222P CAN EXOGENOUS OESTROGEN SCAVENGE SUPEROXIDE ANIONS IN STROKE-PRONE RATS?

H.V.O. Carswell*, S.J. Groves, E. Jardine, C.A. Hamilton & A. Dominiczak Department of Medicine and Therapeutics and *Wellcome Surgical Institute, University of Glasgow, Glasgow

Superoxide anion radicals ($O_2^{\cdot-}$) are believed to be a major cause of oxidative damage to the brain with implications for a role in stroke. In addition, $O_2^{\cdot-}$ scavenges nitric oxide (NO), lowering NO bioavailability (McIntyre *et al*, 1999). Blood vessels in stroke-prone spontaneously hypertensive rats (SHRSP) have increased $O_2^{\cdot-}$ levels and reduced NO bioavailability compared to normotensive Wistar Kyoto (WKY) rats (Hamilton *et al*, 1997). In addition, males have reduced NO bioavailability compared to females (McIntyre *et al*, 1999). 17β-oestradiol has a proven protective role in experimental stroke (Simpkins *et al*, 1997) and supraphysiological levels (40μg/kg/day for five days) reduce $O_2^{\cdot-}$ production and increase NO bioavailability (Barbacanne *et al*, 1999). We examined the effects of physiological doses of 17β-oestradiol on $O_2^{\cdot-}$ production and NO bioavailability in SHRSP and WKY.

3 month old female SHRSP (180-200g) and WKY (200-220g) rats were ovariectomised under halothane anaesthesia and a 17β-oestradiol (21 day release, 0.025mg) or placebo (containing vehicle and matrix only) pellet was sub-cutaneously implanted. 14 days later descending thoracic aortas, carotid arteries and cerebral tissue (cortex, striatum and hippo-campus) were dissected out. Superoxide production in brain homogenates and aortas was measured by lucigenin chemilumi-nescence on a scintillation counter using a xanthine/xanthine oxidase calibration curve. Bioavailability of NO was tested in carotid artery rings by constriction with L-phenylephrine (10^{-8}mol/l to 10^{-5}mol/l) in the presence or absence of N-ϖ-nitro-L-arginine methyl ester (L-NAME (10^{-4}mol/l)). The difference between the areas under the two constriction curves is a measure of basal NO bioavailability.

Unpaired t-tests were employed (mean±SEM).

Plasma levels of 17β-oestradiol were 30±10.6pg/ml (n=4) in the intact WKY compared to 20±4.3pg/ml (n=6) in 17β-oestradiol-treated and 9.8±2.1pg/ml (n=7) in placebo-treated ovariectomised rats. There were no significant differences in $O_2^{\cdot-}$ production (nmol/min/mg) in the aorta or brain in 17β-oestradiol (n=6) versus (vs) placebo (n=6) treated SHRSP: aorta= 2.75±0.77 vs 3.41±0.85; cortex= 1.26±0.33 vs 1.30±0.30; striatum= 1.04± 0.24 vs 1.15±0.23; hippocampus = 1.0±0.19 vs 1.14±0.25. Similarly, in the WKY there were no differences in $O_2^{\cdot-}$ production (nmol/min/mg) in 17β-oestradiol (n=6) vs placebo (n=6) groups: aorta= 2.7±0.62 vs 2.29±0.56; cortex= 0.92±0.09 vs 1.14±0.24; striatum = 0.8±0.12 vs 0.91±0.16; hippocampus = 0.7±0.1 vs 0.88±0.21. 17β-oestradiol did not significantly affect NO bioavailability compared to placebo although there was a trend for an increase in SHRSP: AUC= 1.38±0.27g/g (n=5) vs 0.79±0.12g/g (n=6) (p=0.113, 95%CI-134,18) and in WKY: AUC= 2.13± 0.42g/g (n=6) vs 1.17±0.2g/g (n=5) (p=0.106 95%CI-219,26). 17β-oestradiol did not affect vessel sensitivity to carbachol compared to placebo in SHRSP (EC_{50}= 2.67 $\times10^{-7}$mol/l vs 1.2$\times10^{-7}$mol/l, n=5 and E_{max} = 71±7% vs 70±15%, n=5) or in WKY (EC_{50}= 2.75$\times10^{-7}$mol/l vs 3.3$\times10^{-7}$mol/l, n=5 and E_{max}=78±11% vs 70±15%, n=5).

The results indicate that physiological levels of 17β-oestradiol do not affect basal $O_2^{\cdot-}$ production or NO bioavailability in ovariectomised rats.

Barbacanne, *et al,* 1999 Cardiovascular Research, 41, 672-681

Hamilton, *et al*, 1997 Hypertension, 30, 1517-1524

McIntyre *et al*, 1999 Hypertension, 34, 539-545

Simpkins *et al*, 1997 J. Neurosurg., 87, 724-730.

223P SEX DIFFERENCE IN THE EFFECTS OF 6-HYDROXYDOPAMINE LESIONS IN THE NIGROSTRIATAL DOPAMINERGIC PATHWAY OF ADULT RATS AND SEXUALLY DIMORPHIC ACTIONS OF 17 β-ESTRADIOL.

*H.E. Murray, *+S.R. McArthur, +D.T. Dexter & *G.E. Gillies. Departments of *Neuroendocrinology and +Neuroinflammation, Faculty of Medicine, Imperial College, Hammersmith Hospital, LONDON, W12 0NN.

Parkinson's Disease (PD) exhibits a striking sexual dimorphism in the human population with men being more susceptible than women (Schrag *et al*, 2000). Using a partial unilateral 6-hydroxydopamine (6-OHDA)-induced lesion of the nigrostriatal dopaminergic (NSDA) pathway to generate a rat model of PD, this study aimed to determine the influence of physiological levels of sex steroid hormones on the loss of striatal dopamine (DA) in males and females. Further studies in gonadectomised rats ± hormone replacement therapy investigated the proposed neuroprotective effects of estrogen.

Adult male and female Sprague-Dawley rats (60±5 days) were employed, housed under controlled lighting and temperature (lights on 08.00-20.00hr, 22± 1°C), with food and water *ad libitum*. *Experiment 1.* Gonad-intact male rats and regularly cycling female rats at proestrous (n=6) received a unilateral injection of either 6-OHDA (1μg dissolved in 4μl 0.9% NaCl containing 0.01% w/v ascorbic acid) or vehicle, to the medial forebrain bundle. Two weeks later the animals were sacrificed, the brains removed and both striata dissected out for analysis of DA content using HPLC-ECD. *Experiment 2.* Under halothane anaesthesia, rats were gonadectomised (GDX) and received s.c. slow release implants containing either 0.5mg 17 β-estradiol, E_2, or 1.5mg 5 - α dihydrotestosterone, DHT (to provide physio-logical levels of androgen and proestrous levels of estrogen over a 21 day period). Sham-operated controls were implanted with a placebo pellet containing vehicle only.

After 1 week the rats received a unilateral 6-OHDA lesion and striatal tissue was collected 2 weeks following.

DA levels in the lesioned striatum (LS) were compared with the non-lesioned striatum (NLS) as an index of the severity of the neuronal assault (i.e.percentage loss of DA content). Statistical analysis was performed using ANOVA followed by Student Neuman Keuls test for multiple comparisons. *Experiment 1.* The 6-OHDA caused a significant reduction in striatal DA content (110.1 ± 5.0ng/mg tissue, NLS *vs* 56.3 ± 4.1 ng/mg tissue, LS; $p < 0.01$). By contrast, the severity of the lesion in females was attenuated compared to the males (113.0 ± 6.6 ng/mg tissue, NLS *vs* 77.3 ± 7.4 ng/mg tissue, LS; $p< 0.05$). Injection of vehicle alone had no effect on DA levels. *Experiment 2.* The percentage loss of striatal DA content was significantly less ($p<0.05$) in GDX (30.5±1.2%) *vs* sham operated (54.6±6%) males. Treatment with DHT was without effect in GDX males, whereas E_2 replacement increased the magnitude of the lesion to a value (56±1.9%) similar to that seen in the sham-operated male. By contrast, in the female, GDX significantly increased the loss of striatal DA from 17.05 ± 1.2% in the sham-operated female to 49.3 ± 6.1% ($p <0.01$). The effects of female GDX were unaltered by DHT, but were completely reversed by replacement with E_2.

The results of the present study provide novel evidence for a gender related difference in the neurotoxic effect of 6-OHDA on DA neurones in a rat model of PD and also suggest that this difference is mediated via the sexually dimorphic actions of 17 β-estradiol in the male and female rat CNS

Schrag *et al*, BMJ. 321; 21-22, 2000.

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224P EFFECTS OF NEONATAL DEXAMETHASONE TREATMENT ON THYMIC NOS-2 EXPRESSION IN THE ADULT RAT

H. Lamfon, G. Gillies, E.Theogaraj, C. John, [1]S. F. Smith and J.C. Buckingham. Divisions of Neuroscience & Psychological Medicine and [1]National Heart and Lung Institute, Faculty of Medicine, Imperial College, London W12 ONN.

Recent studies from our laboratory have shown that the inducible form of nitric oxide synthase (NOS-2) is expressed constitutively in the thymus and that the NO it generates is involved in the negative selection of double positive (DP) thymocytes (Downing *et al.*, 1998). Little is known of the factors which influence NOS-2 expression in the thymus although there is evidence that it is down-regulated by glucocorticoids (GCs), as it is in other tissues, possibly via an annexin 1 (ANXA1) dependent mechanism. A number of recent studies have suggested that GC-dependent mechanisms in adulthood may be compromised by exposure to high levels of endogenous or exogenous GCc at critical stages in development with potentially detrimental consequences for immune function (Bakker *et al.*, 2001). As a prelude to studies in this area, we have examined the effects of neonatal dexamethasone (DEX) on thymic mass and on the expression of NOS-2 and ANXA1 at maturity. Female Sprague-Dawley rats were treated with DEX (1μg/10μl/g body weight, i.p.) on postnatal days 3,5 & 7; controls received equal volumes of the saline vehicle (10μl/g body weight, i.p.). The animals were then allowed to mature without further intervention apart from regular weight checks. At 3 months the animals were killed humanely, the thymus was weighed and bisected for subsequent analysis of (a) NOS-2 expression by diaphorase histochemistry (Downing *et al.*, 1998) and (b) ANXA1 by western blot analysis (Taylor *et al.*, 1993).

The results were analysed by one way ANOVA followed by Tukey test or Dunnet's method. The data (Table 1) showed that neonatal DEX treatment caused a significant reduction in body weight ($P<0.001$) and proportional reduction in thymus weight ($P<0.001$) as reflected by a decrease in thymus weight but not in thymus to body weight ratio. Neonatal DEX treatment also caused a significant reduction in the number of NOS-2^+ cells per unit area ($P<0.01$) but increased intracellular ANXA1 expression ($P<0.01$).

Table 1. *Measures of body and thymic weight and of NOS-2 and ANXA1 expression (optical density) in adult rats treated neonatally with saline or DEX. $^{**}p<0.01$, $^{***}p<=0.001$, (n=4-8 animals).*

	Saline	**DEX**
Body weight (g)	270.7 ± 5.4	233.18 ± 4.3***
Thymus weight (g)	0.388 ± 0.01	0.314 ± 0.01***
Thymus:body weight ratio	0.144 ± 0.01	0.136 ± 0.01
NOS-2+ cell number (mm^2)	2.1 ± 0.2	1.2 ± 0.07**
Extracellular ANXA1	42.36 ± 9.7	64.44 ± 16.8
Intracellular ANXA1	28.87 ± 3.1	52.01 ± 4.9**

These results suggest that neonatal DEX treatment exerts long-term effects on the expression of two regulatory proteins within the thymus and thus support the view (Bakker *et al.*, 2001) that such treatments may compromise immune function.

Bakker J et al.2001 Neuroimmunology 112:47-54
Downing J et al.1998, Immunology 95:148-155
Taylor A.D et al., 1993 Neuroendocrinology 12:1014-1021

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225P THE PROGRAMMING ACTIONS OF GLUCOCORTICOIDS ON ANXA-1 EXPRESSION IN THE HYPOTHALAMIC-PITUITARY ADRENAL AXIS OF ADULT RATS TREATED PERINATALLY WITH DEXAMETHASONE

E. Theogaraj, C.D. John, [1]S.F. Smith & J.C. Buckingham Divisions of Neuroscience and Psychological Medicine and [1]National Heart and Lung Institute, Faculty of Medicine, Imperial College, London W12 ONN.

Exposure to both exogenous and endogenous factors in early life can modulate foetal development; the foetus adapts by optimising its physiology to its environment, thus permanently 'programming' physiological processes. However, if the foetus grows under conditions of malnutrition or is exposed inappropriately to stress hormones, these adaptations may be deleterious in a less challenging environment and may cause functional pathologies during later life. Animal studies have shown that the administration of glucocorticoids (GCs) perinatally to rats can cause long-term disturbances in the hypothalamo-pituitary-adrenal (HPA) axis (Felszeghy *et al.*, 2000). This is a matter of concern as perinatal GCs are often used clinically during times of threatened premature delivery.

The aim of this study was to examine the programming actions of GCs and in particular the GC-inducible protein annexin 1 (ANXA-1) on the rat neuroendocrine system. Male CFY (derived from the Sprague-Dawley strain) rats were treated with dexamethasone (dex) i.p. 1μg/10μl /g body weight on post-natal days (P) 3, 5 & 7. They were then allowed to grow without any further intervention. Tissues were collected on postnatal day 60 and incubated in Earle's balanced salt solution (Taylor *et al.*, 1993) prior to Western blotting for ANXA-1. The Western blot results were analysed by one-way ANOVA followed by Dunnet's (parametric) or Dunn's (non-parametric) multiple comparison test as appropriate (See table 1).

