a sub-maximal concentration of BK (1 μM, 2 min, n=3). Exposure (30 min) to the B_2 receptor antagonist, HOE140 significantly reduced responses to BK (1 μM) to 79.8 ± 14.4 % at 1 nM (n=3), 20.8 ± 4.3 % at 3 nM (n=3) and 6.2 ± 3.7 % at 10nM (n=4), whereas the B_1 receptor antagonist, [des-Arg^{10}]HOE140 was without effect (95.1 ± 3.4 % at 100nM, n=3). Following blockade of cyclooxygenase activity the response to BK was significantly reduced to 30.5 ± 1.4 %, 25.6 ± 4.7 % and 24.4 ± 4.9 % in the presence of indomethacin (10μM, n=4), flurbiprofen (10μM, n=4) and naproxen (10μM, n=6), respectively. In 3 recordings, where naproxen reduced the response to BK to 27.2 ± 10.6 %, further addition of prostaglandin E_2 (1 μM) had no direct effect on afferent activity, but restored the BK response to 93.4 ± 1.2 %.

These data indicate that the BK-induced activation of serosal afferents is mediated via the kinin B_2 receptor subtype. The response to BK appears to be dependent on the presence of PGs, since it was reduced by blockade of cyclooxygenase and restored by PGE_2. We suggest that prostaglandins e.g. PGE_2, sensitise the endings of serosal afferent nerves responsive to bradykinin.

Stebbins, C.L., Smith, R.C. and Longhurst, J.C. (1985) *Am.J.Physiol.***249** (Heart Circ Physiol. 18)):H155-163.

79P CLONING OF cDNA ENCODING ARGININE-SPECIFIC MONO(ADP-RIBOSYL) TRANSFERASE IN HUMAN NEUTROPHIL POLYMORPHS: RELEVANCE TO CHEMOTAXIS

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The chemotaxis of human polymorphonuclear neutrophil leucocytes (PMNs) involves continuous re-alignment of the contractile elements of the cytoskeleton in the direction of the chemotactic gradient. The receptors for chemotaxins such as FMLP or PAF are coupled via G proteins to phosphoinositide-specific phospholipase C, but the signalling pathways mediating assembly of filamentous actin are complex and appear to include a requirement also for the activity of an Arg-specific mono(ADP-ribosyl)transferase (Allport *et al.* 1996). The structure and cellular location of the Arg-specific mono(ADP-ribosyl)transferase expressed in human PMNs have not been reported previously, and our experiments that address this issue were prompted by a need to formulate a more secure model for receptor-dependent re-alignment of cytoskeletal microfilaments.

Northern blots of mRNA from human PMNs and skeletal muscle were probed with cDNA corresponding to partial sequence of rabbit mono(ADP-ribosyl)transferase (Zolkiewska *et al.* 1992). This identified a single transcript in PMN mRNA (approx 2.5 kb), which was indistinguishable from that seen in skeletal muscle. We have now cloned the cDNA from PMNs encoding mono(ADP-ribosyl)transferase, and the enzyme is identical to that expressed in human skeletal muscle (Okazaki et al. 1994). Structural analysis of the deduced amino acid sequence reveals a 327 amino acid pro-enzyme, from which are cleaved both the N-(amino $acids_{1-15}$)and C-terminal (amino $acids_{297-327}$) hydrophobic domains to yield the active enzyme. This pattern of processing is typical of an enzyme which is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) side chain. Previous research by this group has identified Arg-specific mono(ADP-ribosyl)transferase activity on the outer surface of PMNs, and we have shown also that the enzyme may be released from the plasma membrane by phosphoinositide-specific phospholipase C (Donnelly *et al.* 1996).

We now propose a model for cytoskeletal re-alignment involving Ca^{2+}-dependent translocation of the mono(ADP-ribosyl)transferase to the cell surface following exposure of PMNs to chemotaxin. Thereafter, the enzyme would be available to mediate mono(ADP-ribosyl)ation of one or other of the membrane-bound proteins that comprise the anchoring point of cytoskeletal microfilaments. A modification of this sort, with cyclical addition and removal of ADP-ribose from the anchoring point, might allow control of (i) actin assembly and (ii) sequential adhesion or release of the cell surface from extracellular matrix.

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Allport, J.R., Donnelly, L.E., Hayes, B.P. *et al.* (1996) *Br. J. Pharmacol.* 118, 1111-1118.
Donnelly, L.E., Rendell, N.B., Murray, S. *et al.* (1996) *Biochem J.* 315, 635-641.
Okazaki, I.J., Zolkiewska, A., Nightingale, M.S. *et al.* (1994) *Biochemistry.* 33, 12828-12836.
Zolkiewska, A., Nightingale, M.S. & Moss, J. (1992) *Proc. Natl. Acad. Sci. USA.* 89, 11352-11356.