Table 1: Western blot analysis of ANXA-1 expression in the adult HPA axis of rats treated perinatally with dexamethasone

Tissue	Extracellular ANXA-1 (% control)	Intracellular ANXA-1 (% control)
Hippocampus	255±40[a]	107±8
Hypothalamus	92±6	109±8
Anterior pituitary	71±17[b]	82±7[b]
Adrenal Gland	63±8[b]	71±5[b]

Data are represented as mean ± SEM (n=4-7); [a] > ANXA-1 from control tissue; [b] < ANXA-1 from control tissue; $p < 0.05$

In a subsequent experiment, male rats were exposed to dex *in utero* by treatment of dams with 1μg/ml in drinking water during days 18-21 of gestation. At postnatal day 60, the rats were sacrificed and the anterior pituitary gland (n=6/group) removed and incubated ± forskolin (FSK 100μm) and ± dex (0.1μm). Resting and FSK stimulated release of ACTH was determined by radioimmunoassay as previously described (Taylor *et al.*, 1993). FSK induced a significant increase in ACTH release in naïve animals (basal 95±18 pg/ml vs FSK alone 206±24 pg/ml; $p<0.01$); this was reduced significantly by dex (FSK + dex 106±25 pg/ml; $p<0.05$). However, the inhibitory effect of dex was blunted in the anterior pituitaries from animals treated with dex perinatally (basal 137±24 pg/ml vs FSK 198±52 pg/ml vs FSK +dex 173±13 pg/ml). This study provides evidence that perinatal dex exposure, results in long-term changes in ANXA-1 expression and also has functional consequences in the HPA axis. We are currently examining whether these effects are causally linked.

Taylor, A.D et al., 1993. Neuroendocrinology 58: 430-439.
Felszeghy, K et al., 2000. Neuroendocrinology 12: 1014-1021

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226P EVIDENCE FOR PRO-SECRETORY AND ANTI-SECRETORY ACTIONS OF GLUCOCORTICOIDS AND ANNEXIN 1 IN GH_3 CELLS AND RAT ANTERIOR PITUITARY PRIMARY CULTURES.

T. Tierney & J.C. Buckingham. Department of Neuroendocrinology, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Hammersmith Hospital Campus, Du Cane Road, London. W12 0NN.

Previous work in our laboratory has shown that the inhibitory actions of dexamethasone (Dex) on secretagogue-stimulated pituitary hormone release are partly mediated by annexin 1 (ANXA1). In this study, we used rat anterior pituitary cultures and the GH_3 somatomammotroph cell line to investigate further the role of Dex and ANXA1 in growth hormone (GH) and prolactin (PRL) release.

Primary anterior pituitary cells (from male CFY rats; 200-220g) and GH_3 cells were maintained in D-MEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were plated in 24 well plates 5 days (primary cultures equivalent to ¼ pituitary/well) or 2 days (GH_3: 250,000 cells/well) before the experiment. In some cases, the cells were serum-starved overnight (by replacing the medium with unsupplemented D-MEM) prior to treatment. In others, the cells were maintained in full-medium until the beginning of the treatments. The treatments comprised pre-incubation in the presence or absence of Dex (0.1-1μM: 2.5h) or an active recombinant N-terminal fragment of ANXA1 ($ANXA1_{1-188}$: 0.01-1ng/ml: 2.5h) and subsequent stimulation for 1h with the adenylate cyclase activator forskolin (FSK: 1-100μM) or vasoactive intestinal peptide (VIP: 1-100nM) with continued presence of Dex or $ANXA1_{1-188}$. GH and PRL released during the second incubation were determined by radioimmunoassay. Statistical analysis was carried out by ANOVA and Tukey's *post-hoc* test (data are mean ± SEM; N=6 wells per group in all cases).

FSK caused concentration dependent release of GH and PRL (ng/well.h) from serum-starved GH_3 cells (GH: basal 0.29 ± 0.03 *vs.* 100μM FSK 98.13±27.80: $P<0.001$; PRL: basal 1.28 ± 0.27 *vs.* 100μM FSK 9.02±1.52, $P<0.001$). In serum-starved GH_3 cells, Dex (0.1μM) inhibited the effects of FSK on GH (~50% reduction: $P<0.01$) and PRL (~80% reduction: $P<0.01$) release as too did $ANXA1_{1-188}$, 1ng/ml, (GH ~50% reduction, $P<0.01$; PRL ~80% reduction, $P<0.01$). In complete contrast, in GH_3 cells that had not been serum starved both Dex (1μM) and $ANXA1_{1-188}$ potentiated FSK-stimulated GH and PRL release (FSK alone: 2-4 fold basal; FSK + Dex: 15 fold basal; FSK + $ANXA1_{1-188}$: 5-30 fold basal $P<0.01$). A similar profile of data was observed in primary cultures, thus in the serum-starved cells Dex and $ANXA1_{1-188}$ inhibited FSK-stimulated GH and PRL release ($P<0.05$) while in non serum-starved cells they had a positive effect ($P>0.05$). In the same vein, in both preparations Dex and $ANXA1_{1-188}$ inhibited the VIP-evoked release of GH and PRL from serum-starved cells but potentiated the secretory responses in non serum-starved cells.

These results provide novel evidence that serum factors modify the regulatory actions of glucocorticoids and ANXA1 on hormone release from primary and clonal rat pituitary cells in culture and thus have important implications for the design of further studies and our understanding of steroid hormone action.

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227P EFFECTS OF PERINATAL DEXAMETHASONE TREATMENT ON IMMUNE CELL DISTRIBUTION IN THE ADULT RAT.

C.D. John, E. Theogaraj, [1]A. Dewar, [1]S.F. Smith & J.C. Buckingham; Divisions of Neuroscience and Psychological Medicine and [1]National Heart and Lung Institute, Faculty of Medicine, Imperial College, London W12 ONN.

The distribution of immune cells between the blood and other immune compartments provides an important index of the ability of the host-defence system to respond to potential or ongoing immune challenge. Glucocorticoids (GCs) play a key role in regulating immune cell trafficking (Dhabhar et al., 1995) causing a decrease in the lymphocyte: neutrophil ratio by inhibiting neutrophil margination and decreasing lymphocyte numbers. GCs have been used for many years to reduce mortality of premature babies from respiratory distress syndrome. Their long-term safety has recently been questioned following reports that inappropriate exposure to GCs at critical stages of development may compromise health in adulthood. This study aimed to determine whether perinatal GC treatment affected the blood lymphocyte: neutrophil ratio in adulthood before and after treatment with dexamethasone (DEX).

Pregnant Sprague-Dawley rats were treated with DEX (1μg/ml in the drinking water) on gestational days 18-21; controls received normal tap water. Subsequent litters matured without further intervention. At maturity, male rats (300-350g) were treated with DEX (20μg/ml, i.p.) or saline (100μl/100g, i.p.), the blood was collected 2h later and processed for fluorescent activated cell analysis to determine lymphocyte: neutrophil ratios. Animals treated prenatally with DEX, showed a significant reduction in serum corticosterone (55.1±2.1pg/ml vs. 66.1±3.6pg/ml; $p<0.05$, determined by radio-immunoassay) and a parallel pronounced increase in the lymphocyte: neutrophil ratio (proportion of lymphocytes = 90.6±1.0% vs. 76.7±1.4% in controls, $p<0.01$, n=10). DEX administered at maturity had no significant effect on the lymphocyte: neutrophil ratio in the control group (no treatment; 76.7±1.4%; saline i.p.; 81.6±0.4; DEX i.p.; 77.6±2.2%; n=7-10). However, when given to animals treated prenatally with DEX, it caused a marked reduction in the proportion of lymphocytes (no treatment; 90.6±1.0%, saline i.p.; 88.6±1.6, DEX i.p.; 74.3±5%; $p<0.01$, n=7-10).

A further experiment assessed the cellular infiltration of the connective tissue sheath enclosing airways and blood vessels. Lactating rats were treated with DEX in the drinking water (1μg/ml) for 7 days; thus pups received oral DEX in milk for the first 7 days of life. At maturity araldite sections from peripheral and central areas of each lung were cut, examined blind and assessed semi-quantitatively for cellular infiltration according to a scale of 0 (not a feature) to 5 (very prominent). A score was given to each animal and the mean scores (n=4) were calculated for each treatment. Cellular infiltration was markedly reduced in animals treated with perinatal DEX in the central and peripheral connective tissue sheath (controls; central = 2.25, peripheral = 1.5 vs. neonatal DEX; central = 1.25, peripheral = 0).

We hypothesize that the reduction in serum corticosterone observed in animals treated neonatally with GCs results in (i) an increase in circulating lymphocytes and a decrease in circulating neutrophils and (ii) a greater sensitivity of the cells to GCs at maturity. The rise in circulating lymphocytes may be associated with the decreased lymphocyte numbers in another immune tissue, the lung.

Dhabhar *et al.*, (1995). J. Immunol., 154, 5511-5527.

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228P INHIBITION OF ISLET PHOSPHODIESTERASE PDE3 INCREASES THE CALCIUM RESPONSE TO GLUCOSE

Cruickshank SF, Drummond RM & Furman BL Department of Physiology and Pharmacology, University of Strathclyde, Strathclyde Institute for Biomedical Sciences, Taylor Street, Glasgow G4 0NR

We (Shaffiee-Nick *et al.*, 1995; Ahmad *et al.*, 2000) and others (Zhao *et al.*, 1997) hypothesised that the cyclic nucleotide phosphodiesterase in pancreatic islet β-cells regulating the cAMP pool relevant to augmentation of insulin secretion is the cyclic GMP inhibited isoform, PDE3B. Selective PDE3 inhibitors augment glucose-induced insulin secretion but the mechanism has not been investigated. cAMP increases $[Ca^{2+}]i$ in the β-cell and the present study was undertaken to investigate the effects of a selective PDE3 inhibitor, Org 9935 (see Shafiee-Nick *et al.* 1995), on the islet $[Ca^{2+}]i$ response to glucose.

Islets were prepared from rat (male Sprague-Dawley) pancreas (Shafiee-Nick *et al.*, 1995), loaded with fura-2 AM, 5μM (HEPES buffer, 3 mM glucose) and $[Ca^{2+}]i$ was measured in intact islets using dual wavelength microfluorimetry at 29.5°C, ratio values being converted to $[Ca^{2+}]i$ from *in vitro* calibration. Islets were exposed successively to increasing concentrations of glucose (3, 5.5, 8, 16.7 mM in HEPES buffer, pH 7.4), the next concentration being added when the $[Ca^{2+}]$ signal had plateaued at 15 min. Experiments were performed in the presence of either Org 9935 (10 μM, n=4) or DMSO (0.5%, n=4).

Analyses were performed on the plateau $[Ca^{2+}]i$ values, using two way repeated measures ANOVA (Minitab).

ANOVA showed a highly significant effect of glucose ($P<0.001$) and Org 9935 ($P<0.01$), as well as a highly significant interaction between glucose and the drug ($P<0.001$). In DMSO treated islets increasing concentrations of glucose produced small, slow concentration-dependent increases in $[Ca^{2+}]$ ($[Ca^{2+}]i$ nmol/l; 3mM glucose, 91±19; 16.7 mM, 127±14). In islets exposed to Org 9935 there were no differences from the controls in the $[Ca^{2+}]i$ at 3 mM or 5.5 mM glucose but the $[Ca^{2+}]i$ at 8 (174±21 nmol/l) or 16.7 mM (277±42 nmol/l) glucose was increased compared to control (respectively 127±14 and 138±17 nmol/l). The slow responses to glucose were probably due to the low temperature, because the response to 60 mM K^+ was very rapid (maximum attained within sec).

These are the first data showing effects of selective PDE3 inhibition on islet $[Ca^{2+}]i$ and are consistent with increases in β-cell cAMP content.

Ahmad M *et al.* (2000) *Br J Pharmacol* 129: 1228-34

Shafiee-Nick R *et al.* (1995) *Br J Pharmacol* 115: 1486-92

Zhao AZ *et al.* (1997) *Proc. Natl. Acad. Sci* 94: 3223-8

229P NEURONAL APOPTOSIS INDUCED BY HIV-1 GP120 IN THE NEOCORTEX OF RAT IS PREVENTED BY SYSTEMIC ADMINISTRATION OF 17β-ESTRADIOL

M.T. Corasaniti[1], S. Piccirilli[2], C. Del Duca[2], R. Nistico[3], A. Costa[3] & G. Bagetta[2,3], [1]Faculty of Pharmacy, University of Catanzaro, Italy; [2]Institute of Neurology C. Mondino, Experimental Neurobiology Center "Mondino-Tor Vergata-Santa Lucia", 00133 Rome and [3]Department of Pharmacobiology, University of Calabria, 87036 Arcavacata di Rende (CS) Italy.

Cognitive impairment, postural disorders and tremor are among the most common symptoms encountered in AIDS patients suffering from HIV-associated dementia (HAD) (see Corasaniti *et al.*, 2001). An important neuropathological feature of the brain described at *post-mortem* is cortical neuronal cell loss (see Everall *et al.*, 1994). Processing of the virus by cells of the myelomonocytic lineage yields host and viral products known to initiate a complex network of events, which may lead neurones to death and to the development of cerebral atrophy in AIDS patients (see Corasaniti *et al.*, 2001). Previously, we have reported that intracerebroventricular (i.c.v.) administration of the HIV-1 coat protein gp120 causes neuronal apoptosis in the neocortex of rat (see Corasaniti *et al.*, 2001) suggesting that this may be implicated in the development of HAD (Jones *et al.*, 2000). Here we report the original observation that systemic treatment with 17β-estradiol ($17\beta E_2$) prevented neuronal death caused by gp120. This implicates estrogen receptors since tamoxifen, which acts predominantly as an antagonist in the brain (McKenna *et al.*, 1992), abolished neuroprotection.

Male Wistar rats (250-280 g) received a single daily dose (100 ng i.c.v.) of gp120 (INTRACEL, Catalog n° 120011; DBA, Milan) given for seven consecutive days alone or preceded (1 h beforehand) by $17\beta E_2$ (0.2 $mgkg^{-1}$ i.p.; Sigma, Milan). Administration of gp120 was made using a 5 μl Hamilton syringe (2 μl volume of injection; 1 $\mu lmin^{-1}$ rate). In the antagonism study, tamoxifen (0.25 $mgkg^{-1}$; Sigma, Milan) was administered (i.p.) daily for seven consecutive days 1h before $17\beta E_2$ and 6 h after gp120. Neuronal apoptosis was assessed in coronal tissue sections (n=6 per brain) from the brain of rats sacrificed 24 h after the last injection of gp120 by using transmission electron microscopy, the TUNEL technique and haematoxylin and eosin staining (see Corasaniti *et al.*, 2001). Apoptotic cells (TUNEL + cells $[1161\ \mu m^2]^{-1}$) were counted as previously described (see Corasaniti *et al.*, 2001). Subchronic i.c.v. administration of gp120 causes ultrastructural changes (e.g. nuclear chromatin clumping, marginalisation and condensation) typical of apoptosis in the neocortex of rats (n=3). Interestingly, the number of apoptotic cells (mean±sem) counted in the brain neocortex of rats (n=4) treated with gp120 alone (7.37±0.29) was significantly reduced in rats (n=5) pretreated with $17\beta E_2$ (1.25±0.20) ($P<0.001$; ANOVA followed by Tukey-Kramer multicomparisons test). Quite importantly, pretreatment with tamoxifen (n=6 rats) abolished the neuroprotection afforded by $17\beta E_2$ (7.17±0.27; $P<0.001$). The mechanism underlying $17\beta E_2$ neuroprotection is under current investigation in our laboratory.

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Corasaniti M.T., *et al.* (2001) *J. Neurochem.*, **79**, 1-8.
Everall I.P., *et al.* (1994) *Acta Neuropathol.*, **88**, 538–544.
Jones M.V., *et al.* (2000) *AIDS*, **14**, 2709–2713.
McKenna S.E., *et al.* (1992) *Horm. Behav.*, **26**, 536-544.

230P EXPRESSION PROFILE OF PHOSPHODIESTERASES 4, 3 AND 7 IN RESTING AND ACTIVATED EPITHELIAL CELLS

Rachael Seamons, Alexandre Hinzpeter, Carolynn MacKenzie*, Simon MacKenzie*, Miles Houslay*, Gino van Heeke. Novartis Horsham Research Centre, Horsham, UK. *Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, University of Glasgow, Glasgow, Scotland, UK.

cAMP acts as a key second messenger in mammalian cells. The sole route of degrading cAMP is through a large family of phosphodiesterases (PDEs) (Houslay *et al.*, 1998). Of these, members of the four-gene PDE4 family have attracted considerable attention because selective inhibitors have potential therapeutic use in a wide variety of diseases including asthma and COPD.

Lung epithelial cells act not only as a physical barrier to protect the lungs from harmful inhaled agents, they also play a key role in modulating airway inflammation. As such, they can respond to a wide range of stimuli to produce inflammatory mediators and express adhesion molecules. Since the PDE4 family is involved in regulating the release of inflammatory cytokines in inflammatory cells, it may play a similar role in epithelial cells.

The purpose of this study was to establish the expression profile for the PDE4 family, as well as for PDE3A, 3B and 7A in primary bronchial epithelial cells (HBECs, Clonetics, USA) as well as for the commonly used epithelial cell lines A549 and NCIH292 (ATCC). Furthermore, the effect of treating HBECs with either LPS or a combination of rolipram and isoprenaline on the transcription of select PDE4 subtypes was investigated.

Total RNA was extracted from either resting or stimulated epithelial cells. HBECs were stimulated with either 100 ng/mL LPS for 2, 6, and 18 hr, or with 50μM rolipram plus 1 μM isoprenaline for 2, 6, and 18 hr, each in triplicate. PDE transcripts were detected by RT-PCR using specifically optimised primer sets that could distinguish between 12 different PDE4 splice variants, PDE3A, 3B and 7A. All PCRs were performed in triplicate. Transferrin and β-actin were used as controls. Semi-quantitative RT-PCR was performed for PDE4D3 and 4D5 by halting the amplification during the exponential phase and normalizing intensities of ethidium bromide-stained fragments on agarose gels vs transferrin. PDE4 enzyme activity was measured as described (Marchmont & Houslay, 1980) following selective immunoprecipitation of each PDE4 subtype.

In resting HBECs, mRNA was clearly detected for PDE4C, 4D1, D2, D3, D5, 3B and 7A. Only very weak signals were detected for PDE4A4B, 4B1, 4B2 and 3A, even after 40 cycles of amplification. Qualitatively, this pattern did not change following treatment with LPS or rolipram/isoprenaline. The PDE4 enzyme activity profile mirrored the transcript data, in that about 80% of the total PDE4 activity was due to the 4D subtype, with the remainder mainly due to PDE4A and 4B.

Semi-quantitative RT-PCR analysis revealed that PDE4D3 mRNA was transiently upregulated 2.3-fold 2 hr post-treatment with rolipram/isoprenaline ($p<0.05$), and returned to baseline after 6 hr. In contrast, PDE4D5 was downregulated 2-fold after 2 hr of LPS stimulation ($p< 0.001$) and returned to baseline by 18 hr. Qualitatively, the PDE profile of NCIH292 cells resembled that of HBECs, while A549 cells showed a different profile with a clear and unique presence of PDE4B3 and stronger presence of PDE3A.

This study established a detailed expression profile for the PDE4 family, PDE3A, 3B and 7A in HBECs and revealed differential transcription of PDE4D3 and 4D5 in response to rolipram/isoprenaline or LPS treatment, respectively.

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231P EFFECTS OF VERATRIDINE UPON ELECTROGENIC ION TRANSPORT ACROSS NPY KNOCKOUT AND WILDTYPE MOUSE DESCENDING COLON MUCOSA

N. P. Hyland & H. M. Cox, Centre for Neuroscience Research, KCL, Guy's Campus, London SE1 9RT

Neuropeptide Y (NPY) is a prominent enteric neuropeptide (Sang & Young, 1996) with prolonged antisecretory effects in mouse large intestine (Holliday *et* al., 2000). The Y_1 receptor which NPY stimulates is located both pre- and post-junctionally in rat colon mucosa (Tough & Cox, 1996). NPY deficient ($NPY^{-}/_{-}$) mice are more susceptible to seizures (Erickson *et al.*, 1996), however little is known of their gastrointestinal phenotype. Veratridine, a *Veratrum* alkaloid, has previously been reported to stimulate intrinsic enteric nerves and consequently alter mucosal ion transport (Sheldon *et al.*, 1990). Our aim in this study was to investigate the effects of veratridine in wildtype ($NPY^{+}/_{+}$) and $NPY^{-}/_{-}$ colon either in the presence or absence of the Y_1 receptor antagonist BIBO3304 (Wieland *et al.*, 1998).

Descending colon was obtained from male adult $NPY^{+}/_{+}$ or $NPY^{-}/_{-}$ mice on a 50% C57/BL6-50% 129SvJ background. Overlying smooth muscle was stripped away and mucosae placed in Ussing chambers and voltage-clamped at 0 mV (Holliday *et* al., 2000). Tissues were maintained at 37°C in oxygenated Krebs-Henseleit solution and once stable, veratridine (30μM) either in the presence or absence of BIBO3304 (300nM, 15mins) was added to the basolateral reservoir only, recording short-circuit current (I_{sc}) continuously. Any remaining neurogenic component was inhibited with tetrodotoxin (TTX; 100nM). Data was pooled and means ± 1 s.e.m. were compared using Student's unpaired *t*-test with a significance level of $p\leq0.05$.

	$NPY^{+}/_{+}$ ($\mu A.cm^{-2}$)	$NPY^{-}/_{-}$ ($\mu A.cm^{-2}$)
Basal I_{sc}	33.2 ± 2.8 (26)	25.4 ± 3.4 (26)
Veratridine	+ 46.5 ± 6.9 (9)	+ 29.8 ± 10.2 (6)
BIBO3304	+ 3.5 ± 0.8 (8)	+ 3.1 ± 0.6 (9)
BIBO3304 + Veratridine	+ 66.3 ± 14.8 (9)	+ 35.1 ± 7 .7 (9)

Table 1. Basal I_{sc} and responses to veratridine, BIBO3304 and veratridine plus BIBO3304 in $NPY^{+}/_{+}$ and $NPY^{-}/_{-}$ colon. *n* numbers are given in parenthesis. There were no significant differences between respective $NPY^{+}/_{+}$ and $NPY^{-}/_{-}$ groups.

Veratridine resulted in a sustained increase in I_{sc} which reached a maximum within 5 minutes. The absence of NPY did not significantly alter the veratridine response compared to $NPY^{+}/_{+}$ tissue, nor did selective blockade of the Y_1 receptor by BIBO3304. Veratridine responses were significantly inhibited to the same extent by TTX ($p\leq0.05$) in $NPY^{+}/_{+}$ and $NPY^{-}/_{-}$ colon. Our data suggests that NPY is not the predominant inhibitory neurotransmitter released in response to veratridine. Neither NPY nor its Y_1 receptor contribute significantly to the chemical stimulation of enteric nerves in $NPY^{+}/_{+}$ or $NPY^{-}/_{-}$ colon.

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232P A STUDY OF MUSCARINIC AND NEUROKININ COMPONENTS UNDERLYING PANCREATIC POLYPEPTIDE MOTOR RESPONSES IN MOUSE PROXIMAL COLON

N. Singh, S.D. Brain* & H.M. Cox, Centre for Neuroscience Research and *Centre for Cardiovascular Biology and Medicine, KCL, Guy's Campus, London SE1 9RT.

Pancreatic polypeptide (PP) is a potent antisecretory agent in mouse colon mucosa and recent studies have shown that the peptide also stimulates Y_4 receptor-mediated smooth muscle contraction in wild type ($Y_4^+/_+$) but not knockout ($Y_4^-/_-$) colon (Tough *et al.*, 2001). PP contractile effects have also been observed in rat proximal colon and were partially sensitive to atropine and tetrodotoxin (TTX; Pheng *et al.*, 1999). These authors and Ferrier *et al.*, (2000) proposed a neurogenic Y_4 receptor-mediation of PP contractions in rat intestine. Our aims were first to establish whether cholinergic and neurokinin (NK) contractile components were involved in PP responses in wild type mouse colon, and secondly to determine whether PP responses were altered in NK_1 receptor knockouts ($NK_1^-/_-$).

Segments of ascending colon from either NK_1 wild type ($NK_1^+/_+$), knockout ($NK_1^-/_-$, Cao *et al.*, 1999) or $Y_4^+/_+$ adult mice (>12 weeks of age, of either sex but the same genetic background; 50% C57/Bl6 – 50% 129Sv) were washed, attached with thread and suspended in 10 ml organ baths, containing oxygenated (95% O_2/ 5% CO_2) Krebs-Henseleit solution at 37°C. Tissues were stretched to a basal tension of 1g and allowed to equilibrate (45 min) with three intermittent washes. Isometric changes in basal tension were recorded to porcine (p) PP (30 nM) in tissues ± inhibitors (15 min). Values are the mean ± 1 s.e. mean throughout and statistical comparisons were performed using Student's unpaired *t*-test.

As previously observed, responses to pPP in $Y_4^+/_+$ colon were biphasic (Tough *et al.*, 2001). The initial peak increase in tension (within 5 min: 0.22 ± 0.14g, n=15) was followed by a prolonged phase where tension was maintained and the amplitude of spontaneous contractions increased. All parts of the pPP response were abolished by nifedipine (1μM, n=4, p = 0.01). Pretreatment with atropine (10μM) did not significantly alter pPP responses in $Y_4^+/_+$ colon (0.14 ± 0.06 g, n=6) but the muscarinic antagonist significantly attenuated 1μM carbachol responses (controls, 0.32 ± 0.21 g, n=14; 0.02 ± 0.03 g plus atropine, p=0.003, n=6). Furthermore pPP responses were not significantly different in $NK_1^+/_+$ (0.17 ± 0.13g, n=8) and $NK_1^-/_-$ colon (0.17 ± 0.11g, n=8). Desensitisation of wild type tissue to the contractile effects of substance P (SP, 3μM, confirmed by loss of sensitivity to a second SP addition) also had no effect upon pPP responses (0.22 ± 0.06 g, n=4) compared with untreated controls above.

We conclude that pPP stimulates Ca^{++}-mediated biphasic contractile responses that are not mediated by muscarinic mechanisms. NK_1 receptors are also not involved in the pPP response in mouse ascending colon. This is in contrast with the rat proximal colon where atropine and an NK_1 receptor antagonist both partially inhibited Y agonist induced contractions (Ferrier *et al.*, 2000). Other NK receptors are unlikely to be involved since SP desensitisation was without effect upon pPP-stimulated Y_4 responses.

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233P CHARACTERISATION OF NEUROKININ A INDUCED CONTRACTION IN RAT AND GUINEA-PIG ISOLATED ILEUM

M. Maginn, K. Rea & J. Matz. Department of Safety Pharmacology. H.Lundbeck A/S. Ottiliavej 9. Copenhagen. Denmark DK2500.

Tachykinins are a group of neuropeptides that are distributed widely in the mammalian central and peripheral nervous systems producing a wide range of biological effects including smooth muscle contraction via the activation of the NK_1 or NK_2 or NK_3 receptor subtypes (Regoli *et al.*, 1989). Neurokinin A (NKA) is one of the five-mammalian tachykinins suggested to predominantly activate NK_2 receptors in the intestine contractile response (Kerr et al., 2000). The aim of this study was to characterise further the NKA mediated contractile response in two rodent species.