80P cAMP ELEVATION MODULATES fMLP-STIMULATED Ca^{2+} AND Mn^{2+} INFLUX IN HUMAN NEUTROPHILS

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Inhibitors of leukocyte activation, such as phosphodiesterase inhibitors (PDEIs, Nielson *et al.*, 1990) and E-type prostaglandins (PGE_1 and PGE_2, Hecker *et al.*, 1990) elicit an increase in cAMP levels in the target cell. These agents also inhibit human neutrophil activation and simultaneously elevate cAMP levels. The latter has been proposed as a common mechanism of action to explain their inhibitory activities in the neutrophil. Neutrophil activation by chemoattractants, such as formyl methionyl-leucyl-phenylalanine (fMLP), are accompanied by an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$).

Human neutrophils were isolated from venous blood by centrifugation (450-470g) with Polymorphprep (Nycomed) and incubated with cytochalasin B (5µg ml^{-1}) and inhibitors or vehicle in phosphate buffered saline containing 0.9mM $CaCl_2$, 0.5mM $MgCl_2$ and 11mM glucose for 5 min (37°C). Superoxide (O_2^-) production (5 min, 37°C) was then stimulated by the addition of fMLP (0.1µM), measured as superoxide dismutase (90U ml^{-1})-inhibitable reduction of ferri-cytochrome C (85µM). Alternatively, the neutrophils ($5x10^7$ ml^{-1}) were loaded with Fura-2am (2.5µM, 30 min, 37°C) in Ca^{2+}/Mg^{2+}-free Hanks buffered salt solution. $[Ca^{2+}]_i$ (λ_{ex} 340 and 380nm) and Mn^{2+} (a Ca^{2+} surrogate, λ_{ex} 360nm) influx was measured spectrophotometrically at λ_{em} 510nm (Murphy & Westwick, 1994). Statistical analysis was performed on paired sets of data using ANOVA and Bonferroni's test for multiple comparisons.

PGE_2 inhibited fMLP-stimulated O_2^- production ($p[A]_{50}$=7.2±0.1, mean±s.e.mean, n=5); PGE_2 maximum inhibition was observed at 10µM (89±3%). The PDEIs, IBMX (non-selective), RO 20-1724 and rolipram (both type IV selective), inhibited the fMLP O_2^- response with $p[A]_{50}$ values of 5.0±0.2 (75±4% at 500µM), 6.9±0.1 (72±3% at 30µM) and 7.4±0.1 (73±3% at 10µM); respectively (n=4-8, maximum inhibition indicated in parentheses); whilst milrinone (type III selective) only achieved 50±2% inhibition at 100µM (pIC_{50}=4.2±0.1, n=3). fMLP-stimulated O_2^- generation was also attenuated in the presence of the divalent cation chelator, EGTA (0.95mM) by 62±4% (n=3, P<0.005).

Rolipram (0.01-1µM) or PGE_2 (10µM) pre-incubation (5 min, 37°C) with human neutrophils did not inhibit the peak increase in $[Ca^{2+}]_i$ stimulated by fMLP (0.1µM). However the post-peak $[Ca^{2+}]_i$ increase (measured 30s after fMLP addition) was attenuated by rolipram (39±5% at 1µM, n=7, P<0.01) and PGE_2 (52±5% at 10µM, n=9, P<0.01). In addition, fMLP-stimulated Ca^{2+} influx-dependent increase in $[Ca^{2+}]_i$ was inhibited by rolipram (1µM, 86±6%, n=3, P<0.01) and PGE_2 (10µM, 81±11%, n=3, P<0.01). Furthermore, in the presence of rolipram (1µM), fMLP-stimulated Mn^{2+} influx was reduced by 27±12% (n=4, P≈0.06). PGE_2 (10µM) alone had no effect (8±17% inhibition, n=4); but the combination of rolipram and PGE_2 was more effective than either agent alone (40±11% inhibition, n=4, P<0.05).

Our data suggests that cAMP elevating agents suppress human neutrophil activation by attenuating increases in $[Ca^{2+}]_i$. The present findings indicate that cAMP elevation modulated Ca^{2+} influx *per se*; however cAMP appears to have additional effects on the regulation of $[Ca^{2+}]_i$ such as potentiation of Ca^{2+} sequestration and/or extrusion. The importance of these other mechanisms of modulating $[Ca^{2+}]_i$ by cAMP-elevating agents warrants further investigation.

Hecker, G., Ney, P. & Schrör, K. (1990) *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 341, 308-315.
Murphy, C.T. & Westwick, J. (1994) *Biochem. J.*, 103, 531-537.
Nielson, C.P., Vestal, R.E., Sturm, R.J. & Heaslip, R. (1990). *J. Allergy Clin. Immunol.*, 86, 801-808.