Segments of ileum (1.2 cm) were dissected from guinea-pig (male Duncan-Hartley, 250-350g) and rat (male Wistar, 200-250g) and mounted under isometric conditions in a modified Krebs solution at 37°C and bubbled with 95% O_2/5% CO_2. All compounds were dissolved in 10% aq. 2-OH-Pr-ß-cyclodextrin (HPβC) and subsequently diluted in modified Krebs solution with a maximal HPβC concentration of 0.01%. All values are expressed as the mean ± SEM.

Following 60-min incubation at 1.5 g tension, NKA was administered non-cumulatively from 1 nM to 3 μM to avoid receptor desensitisation. A total drug contact of 2-min was used with a 10-min antagonist equilibration time and washout between each dose. NKA produced a concentration-dependent and reproducible contractile response in both the rat and guinea-pig. The E_{max} were 4.3 ± 0.4g (guinea-pig) and 2.0 ± 0.3g (rat). The EC_{50} were 43.9±7.2 nM (n=17) in the guinea pig and 46.6±5.4 nM (n=10) in the rat. The NK_1 receptor–selective antagonist L742694 (Hale et al., 1996) antagonised the NKA induced contraction in both the rat and the guinea pig (K_i=1069±396 nM, n=5 and 59±73 nM, n=6 respectively). L742694 more potently inhibited NKA induced contraction in the guinea-pig P<0.05 (Student t-test). L742694 inhibited the maximal NKA induced contraction by 23.1±17.4% (n=5) in the rat and to 23.7±16.0% (n=6) in the guinea pig. In the presence of the concentration of L742694 producing maximum inhibition (300 nM in the guinea pig, 3 μM in the rat) the NKA induced contraction was further studied by addition of SR 48968, a NK_2 antagonist in both the rat and guinea-pig (Petitet et al., 1993). In the presence of L742694, SR 48968 antagonised the remaining NKA induced contraction in both the rat and the guinea pig (K_i=0.62±18 nM, n=3 and 0.70±9 nM, n=6 respectively). SR 48968 (300nM) maximally inhibited the NKA induced contraction by 93.7±1.4% (n=6) in the guinea pig. Both NK_1 and NK_2, and possibly NK_3 receptor activation, mediate the NKA contractile response in both the rat and the guinea pig. The majority of the response is mediated by NK_2 activation, whilst the different K_i values for L742694 suggests that the there is a variation in the receptor binding between the two species for the NK_1 receptor.

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234P SUBSTANCE P-INDUCED TONIC CONTRACTION OF THE RAT ISOLATED ILEUM INVOLVES ACTIVATION OF L-TYPE CALCIUM CHANNELS AND INOSITOL TRISPHOSPHATE RECEPTORS

C. Coltman, R.I. Prince, W.M. Purcell & R.H. Osborne, Centre for Research in Biomedicine, University of the West of England, Coldharbour Lane, Bristol BS16 1QY

Substance P (SP)-induced contraction of rat ileum tissue is reduced in the presence of nitrendipine and Ca^{2+} - free buffer (Willis, *et al.*, 1993). However, SP receptors are believed to activate inositol trisphosphate ($InsP_3$) systems that increase intracellular Ca^{2+} levels. Here we report the effects of the NK_1 receptor antagonist NATBE (N-acetyl-L-tryptophan-3,5-bis (trifluoromethyl)benzyl ester (MacLeod, *et al.*,1993), the L-type Ca^{2+} channel blocker verapamil (Lee & Tsien, 1983), the $InsP_3$ receptor antagonist decavanadate (Föhr *et al.*, 1989) and a membrane-penetrable modulator of IP_3-induced Ca^{2+} release, 2-aminoethoxydiphenylborate (2-APB) (Maruyama *et al.*, 1997) on SP-induced contraction of rat isolated ileum tissue.

Ileal segments (4 sections taken 15-30 cm from the ileo-caecal junction) were obtained from male Wistar rats (220 – 250g) and mounted in 10ml organ baths containing Tyrode's solution (plus 1 μM atropine, gassed with 95% O_2 : 5% CO_2) under a tension of 1g. Tissues equilibrated for 20 min before non-cumulative dose response curves to SP were obtained using a 4 min cycle with 1 min contact time using an isotonic transducer. Antagonists were added to the buffer and tissues equilibrated for 20 min before retesting the effects of SP. Membrane permeability of decavanadate was improved by the addition of 1% DMSO to the buffer in control and test situations. Data are mean ± s.e. and statistical significance was determined using Student's *t*-test of pair differences.

SP (10 nM – 1 μM) caused dose-dependent tonic contractions with an EC_{50} value of 95 ± 9 nM (n = 12). Inclusion of 1 μM NATBE in the buffer increased the SP EC_{50} value to 650 ± 80 nM ($P<0.001$, n = 12) and reduced the maximum response by 35 ± 5%. At doses of 2 and 5 μM the antagonist reduced the maximum response to applied SP by 52 ± 7% (n = 8; $P<0.01$) and 79 ± 9% (n = 8; $P<0.001$) respectively. The maximum response caused by SP was reduced by 66 ± 8% (n = 8; $P<0.001$) and 95 ± 2% (n = 8; $P<0.0001$) in the presence of 1 μM and 10 μM verapamil respectively. 1μM 2-APB increased the EC_{50} value of SP from 90 ± 11 nM to 280 ± 40 nM (n = 8; $P<0.001$) while the maximum response was reduced by 47 ± 7% ($P<0.01$). The contractile effect of SP was reduced by 88 ± 12% (n = 8; $P<0.0001$) in the presence of 10 μM and abolished by 50 μM 2-APB (n = 8; $P<0.0001$). Decavanadate (50 μM) caused a 59 ± 7% fall (n = 8; $P<0.001$) in the maximum response to applied SP.

These data suggest that the rat ileum contains NK_1 receptors which, when activated by SP, cause contraction by opening L-type calcium channels. However, the inhibitory effects of decavanadate and 2-APB clearly demonstrate that SP-induced tissue contraction also involves release of Ca^{2+} from $InsP_3$-activated stores.

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235P SEROTONERGIC EFFECTS OF *MYO*-INOSITOL HEXAKISPHOSPHATE IN ISOLATED GUINEA-PIG ILEUM PREPARATIONS

S. Arkle, S. Jaura, A. Kalogirou & S. Nawaz. School of Pharmacy & Biomedical Sciences, University of Portsmouth, Portsmouth, UK. P01 2DT.

Myo-inositol hexakisphosphate (IP_6) has been reported to alter cellular and tissue function through a diverse range of actions (Shears, 2001). These include stimulation of contractile responses in the isolated guinea-pig ileum (GPI) (Arkle *et al.*, 1992). The aim of the study reported here was to investigated the relationship of IP_6-stimulated contraction in GPI to serotonergic mechanisms.

Isometric recordings were made in GPI and coaxially stimulated GPI (csGPI) and isotonic recordings in isolated longitudinal muscle strips of GPI (GPIILM) according to the method of Magnus (1904). Ileum from adult male Purbright guinea-pigs (400-600g) was incubated at 32°C in Tyrode solution. Preload was 2-4g for GPI and csGPI and 250mg for GPIILM. csGPI was stimulated at 0.2Hz with 70V pulses of 2 msec. Agonist were added for 1 min contact followed by 2 min wash and recovery in a 3 min cycle. Contractile responses were measured as peak active tension and normalised against the response to 10μM acetylcholine (ACh).

Whereas 5HT and IP_6 stimulated dose-dependent contractions in GPI; pD_2 3.8±0.3 (12) (mean±s.e.m. (n)) for IP_6, 5.3±0.2 (12) for 5HT, the response to 1mM *myo*-inositol hexasulphate (IS_6), used as a negative control, was 12.7±3.6% (5) of that to IP_6. The pD_2 of IP_6 was not altered by preincubation of GPI for 20 min with up to 100nM ondansetron, methiothepin or methysergide. (n=5, $P>0.05$) but was increased by the $5HT_4$-selective antagonist SB203186 (pD_2 increased by 0.45±0.09, 0.84±0.1, 1.23±08 and 1.55±06. (5), $P<0.05$ at 3, 10, 30 and 100 nM SB203186 respectively). Schild analysis of these data revealed a pK_B of 8.9±0.5 and Schild slope of 0.9±0.3. Similar analysis of the effects of SB203186 on 5HT responses yielded a pK_B of 8.3±0.2 and slope of 1.0±0.1 (5). To test whether this apparent serotonergic effect of IP_6 is due to a direct action on the gut smooth muscle or an indirect effect via release of 5HT the effect of IP_6 was tested in anatomically and pharmacologically denervated preparations (GPIILM and anaesthetised csGPI respectively). Responses to IP_6 were significantly reduced ($P<0.05$) in GPIILM compared to GPI (2±2% of 100μM ACh response at 100μM IP_6 and 6±3% (5) at 1mM IP_6 in GPIILM; 12±4% and 39±4% (5) respectively in GPI. When csGPI was incubated with IP_6 (30μM-3mM) then with 30μM lignocaine, at which concentration the response to ACh was unaltered, then re-incubated with IP_6 the response to the inositol decreased ($P<0.05$) such that the maximal response fell from 41±4% to 9±2% (5) of that to 100μM ACh. The response to IP_6 was restored to 53±8% of that to ACh.

These data support the hypothesis that IP_6 stimulates contraction of GPI via an indirect mechanism involving release of 5HT from within the gut wall and that IP_6 does not have a direct effect on the smooth muscle of the gut.

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236P INVESTIGATION OF THE EFFECT OF WAY-100635 (5-HT_{1A} RECEPTOR ANTAGONIST) ON 5-HT-INDUCED CONTRACTION IN DIFFERENT REGIONS OF THE *SUNCUS MURINUS* INTESTINE

F. A. Javid & R. J. Naylor, School of Pharmacy, University of Bradford, Bradford West Yorkshire, BD7 1DP.

In previous studies we have shown that different 5-HT receptors are involved in mediating the contractile effect of 5-HT in the *Suncus murinus* intestine (Javid & Naylor, 1999a,b). The aim of the present studies is to investigate further the involvement of 5-HT_{1A} receptors in mediating a response to 5-HT in different regions of the *Suncus murinus* intestine.

Segments (1.5 cm length) taken from the intestine (1-2 cm distal to the pyloric sphincter, S1 segment (proximal region), and 1-2 cm proximal to the anal region, S2 segment (terminal region)) of adult Japanese House Musk shrew, *Suncus murinus* (30-82 g) of either sex were mounted in 10 ml organ baths containing Krebs' solution (37 °C, 95% O_2, 5% CO_2). The tissues were allowed to equilibrate for 60 min and washed every 20 min. The resting tension was maintained at 0.5 g and contractions were recorded isometrically. Non-cumulative concentration-response curves to 5-HT (0.1 nM- 30.0 μM) in the absence and presence of WAY-100635 (1.0 μM) (Fletcher *et al.*, 1994) were established, with a 1 min contact time and 20 min intervals, using a paired experimental design. Tension changes were expressed as the mean ± s.e. mean of KCl-induced contraction (120.0 mM) of n=6 and analysed using paired t-test.

5-HT (0.1 nM - 30 μM) produced a concentration-dependent contraction curve in all the regions of the intestine examined. In the presence of WAY-100635 (1.0 μM) the contraction-response curve to 5-HT in the S1 segment was slightly shifted to the right without affecting the maximum response, and that achieved significance ($p<0.01$) only at 10.0 and 30.0 nM of 5-HT (Figure 1). However, in the S2 segment (the terminal region), the contractile responses to 5-HT (0.1 - 30 μM) in the presence of WAY-100635 (1.0 μM) were significantly greater ($p<0.05$ and $p<0.01$) as compared to the control responses to 5-HT (Figure 1).

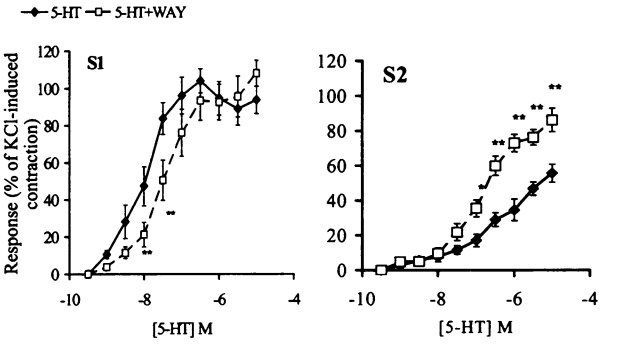

Figure 1. The effect of WAY-100635 on the contractile response to 5-HT in different regions of *Suncus murinus* intestine.

The data suggest that 5-HT_{1A} receptors may be involved in a contraction and a relaxation response to 5-HT in the proximal and terminal regions of the intestine respectively.

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237P FURTHER INVESTIGATION OF 5-HT INDUCED RELAXATION IN THE MOUSE ISOLATED COLON

H. Khan, B.R. Tuladhar & R.J. Naylor, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP.

In a previous study, we have reported a novel 5-HT induced relaxation in the tetrodotoxin (TTX) pre-contracted mouse isolated distal colon (Khan *et al.*, 2001). In this study, we have investigated the differential distribution of the 5-HT response in two regions of the mouse colon and investigated further the 5-HT receptor(s) involved in this response.

Segments of the proximal and distal colon (taken 1 & 4 cm, respectively, from the ileo-caecal junction) were obtained from BKW mice and mounted in organ baths as described previously (Khan *et al.*, 2001). After a 30 minute equilibration period, TTX (0.3 μM) and 5-HT (3 μM) were added in succession. The effects of 5-HT receptor antagonists and pre-treatment of 5-HT itself were examined in the distal segments of colon by adding the antagonists or 5-HT at least 30 min prior to the addition of TTX (and 5-HT). Statistical comparisons were made using the unpaired Students' *t* test and results were expressed as mean± s.e.m.

An addition of TTX produced contraction in all segments of the proximal and distal colon (0.84±0.09g & 1.14 ± 0.17 g, n = 24 respectively). However, subsequent addition of 5-HT produced a relaxation in the distal segments (-0.94± 0.21g, n=24) but not in the proximal segments. The addition of 5-HT (3 or 30 μM) on the untreated distal tissues produced biphasic responses (-0.17±0.04, 1.23±0.23 g and -0.13±0.03, 0.59±0.20g, respectively) which faded to baseline tension within 1-2 min. When TTX and 5-HT were subsequently added, the contraction to TTX was not reduced, but the relaxation to 5-HT was abolished. The relaxation response to 5-HT in the distal colon was, however, not significantly ($P>0.05$) affected by pre-treatment of tissues with various 5-HT receptor antagonists (Table 1).

The ability of prior desensitisation with 5-HT to abolish the 5-HT induced relaxation indicates that the response is mediated via a 5-HT receptor. The inability of 5-HT to produce relaxation in the proximal segments is most likely to be due to the absence of the receptor in that section of the colon. A further study is required to understand the 5-HT receptor(s) mediating this effect as the results indicate the non-involvement of 5-HT_{1A}, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_6 and 5-HT_7 receptors (see also Khan *et al.*, 2001).

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Table1: The effect of 5-HT receptor antagonists on the tension changes (g) due to TTX and 5-HT in the mouse distal colon.

Antagonist	Conc.	n	Control		Treated	
			TTX	5-HT	TTX	5-HT
Methiotepin	1 μM	4	0.84±0.11	-0.56±0.18	0.78±0.11	-0.61±0.20
Ketanserin	10 μM	8	0.75±0.09	-0.49±0.06	0.77±0.16	-0.57±0.08
WAY100635	0.1 μM	4	0.99±0.15	-0.68±0.10	0.77±0.16	-0.60±0.12
SB258585A (Hirst *et al.*, 2000)	1 μM	4	1.55±0.15	-1.67±0.22	1.38±0.17	-1.27±0.23
SB269970A (Lovell *et al.*, 2000)	1 μM	4	1.49±0.21	-1.51±0.26	1.45±0.23	-1.54±0.26

238P EVIDENCE FOR THE ROLE OF NITRIC OXIDE AS AN INHIBITORY NEUROTRANSMITTER IN THE MOUSE PROXIMAL COLON

H. Khan, B.R. Tuladhar & R.J. Naylor, The School of Pharmacy, University of Bradford, Bradford, BD7 1DP.

The present study was undertaken to investigate the role of nitric oxide (NO) as an inhibitory neurotransmitter in the mouse isolated proximal colon.

Segments of the proximal colon (2 cm in length and taken approximately 1 cm from the ileo-caecal junction) were obtained from BKW mice of either sex (25-35g) and mounted in 10ml organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs solution (37 °C) under an initial tension of 1g. The tension changes in the tissues were measured isometrically. Control responses to tetrodotoxin (TTX, 0.3 μm) were determined after 30 min equilibration. The tissues were washed and once the initial baseline tension was re-established, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μm) or N^G-nitro-L-arginine (L-NNA, 100 μm) was added and TTX (0.3 μm) responses were measured after the baseline change due to NO synthase (NOS) inhibitors had dissipated, some 20 minutes afterwards. The procedure was repeated in the third and fourth run with a further addition of L-arginine (L-Arg, 5 mM) and D-arginine (D-Arg, 5 mM) respectively in addition to either L-NAME or L-NNA. All results are expressed as the mean±s.e.mean. The significance of dif-ferences between the values was determined using ANOVA followed by Bonferroni Dunnett's *t* test ($P<0.05$).

All tissues consistently contracted to TTX (Table 1). The contractile response to TTX consisted of an initial phasic followed by a tonic response. The addition of L-NAME or L-NNA produced a short lasting contraction of the tissues (0.80±0.23 g, n=12 with L-NAME and 0.98±0.13 g, n=16 with L-NNA). The contraction of the tissues to TTX were almost abolished in the presence of either L-NAME or L-NNA but recovered with further addition of L-Arg but not D-Arg (Table 1).

The ability of TTX to contract the tissue indicates a blockade of a neuronal relaxant tone in the mouse proximal colon. The ability of NOS inhibitors L-NAME and L-NNA to block the TTX response and the ability of L-Arg but not D-Arg to reverse the effect of L-NAME and L-NNA, indicate that the release of nitric oxide from the enteric neurons mediate this relaxant tone. Thus, the results indicate not only that nitric oxide is an inhibitory neurotransmitter but also, even in the isolated condition, the proximal colon receives a continuous, neuronally mediated, nitrergic relaxant tone.

Table 1. Contraction (g tension) due to tetrodotoxin in the mouse isolated proximal colon in the presence of various drugs.

NOS Inhibitor (concentration)	Control	with NOS Inhibitor	with NOS Inhibitor & L-Arg (5 mM)	with NOS Inhibitor + D-Arg (5 mM)
L-NAME (100 μM)	0.84±0.08 (n=12)	0.02±0.02 (n=12)*	0.77±0.09 (n=12)$	0±0 (n=4)*
L-NNA (100 μM)	1.24±0.09 (n=16)	0±0 (n=16)*	0.80±0.09 (n=16)*$	0±0 (n=16)*

* $P<0.001$ compared to control, $ $P<0.001$ compared to NOS Inhibitor

239P REGULATION OF CHOLINERGIC MEDIATED NITRIC OXIDE PRODUCTION IN RAT GASTRIC EPITHELIAL CELLS.

C.S. Lodge, S. Arkle & J.F. Brown, Institute of Biomedical & Biomolecular Sciences, School of Pharmacy & BMS, University of Portsmouth, Portsmouth, UK. PO1 2DT

Nitric oxide synthase (NOS) activity has previously been reported in rat gastric epithelial cells (Brown *et al.*, 1992). The identity of agonists and intracellular pathways able to stimulate NO production in intact cells has been hindered due to the limitations of the detection systems employed. These systems usually measure end products of NO synthesis such as nitrite or co-products such as L-citrulline. Recently, a NO selective fluorescent dye, 4,5-diaminofluorescein (DAF-2) has been developed, allowing real-time measurement of NO synthesis in living cells and tissue (Kojima *et al.*, 1998). The aim of this study was to identify second messenger pathways involved in regulating cholinergic mediated NO production in rat gastric epithelial cells.

Rat gastric epithelial cells were isolated by pronase digestion coupled with intermittent calcium chelation according to a previously described method (Brown *et al.*, 1992). Cells were suspended at a concentration of 10^6 cells/ml^{-1} and NO production was measured as the rate of change of fluorescence in the presence of extracellular DAF-2 (10 μM). Excitation and emission wavelengths of 495 nm and 515 nm were used respectively with bandwidths of 5 nm and 20 nm.

A summary of the major findings is presented in Table 1. Acetylcholine (ACh; 1-3000 μM) produced a concentration related increase in NO production which was blocked by atropine and potentiated by eserine (Figure 1). In addition, the response to ACh was potentiated by IBMX and inhibited in the presence of protein kinase A and C inhibitors (Table 1)

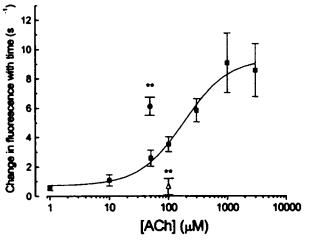

Figure 1. Effect of eserine (●) and atropine (Δ) both at 10 μM on ACh (■) stimulated NO production in gastric epithelial cells, measured as the rate of change in fluorescence intensity with time. Data are mean±SEM (n=6), where **P<0.05 by analysis of variance and Dunnett's test for difference from ACh (100 μM)

Table 1. Effect of agents on acetylcholine stimulated NO production. Data are mean±.SEM (n) where ##P<0.05 for difference from response to ACh (50 μM)/alone and **P<0.01 for difference from ACh (100 μM)/alone by analysis of variance and Dunnett's post-hoc test.

Agonist/Antagonist(concentration)	#Inhibits	Change in fluorescence with time (s^{-1})
ACh(100 μM)/alone	------	5.63±0.19 (6)
ACh(100 μM)/Gö6976 (8 μM)	PKC	0.38±0.08 (6)**
ACh(100 μM)/Staurosporine (1 nM)	PKC	0.52±0.10 (6)**
ACh(100 μM)/H-89 (48 nM)	PKA	4.94±0.80 (6)
ACh(100 μM)/H-89 (1 μM)	PKA	0.25±0.07 (6)**
ACh(100 μM)/H-7 (1 μM)	PKA	0.55±0.17 (6)**
ACh(50 μM) alone	------	2.58±0.55 (6)
IBMX (1 μM) alone	PDE	0.11±0.05 (6)
ACh(50 μM)/IBMX (1 μM)	PDE	7.15±1.37 (6)##

#PKC=protein kinase C; PKA=protein kinase A; PDE=phosphodiesterase

These results suggest that protein kinase A and C are involved in the intracellular regulation of ACh mediated NO production in rat gastric epithelial cells.

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Kojima, H., *et al.*, (1998). *Chem. Pharm. Bul.*, **46**, 373-375.

240P PREVIOUS EXPOSURE TO CHOLECYSTOKININ POTENTIATES THE CONTRACTILE EFFECTS OF ACETYLCHOLINE ON THE GUINEA PIG ISOLATED PYLORIC SPHINCTER MUSCLE

J.D. Patel & I.S. Ebenezer. School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, Hants, PO1 2DT.

It is well established that acetylcholine (ACh), acting on ACh muscarinic receptors, or cholecystokinin (CCK), acting on CCK_1 receptors, contract the pyloric sphincter muscle, and are responsible for inhibiting gastric emptying (Green et al., 1988, Moran et al., 1994). Based on unusual results obtained in pilot experiments, the present study was undertaken to investigate the effects of ACh on the contraction of guinea-pig pylorus muscle that had previously been exposed to CCK.

Adult male guinea-pigs were euthanised by exposure to CO_2 and cervical dislocation. The proximal duodenum and stomach was rapidly removed and placed in Tyrode buffer. The pyloric sphincter muscle was removed, set up in a 10 ml isolated organ bath and attached to an isometric force transducer, as described by Moran et al. (1994). Experiment 1. Concentration-response curves were constructed for ACh (n=10; Ach concentration range: $1x10^{-11}$ to $3x10^{-4}$M)) and CCK (n=5; CCK concentration range $1x10^{-9}$ to $2x10^{-5}$M). In all cases the tissue was exposed to a particular concentration of the drug for 30s before it was washed out. The tissue was allowed to recover for 60s before it was exposed to the next concentration of the drug. Experiment 2. The pyloric sphincter muscle was exposed to CCK ($1x10^{-6}$M) for 60s before it was washed out. 60s later the tissue was exposed to either (a) ACh $1x10^{-7}$M (n=3) or (b) ACh $1x10^{-6}$M (n=4). This procedure was repeated 5 times for each tissue. The contractile response of the muscle to ACh ($1x10^{-7}$M or $1x10^{-6}$M) measured prior to exposure to CCK was compared with those recorded with CCK after exposure. Experiment 3. A similar procedure as that used for Expt. 2a was used except that 30s prior to exposure to ACh, atropine was added to the bath. The results obtained in this study were analysed by repeated measures ANOVA followed by post-hoc tests.

Both ACh and CCK produced concentration-dependent increases in the contractile responses of the pyloric sphincter muscle (EC50 = $5.1x10^{-6}$M and $7.6x10^{-6}$M respectively), which are consistent with previous observations (see e.g. Moran et al., 1994). The results obtained in Experiment 2 are shown in Fig. 1A & B. The contractile responses to ACh ($1x10^{-6}$M or $1x10^{-7}$M) were significantly increased in tissue that have been previously exposed to CCK ($1x10^{-6}$M). The response to ACh (1x10-6M) after previous exposure to CCK ($1x10^{-6}$M) was reduced in a dose-related manner by atropine. For example, atropine 10^{-8}M reduced the response by 39.3%, and atropine 10-6M totally abolished the contractile response of the tissue to ACh.

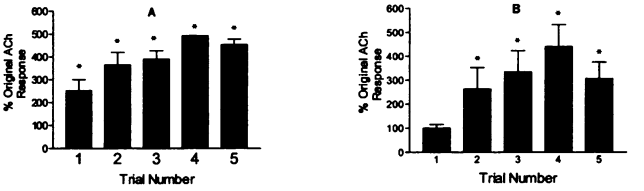

Figure 1. Effects of (A) ACh ($1x10^{-6}$M) or (B) ACh ($1x10^{-7}$M) on contractile responses of pyloric sphincter muscle that has been previously exposed to CCK ($1x10^{-6}$M). Verticle lines rep. +s.e.mean, *P<0.05.

The results of this study indicate that if the pyloric sphincter muscle is previously exposed to CCK, the responses to ACh are greatly enhanced. This enhancement appears to be mediated by post-synaptic ACh muscarinic receptors as it can be totally abolished with atropine. The mechanisms involved are not known, but may result from "communication" between intracellular processes induced by exposure to CCK and ACh. This phenomenon may represent a novel physiological mechanism involved in gut contractility.

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241P COMPARISON OF MUSCARINIC RECEPTOR SUBTYPE NUMBER AND FUNCTION IN MALE AND FEMALE RAT URINARY BLADDER

C. Kories, C. Wecking, C. Fetscher & M. C. Michel, Dept. of Medicine, Univ. Essen, 45122 Essen, Germany

Since muscarinic receptors are the main pathway responsible for contraction of the urinary bladder and since symptoms of overactive bladder are more prevalent in women than in men (Michel et al., 2001), we have compared the number and function of muscarinic M_2 and M_3 receptors in the urinary bladder of male and female rats.

Urinary bladders were obtained from 10-12 week old male and female Wistar rats (body weight 341 ± 3 vs. 228 ± 2 g, $p < 0.0001$; bladder weight 85 ± 1 vs. 65 ± 1 mg, $p < 0.0001$; n = 137 and 142, respectively). Muscarinic receptor number and subtype distribution was determined in saturation and competition binding experiments, respectively, using [^{3}H]-l-quinuclidinylbenzylate as the radioligand and methoctramine and darifenacin as competitors. Phospholipase C activation was determined as [^{3}H]-inositol phosphate formation in bladder slices. G-protein α-subunits were quantified by immunoblotting. Force of contraction was determined in bladder strips. Data are means ± SEM of n experiments. Statistical significance of differences was assessed by unpaired two-tailed t-tests with $p < 0.05$ considered significant.

Male and female rats expressed a similar number of muscarinic receptors (144 ± 5 vs. 140 ± 6 fmol/mg protein, n = 8 each). While methoctramine competition binding curves were not consistently better fitted by a two-site model, most darifenacin competition curves were biphasic and yielded 29 ± 10% and 31 ± 7% high affinity sites (corresponding to M_3 receptors) in male and females, respectively (n = 6 each).

Immunoreactivity of α-subunits of the G-proteins $G_{q/11}$, $G_{i1/2}$, G_{i3} and G_s was similar in both genders.

Carbachol concentration-dependently stimulated inositol phosphate accumulation in bladder slices from male and female rats with calculated maximum responses of 69 ± 17 and 77 ± 18% over basal and pEC_{50} values of 4.90 ± 0.45 and 4.40 ± 0.46, respectively (n = 7-8 each). Both methoctramine and darifenacin were similarly potent inhibitors of carbachol-stimulated inositol phosphate formation in both genders, with darifenacin being approximately 100-fold more potent (pIC_{50} 8.55 ± 0.21 vs. 8.60 ± 0.21) than methoctramine (pIC_{50} 6.57 ± 0.51 vs. 6.47 ± 0.19).

Carbachol concentration-dependently contracted bladder strips with a pEC_{50} of 5.66 ± 0.05 and 5.72 ± 0.06 and maximum effects of 0.43 ± 0.01 and 0.42 ± 0.02 mN mg^{-1} wet weight in male and female rats, respectively (n = 28 and n = 24, respectively). The contractile effect of carbachol was concentration-dependently antagonised by the non-selective atropine (1-30 nM), the M_1-selective pirenzepine (1-30 μM), the M_2-selective methoctramine (1-10 μM) and the M_3-selective darifenacin (10-100 nM); while the latter exhibited an unsurmountable antagonism, the inhibitory effect of each antagonist was similar in male and female rats.

We conclude that number und function of muscarinic receptors and the relative roles of their M_2 and M_3 subtypes do not differ between urinary bladders of male and female rats.

Michel, M. C. et al. (2001) *This Meeting*

242P A COMPARISON OF NKA RESPONSES IN THE HUMAN AND PIG DETRUSOR

D.J. Sellers[1], L. Templeman[2], C.R. Chapple[3], D.W.P. Hay[4] & R. Chess-Williams[2]. Division of Biomedical Sciences, Sheffield Hallam University, UK[1]; Department of Biomedical Science, University of Sheffield, UK[2]; Department of Urology, Royal Hallamshire Hospital, Sheffield, UK[3], GlaxoSmithKline, 709 Swedeland Road, King of Prussia, Pennsylvania, USA.[4]

The biological actions of tachykinins such as substance P and neurokinin A (NKA) are diverse and include smooth muscle contraction (Maggi et al., 1993). These actions are mediated by three distinct receptor subtypes, namely NK-1, NK-2 and NK-3 receptors (Maggi et al., 1993). Specific tachykinin receptors have been found to be present in the detrusor muscle of the urinary bladder of several species, including man. The rat and guinea pig bladder have been shown to contain both NK-1 and NK-2 receptor subtypes (Burcher et al., 1986), whereas only the NK-2 receptor seems to mediate contraction in the human bladder (Zeng et al., 1995). The pig is an often used animal model for the study of bladder physiology and pathophysiology as it is thought to be similar to humans. The present study investigates the role of NK-2 and NK-3 receptors in mediating the contraction of detrusor muscle strips from human as well as pig, to determine whether the pig is a good model for the study of tachykinin receptors in the human bladder.

Strips of detrusor muscle were taken from the bladder dome of female pigs (70-90 kg) and detrusor muscle was obtained from patients undergoing cystectomy for bladder cancer or colposuspension. The urothelium and serosa were removed and the strips suspended in gassed Krebs at 37°C under a resting tension of 1.0 g. Cumulative concentration-response curves to neurokinin A (NKA) were obtained in the absence and presence of either the NK-2 receptor-selective antagonist SR 48968 (3 - 100 nM) or the NK-3 receptor-selective antagonist SB 223412 (3 - 30 μM) with a 30 min incubation period. Experiments were performed in the presence of phosphoramidon (10 μM) to inhibit endopeptidase, CP99,994 (0.1 μM) to antagonise NK-1 receptors and meclofenamic acid (1 μM) to inhibit cyclo-oxygenase.

In human detrusor muscle NKA produced concentration-dependent contraction with mean pEC_{50} and maximum responses of 7.47±0.19 and 3.48±0.67 g respectively (n=4 patients). These curves were shifted potently to the right by the NK-2 receptor-selective antagonist SR 48968 (pK_B 8.85±0.08, n=14) whereas the NK-3 receptor-selective antagonist SB 223412 had a minimal effect (pK_B 5.81±0.11, n=12). The slope of the Schild plot for SB 223412 was close to unity (1.07±0.27), whilst that for SR 48968 was significantly different from 1 (slope = 0.50±0.06, $P<0.001$). Maximum responses were not affected by either of these antagonists. In pig detrusor muscle, NKA produced concentration-dependent contractions, with a pEC_{50} and maximum responses of 7.07±1.23 and 2.38±0.88 g (n=8). In pig detrusor muscle SR 48968 had a similar potency for antagonism of NKA-induced contraction (pK_B of 8.45±0.09, n=6, Schild slope 0.34±0.02, $P<0.001$) as in human bladder, whereas, SB 223412, at a concentration of 10 μM, was without effect on the NKA concentration-response curves.

These data confirm that NKA-induced contraction of human detrusor muscle is mediated by the NK-2 receptor subtype. The NK-2 receptor also appears to mediate contraction of the pig detrusor muscle. The NK-3 receptor appears to play no role in detrusor contraction.

Burcher et al., (1986) *Eur. J. Pharmacol.*, 128, 165.
Maggi C.A. et al., (1993) *J. Autonom. Pharmacol.*, 13, 23-93.
Zeng et al., (1995) *J. Urol.*, 153, 1688-1692.

243P RELAXANT EFFECT OF KETOTIFEN ON RAT ISOLATED BLADDER CONTRACTIONS

Sadraei H. & Hajhashemi V. Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences. Isfahan University of Medical Sciences, Iran

Ketotifen is a benzocycloheptathiophen which was initially designed as an antiasthmatic drug (Grant *et al.*, 1990). However, this drug has other pharmacological activities including a relaxant effect on guinea-pig intestinal smooth muscle (Abu-Dalu *et al.*, 1996) and rat isolated uterus (Sadraei, 2000). In this study we have examined the inhibitory effect of ketotifen on rat isolated bladder contractions in comparison with atropine and diazoxide.

Male Wistar rats (210-260g) were killed by a blow on the head and their bladders were removed, secured in Tyrode's solution in an organ bath at 37°C and gassed with O_2. Isotonic contractions induced by acetylcholine (ACh) and KCl were recorded before and after the addition of drugs (ketotifen, atropine, diazoxide). Each drug concentration was in contact with the tissue for at least 10 min before its effect was evaluated. In the case of Ach, the maximum amplitude of contraction and in the case of KCl, the area under the curve in the last 2.5 min were measured and expressed as % of maximum response for each tissue.

Ketotifen at 500nM and 5μM bath concentration shifted the concentration-response curve for ACh to the right with dose ratios (x) of 4 and 16 respectively. Atropine at 50nM concentration caused a dose ratio of 65.

Diazoxide at 50μM had no significant effect on the ACh concentration-response curve, but at 500μM bath concentration shifted the ACh concentration-response curve to the right (x= 3). In the case of the KCl concentration-response curves (cumulative), ketotifen attenuated responses to all concentrations of KCl (10, 20, 40 and 80mM). For example, at 500nM ketotifen the response to 80mM KCl was reduced from 97±1.6% (mean±s.e.mean) to 90±1.7%, and to 55±2.9% ($P<0.001$, Student's t-test) with 5μM ketotifen (n=6). Atropine at 50 & 500nM concentrations had no effect on KCl concentration-response curves (n=4) but diazoxide affected the responses to 10 and 20mM KCl. At 50μM diazoxide, the response to 20mM KCl was reduced from 43±3.8% to 31±3.5% ($P<0.01$) and to 14±1.2% ($P<0.001$) with 500μM diazoxide (n=5).

From these results it can be concluded that ketotifen inhibits the contractions due to ACh and KCl, while atropine only inhibits the contractions due to ACh. Ketotifen has some anti-muscarinic receptor activity (Etze, 1992) and this may explain its effect against ACh, but inhibition of the response to KCl indicates that other mechanisms may also be involved. If ketotifen has a similar inhibitory effect on bladder contraction *in vivo*, it might be a suitable drug for the control of unstable bladder.

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Eltze, M. *et. al.*, (1992). *Eur. J. Pharmacol.*, **211**, 283-293.
Grant, S.M. *et. al.*, (1990). *Drugs*, **40**, 412-448.
Sadraei, H. (2000). *Shahrekord Uni. Med. Sci. J.*, **1(4)**, 19-26.

244P EFFECTS OF SPHINGOSINE-1-PHOSPHATE IN HUMAN PROSTATE CANCER CELL LINES

Y. Li, S. Nau, C. Altmann, P. Czyborra, M. C. Michel, Depts. of Medicine and Pharmacology, Univ. Essen, 45122 Essen, Germany

Sphingosine-1-phosphate (SPP) can stimulate cellular growth in many cell types but also may cause cell death in others (Spiegel & Milstien, 2000). Since it has been reported that prostate cancer-derived cell lines express receptors for SPP (Gibbs et al., 2001), we have determined the effects of SPP on intracellular Ca^{2+}, cell proliferation and cell death in two of these cell lines, PC3 and DU 145 cells.

Alterations of intracellular Ca^{2+} concentrations were determined using the fluorescent dye Fura-2. Effects on cell proliferation were determined by cell counting and by quantification of [^{3}H]thymidine incorporation. Induction of apoptosis was determined by staining with Hoe 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole; 5 μg ml^{-1}). Since induction of apoptosis can involve a p38 member of the family of mitogen-activated protein kinases, effects of the p38 inhibitor SB 203,580 (4-(4-fluorophenyl)-2-(4-methylsulfinyl-phenyl)-5-(4-pyridyl)1H-imidazole) were also determined. Data are means ± SEM of n experiments.

Basal intracellular Ca^{2+} concentrations were 439±8 nM and 460±9 nM (n=28 each) in DU 145 and PC3 cells, respectively. SPP (0.1 nM -100 μM) concentration-dependently increased Ca^{2+} concentrations with a pEC_{50} of 6.66±0.28 and 6.94±0.21 and maximum elevations of 167±21 and 322±35 nM (n=4 each) in DU 145 and PC3 cells, respectively.

Low concentrations of SPP (1 μM) had little effect on [^{3}H]thymidine incorporation, cell counts or Hoe 33342 staining in either cell line. Intermediate concentrations, (e.g. 10 μM) SPP caused minor enhancements of [^{3}H]thymidine uptake but these did not translate into any significant increase of cell numbers. The [^{3}H]thymidine uptake in DU 145 (but not PC3) cells was fully blocked by 10 μM SB 203,580 (42±4% vs. 2± 10% over basal, n=4, $p<0.05$ in a paired t-test). High SPP concentrations (e.g. 100 μM) markedly reduced [^{3}H]thymidine incorporation to 32±4% and 45±10% of control (n=5, $p<0.05$) and detectable cell numbers to 8±4% and 35±3% of control (n=3, $p<0.05$) in DU 145 and PC3 cells, respectively. Concomitantly, 100 μM SPP increased the percentage of Hoe 33342-positive cells from 4±1% to 81±12% and from 5±1% to 26± 2% (n=4 each, $p<0.05$ in a repeated measures ANOVA) in DU 145 and PC3 cells, respectively. Induction of apoptosis by SPP was confirmed by immunostaining of bromodeoxyuridine triphosphates labelling. While 10 μM SB 203,580 did not significantly alter the effects of 100 μM SPP on [^{3}H]thymidine uptake or cell numbers, it enhanced the SPP effect on Hoe 33342 staining in PC3 (to 66±7%, n=4) but not in DU 145 cells.

We conclude that DU 145 and PC3 prostate cancer cell lines respond to SPP exposure with concentration-dependent elevations of intracellular Ca^{2+} concentrations. In contrast to many other cell types this does not translate into enhanced cell proliferation but rather, at least at high concentrations, into increased cell death, possibly by apoptosis. This cell death induction does not appear to involve a p38 mitogen-activated protein kinase.

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Spiegel, S. & Milstien, S. (2000) *Biochim. Biophys. Acta* 1484: 107-116

245P COMPARISON OF RENAL EFFECTS OF SYSTEMIC AND INTRARENAL ADMINISTRATION OF SPHINGOSYLPHOSPHORYLCHOLINE

J. Bai, U. Heemann & M.C. Michel, Dept. of Medicine, Univ. Essen, 45122 Essen, Germany

We have recently reported that i.v. infusion of lysosphingolipids such as sphingosine-1-phosphate or sphingosylphosphorylcholine (SPPC) lowers renal blood flow (RBF) and concomitantly increases diuresis and natriuresis in anaesthetised rats in the absence of major alterations of glomerular filtration rate (Bischoff et al. 2001). This pattern is similar to that seen with i.v. infusion of neuropeptide Y, but direct intrarenal (i.r.) infusion enhances the renovascular and concomitantly attenuates tubular neuropeptide Y effects indicating mediation via an extrarenally located receptor (Bischoff & Michel 1998). Therefore, we have compared the effects of i.v. and direct i.r. infusion of SPPC.

Male Wistar rats (300-450 g) thiobutabarbitone-anaesthetised were instrumented as previously described (Bischoff et al. 2001), and an additional catheter was placed into the right suprarenal artery for direct i.r. infusion. Following equilibration, the rats received 10 μg kg^{-1} min^{-1} of SPPC or vehicle via the femoral vein or directly i.r. for 60 min (9-10 per group). Mean arterial blood pressure (MAP) and RBF of the right kidney were determined initially every minute and then in 15 min intervals, whereas urine was collected from both kidneys in 15 min intervals throughout. Data during SPPC or vehicle infusion are expressed as alterations relative to the average of the last three measurements prior to infusion and are shown as means ± SEM of n rats. Statistical significance of differences between groups was assessed by two-way analysis of variance testing for overall treatment effects relative to vehicle with a $p < 0.05$ considered significant.

Prior to infusion, MAP, RBF and right and left kidney urine flow were 102 ± 4 mm Hg, 7.2 ± 0.5 ml min^{-1} and 61 ± 13 and 52 ± 5 μl 15 min^{-1}, respectively, in vehicle-infused rats, and not significantly different in the rats who went on to receive i.v. or i.r. SPPC administration. In confirmation of previous results with i.v. SPPC administration (Bischoff et al. 2001), neither i.v. nor i.r. SPPC infusion significantly affected MAP relative to vehicle-infused animals. Peak reductions of RBF occurred 2-3 min after the start of the SPPC infusion and were 0.49 ± 0.27 and 0.84 ± 0.36 ml min^{-1} (compared to 0.06 ± 0.10 ml min^{-1} in the vehicle group). While RBF gradually returned to baseline values upon continued i.v. SPPC administration, it remained at 0.63 ± 0.42 ml min^{-1} below baseline upon i.r. SPPC infusion. Concomitantly, for the overall time course i.r. SPPC caused significantly greater RBF reductions than i.v. infusion ($p < 0.001$). Upon direct i.r. infusion into the right kidney, urine flow rate of that kidney was significantly greater than with i.v. infusion of SPPC ($p < 0.01$); for example, during the last collection period it was 32 ± 6, 17 ± 13 and 1 ± 3 μl 15 min^{-1} over basal values with i.r. and i.v. SPPC and with vehicle, respectively. In contrast, the opposite was seen with the left kidney in which systemic SPPC infusion increased urine flow whereas direct infusion into the right kidney did not.

We conclude that both renovascular and tubular SPPC effects are enhanced upon direct i.r. administration. Thus, in contrast to neuropeptide Y, SPPC does not primarily alter tubular function via an extrarenal receptor.

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Bischoff, A. & Michel, M. C. (1998) *Pflüger's Arch. – Eur. J. Physiol.* 435: 443-453

246P SODIUM – LITHIUM COUNTERTRANSPORT ACTIVITY IN RAT PROXIMAL TUBULE CELL SUSPENSIONS.

LA Marshall, MJ McLeod & JS McLay Dept Medicine & Therapeutics, University of Aberdeen, Foresterhill, AB9 2ZD

Sodium – lithium countertransport (SLC) represents an *in vivo* form of sodium – sodium exchange, the physiological role of which is still unclear. However, SLC activity has been reported to be elevated in human erythrocytes and more recently skin fibroblasts in patients with essential hypertension (Zerbini *et al*, 2001). It now appears that the presence of SLC activity may not be restricted to the erythrocyte, therefore the aim of this study was to determine whether proximal tubule cells (PTC) also possess SLC activity. The PTC play an important role in the regulation of salt and water homeostasis and within these cells the activity of many other sodium transporters have been linked with experimental models of hypertension (Doris, 2000). PTC thereby represent a functionally appropriate cell type in which to investigate SLC activity.

Male Sprague–Dawley rats (250-350g) were killed by cervical dislocation. The cortex was dissected from the kidney and proximal tubule cells were isolated by collagenase digestion followed by Percoll density gradient centrifugation. SLC activity was measured by loading cells in lithium (150mM for one hour), washing cells twice in choline buffer and measuring the amount of lithium efflux into sodium – containing or sodium–free buffers. This was performed using the Millipore Rapid Filtration technique.

Lithium uptake into cells was shown to be time and concentration–dependent and the MTT assay confirmed that incubation with lithium was non–toxic to the cells.

The rate of lithium efflux was higher into sodium than choline, demonstrating a sodium–dependent lithium efflux (65.2μM lithium mgprotein^{-1} min^{-1} versus 6.7μM lithium mgprotein^{-1} min^{-1}, n = 3). The rate of SLC was decreased in the presence of 100μM ouabain, an inhibitor of the Na^+/ K^+ ATPase (58.5μM lithium mgprotein^{-1} min^{-1} versus 43.8 μM lithium mgprotein^{-1} min^{-1}) and abolished in the presence of 20μM dimethylamiloride, an inhibitor of Na^+ / H^+ exchange. Phloretin, an established although non–specific inhibitor of erythrocyte SLC, also produced a decrease in the rate of SLC (138μM lithium mgprotein^{-1} min^{-1} versus 42.5μM lithium mgprotein^{-1} min^{-1}, n = 3). Intracellular ATP depletion had no effect upon the SLC activity, implying that this transport process is energy – independent. This is in accordance with erythrocyte studies.

The results of this study demonstrate that freshly isolated rat proximal tubular cells display a sodium–lithium counter-transport activity. The lithium efflux from cells is not all due to the action of the Na^+/ K^+ ATPase or Na^+/ H^+ exchangers and appears to be energy–independent. Phloretin (200μM) partially inhibited PTC SLC activity, a property shared with erythrocyte SLC activity.

Proximal tubule cells may therefore represent a potential model for the characterisation of the SLC in a nucleated and functionally appropriate cell type.

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247P Na^+-INDEPENDENT TRANSPORTERS, LAT-2 AND $b^{0,+}$, EXCHANGE L-DOPA WITH NEUTRAL AND BASIC AMINO ACIDS IN OPOSSUM KIDNEY CELLS

P. Gomes & P. Soares-da-Silva. Inst. Pharmacol. & Therap., Fac. Medicine, 4200 Porto, Portugal.

Previous studies showed that opossum kidney (OK) cells take up L-DOPA through a saturable, stereoselective and temperature-dependent process when applied from the apical and basolateral cell border (Soares-da-Silva et al., 1997; Vieira-Coelho & Soares-da-Silva, 1997), this being similar to that occurring in rat renal proximal tubules (Pinto-do-Ó & Soares-da-Silva, 1996). However, the transporters involved in uptake of L-DOPA by renal epithelial cells have not been identified. The present study examined the functional characteristics and regulation of the L-DOPA transport in two functionally different clonal subpopulations of opossum kidney (OK) cells, OK_{LC} and OK_{HC} cells. These cells derive from the same original batch obtained from the American Type Culture Collection (F-12476) and are morphologically identical, but differ markedly on their ability to transport sodium (Gomes & Soares-da-Silva, 2000).

OK cells (ATCC 1840-CRL) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Minimum Essential Medium supplemented with 10% foetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 μg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. L-DOPA and [^{14}C]-L-DOPA were quantified by means of high pressure liquid chromatography with electrochemical detection and liquid scintillation counting, respectively. Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

The uptake of L-DOPA was largely Na^+-independent, though in OK_{HC} cells a minor component (~ 15 %) required extracellular Na^+. At least two Na^+-independent transporters appear to be involved in L-DOPA uptake. One of these transporters has a broad specificity for small (L-alanine, L-serine, L-threonine and L-cysteine) and large neutral amino acids (L-leucine, L-isoleucine and L-phenylalanine), is stimulated by acid pH and inhibited by 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BCH; OK_{LC}, K_i=291 μM; OK_{HC}, K_i=380 μM). The other Na^+-independent transporter binds neutral and basic (L-arginine and L-lysine) amino acids and also recognizes the di-amino acid cystine. The efflux of [^{14}C]-L-DOPA from OK_{LC} and OK_{HC} cells over 12 min corresponded to a small amount of [^{14}C]-L-DOPA accumulated in the cells (fractional outflow = 32±1%). L-leucine, non-labelled L-DOPA, BCH and L-arginine, stimulated the efflux of [^{14}C]-L-DOPA in a Na^+-independent manner.

It is suggested that L-DOPA uses at least two major transporters, systems LAT-2 and $b^{0,+}$. The transport of L-DOPA by LAT-2 corresponds to a Na^+-independent transporter with a broad specificity for small and large neutral amino acids, stimulated by acid pH and inhibited by BCH. The transport of L-DOPA by system $b^{0,+}$ is a Na^+-independent transporter for neutral and basic amino acids that also recognizes the di-amino acid cystine.

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248P INCREASED Na^+,K^+ ATPase ACTIVITY CORRELATES POSITIVELY WITH L-DOPA UPTAKE IN RENAL OK CELLS

P. Gomes & P. Soares-da-Silva. Inst. Pharmacol. & Therap., Fac. Medicine, 4200 Porto, Portugal.

Considerable evidence suggests that uptake of L-DOPA, the precursor of natriuretic hormone dopamine, in renal epithelial cells is dependent on apical-to-basal flux of Na^+ (Pinto-do-Ó *et al.,* 1996). The present study was designed to seek further evidence on the involvement of mechanisms regulating the tubular transport of sodium on the intracellular availability of L-DOPA, in wild type and in OK cells overexpressing Na^+,K^+ ATPase.

In order to generate a cell line that overexpresses Na^+,K^+ ATPase, OK cells were transfected with the wild-type rodent Na^+,K^+ ATPase alpha-subunit (pCMV ouabain vector), and native cells used as a control. Cells were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters (Costar, 3524) in Minimum Essential Medium supplemented with 10% foetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin; transfected cells were grown in selection medium containing 10 μM ouabain. The ability to translocate sodium from the apical to the basal cell side was evaluated by measuring the activities of Na^+,K^+ ATPase and the Na^+/H^+ exchanger, as previously described (Gomes et al., 2001; Vieira-Coelho *et al.*, 2001). In transport studies, L-DOPA was quantified by means of HPLC with electrochemical detection. Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test.

Wild type cells exposed to culture medium containing 10 μM ouabain die in a time dependent manner; in contrast, transfected cells exhibit normal growth under these conditions. The maximal amphotericin B (1.0 μg ml^{-1}) induced increase in short-circuit current (I_{sc}) was significantly higher ($P<0.05$) in transfected cells (36.9±3.9 vs 69.0±2.7 μA/cm^2). In addition, the sensitivity to ouabain was increased ($P<0.05$) in transfected cells (IC_{50} values: wild type, 1.4 μM; transfected, 17.2 μM), which is characteristic of rodent kidney cells. Na^+/H^+ exchanger activity was also found to be increased in transfected cells (0.0034±0.0002 vs 0.0063±0.0005 pH units/s). Non-linear analysis of the saturation curve for L-DOPA uptake revealed a K_m value (in μM) of 184±15 and 320±28 and a V_{max} value (in nmol mg $protein^{-1}$ 6 min^{-1}) of 25.3±0.7 and 48.7±1.7 in wild and transfected cells, respectively. The inhibitory effect of amphotericin B (2.5 μg ml^{-1}) upon L-DOPA accumulation in transfected cells (45.9±2.6 % reduction) was greater than that in wild type cells (23.1±2.1 % reduction).

It is concluded that enhanced ability of transfected OK cells to translocate sodium from the apical to the basal cell side correlates positively with their ability to accumulate L-DOPA, which is in agreement with the role of sodium in taking up the precursor of renal dopamine. It is also suggested that these cells may constitute an interesting cell model for the study of renal epithelial physiology.

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249P EVIDENCE THAT FRACTION IV (FIV), A PURIFIED FRACTION OF *Phoneutria nigriventer* VENOM (PNV), IS RESPONSIBLE FOR THE NEUROGENIC OEDEMA OBSERVED IN RESPONSE TO PNV IN MICE

[1,3]S.K.P Costa, [1]S. Hyslop, [3]A.D. Grant, [2]R.J. Docherty & [3]S.D. Brain. [1]Dept Pharmacol., FCM, UNICAMP, Campinas, Brazil, [2]Sensory Function Group and [3]Centre for Cardiovascular Biology & Medicine, KCL, London SE1 9RT.

Venom from the *Phoneutria nigriventer* snake (PNV) causes pain and oedema in humans (Bucaretchi *et al.*, 2000). In rat skin, intradermal injection (i.d.) of PNV evokes neurogenic plasma extravasation (Costa *et al.*, 2000). In this study, we screened PNV fractions obtained by gel filtration and determine their ability to activate sensory nerves to mediate oedema. The fractions were tested in wild-type (W-T) and NK_1 receptor knockout (KO) mice.

PNV (10 mg) was dissolved in ammonium acetate (0.15 M, pH 4.7) and centrifuged. PNV was applied to Superdex 75 column (HR10/30). The column was eluted with buffer (0.5 ml/min) and the elution profile was monitored at 280 nm. Fractions of 1 ml were collected and those corresponding to the main seven peaks were pooled and lyophilized. The concentration of material used in the assays was based on the absorbance of each peak at 280 nm, where an A280 of 1.0 corresponded to a concentration of 1 mg/ml. Oedema was assessed in the skin of male and female mice. Animals (28 g) were anaesthetised with urethane (7 μg/g, i.p.) and ^{125}I-labelled albumin (BSA, 1.25 μCi) injected by tail vein. Tests agents (50 μl) were injected i.d.. After 30 min, a blood sample was collected, the animals were killed by cervical dislocation and skin sites removed. The oedema formation was calculated as μl plasma per g. Data are mean ± s.e.mean for *n* animals. Statistical analysis was by ANOVA followed by Student's unpaired t-test. $P<0.05$ was taken as significant.

Intradermal injection of substance P (100 pmol/site) induced a marked oedema in the skin of W-T (62 ± 15 μl, n=5) but had no effect in the skin of KO mice (-5.4 ± 6.7, n=4). PNV (1-10μg/site) i.d. injected caused a dose-dependent oedema in the skin of W-T mice (n=5) but not in KO mice (Fig. 1A, n=4). Only FIV (1-30 μg/site, n=7) produced oedema ($P<0.001$) of the 7 fractions tested. FIV (1-10 μg/site) was a potent oedema inducer in W-T mice but not KO mice (Fig. 1B, n=5). The NK_1 receptor antagonist SR140333) (120 nmol/kg, i.v., n=7) (Costa *et al.*, 2000) markedly inhibited the oedema induced by either the NK_1 receptor agonist GR73632 (30 pmol; from 45 ± 9 to 7.6 ± 2 μl; $P<0.001$, n=5-7) (Costa *et al.*, 2000) or FIV (3 μg; from 43 ± 9 to 0.2 ± 5 μl; $P<0.01$, n=5-7).

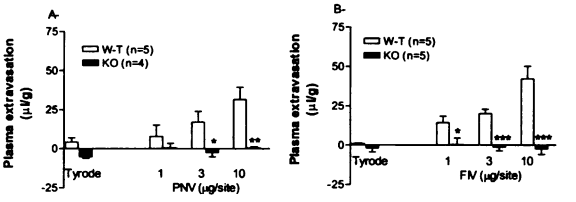

Figure 1. Effect of PNV (panel A) and FIV (panel B) in the skin of W-T and KO mice. Data are mean ± s.e.mean for *n* animals. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to W-T group.

PNV produced oedema in the skin of W-T mice but not in KO mice. FIV is the main oedema-inducing component. The inability of FIV to produce oedema in the skin of NK_1 KO and SR140333-treated mice suggests that FIV is the active component (it is probably the polypeptide PNV3, wt 14,475; Bento *et al.*, 1995) responsible for the PNV-induced neurogenic oedema formation.

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250P CAPSAICIN-EVOKED RELEASE OF SUBSTANCE P FROM THE SPINAL CORD OF NK_1 RECEPTOR WILD TYPE AND KNOCKOUT MICE.

[1]I.J. Lever, [2]A.D. Grant, [2]S.D. Brain & [1]M. Malcangio. [1]Centre for Neuroscience Research & [2] Centre for Cardiovascular Biology & Medicine, GKT School of Biomedical Sciences, King's College London, SE1 1UL.

Substance P (SP) is an excitatory tachykinin transmitter released from peripheral and central terminals of primary afferent nociceptors in response to noxious stimuli. Neurokinin (NK_1) receptors for SP are located peripherally on vascular cells and have a functional role in generating oedema after topically applied capsaicin, (Cao, *et al.*1999). NK_1 (auto)-receptors, may also be located on central terminals of primary afferents, acting to inhibit SP release (Malcangio & Bowery, 1994). We evaluated the release of SP from the spinal cords of NK_1 knockout and wild-type mice and changes after stimulation of central nociceptive terminals by capsaicin *in vitro*.

Adult wild-type (+/+) and NK_1 receptor knockout (-/-) Sv129+C57BL/6 mice (Cao *et al.*, 1999) were anaesthetised with urethane (7μg/g) I.P. Capsaicin solution (20 μl of 33mM) applied topically to the ears of +/+ mice induced oedema ($p<0.001$), and this was not observed in -/- mice or in mice treated with the selective NK_1 antagonist SR140333 (480 nmol/kg) I.V, as expected. In separate experiments, mice from the same breeding colonies (both sexes; 20-30g) were decapitated and the spinal cords removed. Sections (16mm) of the lumbar enlargement region were mounted in chambers and superfused (1ml/min) with oxygenated Krebs' solution. Three superfusate samples (8 min fractions) were collected before cord stimulation and the mean used to determine basal SP release. During collection of the fourth fraction, cord sections were stimulated by superfusion of capsaicin solution (1μM). Three subsequent fractions were collected after stimulation to assess release recovery. Cord sections were then removed, weighed and SP extracted in acetic acid. SP content in superfusate and tissue samples was measured by radioimmunoassay, (sensitivity 0.5 fmol/tube), adapted from Malcangio & Bowery 1994.

Basal SP outflow levels were significantly higher in -/- mice 9.5 ± 1.4 fmol/8ml compared to +/+ animals 5.6 ± 0.6 (mean ± s.e.m, n=11, p=0.037, Mann-Whitney-U test). Capsaicin (1μM) increased the SP content in superfusate samples from both +/+ and -/- animals to 21.2 ± 3.7 and 28.22 ± 4.9 fmol/8ml, respectively ($p<0.05$ ANOVA then Dunnett's test). However, the amount of SP released over basal was not significantly different between -/- and +/+ mice. At the end of release experiments, the total amount of SP extracted from +/+ and -/- mouse cord tissue was 114.1 ± 25.3 and 115.3 ± 24.9 fmol/mg tissue, respectively ($p>0.05$ t-test). These data suggest a role for NK_1 receptors in regulating basal but not capsaicin-evoked release of SP in the mouse spinal cord.

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251P CAPSAZEPINE INHIBITS A RECOMBINANT HUMAN HCN1-MEDIATED CURRENT

C.H. Gill, P.J. Strijbos, S.A. Bates, W.J. Cairns, D. Owen & C.H. Davies. GlaxoSmithKline, New Frontiers Science Park North, Harlow, Essex, CM19 5AW.

Hyperpolarization-activated cyclic nucleotide-gated ion-channels (HCN) play important roles in controlling neuronal excitability. To date, four HCN subunits have been cloned, termed HCN1-4. We have transiently transfected CV-1 cells with the human HCN1 (hHCN1) subunit, cloned from human brain cDNA, and characterized its properties using whole-cell patch-clamp recordings (Shin *et al.*, 2001).

Cells were routinely voltage-clamped at –56 mV and data are expressed as mean ± s.e.mean from 3-8 cells. Drugs were dissolved in water or DMSO (<0.1% final concentration). A series of hyperpolarizing steps (in 10 mV increments and 1 s duration) to –176 mV induced slowly activating inward currents. Ter-minating each step with a depolarizing pulse to + 24 mV produced a series of tail currents of graded amplitudes. A Boltzmann function fit of their magnitudes yielded a half-maximal activation potential ($V_{1/2}$) of –105.8 ± 1.1mV. The current-voltage relationship of the hHCN1-mediated response was determined by fully activating the channel by hyperpolarizing cells to –166 mV and analysing the tail current amplitudes produced by depolarizing to +14 mV, via a series of 10 mV steps. The reversal potential (V_{rev}) for the current carried by hHCN1 was calculated to be – 43 ± 2 mV. Elevating external $[K^+]$ from 5 to 25 mM enhanced (by 378 ± 38 %) the hHCN1-mediated response to hyperpolarizing steps from –66 to –136 mV every 20 s. In contrast, CsCl (5 mM) and the selective HCN channel blocker ZD-7288 (10 μM) inhibited this current by 99 ± 1 % and 59 ± 6 % respectively. Likewise, the vanilloid receptor antagonist capsazepine inhibited the hHCN-1 mediated current. This effect was reversible and concentration-dependent (IC_{50} = 7.9 ± 0.7 μM). Inhibition by capsazepine was not voltage-dependent (55 ± 7 % block at –166mV and 55 ± 10 % block at + 14 mV) and did not result from a change in V_{rev} (43 ± 3 mV). Furthermore, antagonism was not use-dependent as a 7 minute application of 10 μM capsazepine in the absence of hHCN1 activation inhibited the current to a similar extent (71 ± 9 %) to that when the channel was activated every 20 s throughout the application of capsazepine (63 ± 3 %). Interestingly, the speed of inhibition afforded by capsazepine (10 μM) was faster than that by the non use-dependent and equipotent blocker ZD-7288 (10 μM). However, capsazepine (10 μM) did induce a leftward shift in the hHCN1 activation curve ($V_{1/2}$ = -140.3 ± 1.6 mV).

Given that HCN channels and vanilloid receptors are expressed on dorsal root ganglion (DRG) neurones (Wang *et al.*, 1997; Bevan *et al.*, 1992), we investigated whether capsazepine inhibited native HCN channels. Capsazepine (10 μM) inhibited the HCN-mediated current recorded from rat DRG neurones, in response to hyperpolarizing steps from –56 to –156 mV, by 78 ± 7 %. Given these findings, capsazepine may modulate the function of sensory neurones via multiple mechanisms.

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252P MODULATION OF RECOMBINANT RAT VANILLOID RECEPTOR-1 MEDIATED CALCIUM RESPONSES BY PKC AND FORSKOLIN

C. Harrison[1], D. Smart[2], D.J. Rowbotham[1] & D.G. Lambert[1]
[1]University Department of Anaesthesia, Critical Care and Pain Management, LRI, Leicester LE1 5WW, [2]GSK, Harlow, Essex.

The VR1 vanilloid receptor is a Ca^{2+} permeable, ligand gated ion channel that is activated by capsaicin. In rat sensory neurons, increased activity of the cAMP pathway causes sensitisation of the vanilloid receptor (Lopshire & Nicol, 1998). The aim of this study was to investigate the effects of cAMP/PKA and PKC modulation on the capsaicin induced increase in $[Ca^{2+}]_i$ in HEK293 cells stably expressing the recombinant rat VR1 receptor.

HEK293-VR1 cells were cultured as described previously (Sprague *et al.*, 2001). $[Ca^{2+}]_i$ was measured in fura-2 loaded whole cell suspensions in Krebs-HEPES buffer at 37°C on a Perkin-Elmer luminescence spectrophotometer, with excitation at 340/380nm and emission at 510 nm, as described previously (Hirst *et al.*, 1999). Data are mean±s.e.mean for n=3-8, statistical comparisons were made by t-test or ANOVA where appropriate and considered significant when $p<0.05$.

Activation of the VR1 receptor by capsaicin (0.1nM-100μM) caused an increase in $[Ca^{2+}]_i$, characterised by an initial rapid rise, followed by a small rapid decline and a subsequent plateau. The increase in $[Ca^{2+}]_i$ was concentration dependent, with a pEC_{50} value of 7.25±0.16. Pre-treatment of HEK293-VR1 cells with 1μM forskolin for 5min significantly enhanced the maximal increase in $[Ca^{2+}]_i$ (150±7% of control) but significantly reduced the potency, pEC_{50}=6.97±0.17. The shape of enhanced capsaicin response was unaltered.

Forskolin alone elicited a small (50-150nM) increase in $[Ca^{2+}]_i$ in both HEK293-VR1 cells and untransfected HEK293 cells.

The response to capsaicin but not forskolin was sensitive to the VR1 antagonist capsazapine (CPZ, 10μM) (forskolin 55±5nM, forskolin + CPZ 66±2nM, forskolin + capsaicin 358±42nM, forskolin + capsaicin + CPZ 127±25nM). However, direct activation of protein kinase A with 8Br-cAMP (10μM) failed to increase the magnitude of the capsaicin response (capsaicin 514±42nM, capsaicin + 8Br-cAMP 593±58nM). Furthermore, inhibition of protein kinase A with H89 (5μM) failed to reverse the enhancement of the capsaicin response produced by forskolin (capsaicin + forskolin 733±96nM, capsaicin + forskolin + H89 640±65nM). Activation of protein kinase C indirectly with carbachol (1mM), acting through endogenous muscarinic receptors failed to enhance the capsaicin response (capsaicin 262±42nM, capsaicin + carbachol 240±49nM) as did direct activation of protein kinase C with PDBu (1μM) (capsaicin 262±42nM PDBu 236±13nM). However, the shape of the response produced by the combination of protein kinase C activation and capsaicin was altered in that the initial peak phase of the response did not decline.

Collectively, these data suggest that VR1 receptor activity may be modulated by forskolin and protein kinase C.

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253P THE EFFECTS OF CENTRALLY PENETRANT AND PERIPHERALLY RESTRICTED iNOS INHIBITORS, ON CAPSAICIN INDUCED SECONDARY HYPERALGESIA.

R.S. Sargent, S.D. Collins, S.C. Stratton & I.P. Chessell. Neurology, GlaxoSmithKline Medicines Research Centre, Gunnells Wood Road, Stevenage, Herts, SG1 2NY

Intradermal injection of capsaicin in human volunteers is used as a model of central sensitisation in the clinic (Simone et al., 1987), eliciting both allodynia and secondary hyperalgesia. Preclinically, intradermal capsaicin into the heel of the rat produces a secondary hypersensitivity in the footpad distal to the site of injection, which is thought to involve sensitisation of second order dorsal horn neurones in the spinal cord (Gilchrist et al., 1996). Inducible nitric oxide synthase (iNOS) is expressed in inflammation and catalyses the synthesis of nitric oxide (NO), a key inflammatory mediator (Moncada et al., 1991). Here we have examined the effects of GW274150 (Alderton et al., 2000), a centrally penetrant iNOS inhibitor, and GW273629 (Knowles et al., 2000), a peripherally restricted iNOS inhibitor, in the capsaicin-induced model of secondary hyperalgesia in the rat.

Male Random Hooded rats (150-200g) were fasted overnight. GW274150, GW273629 or vehicle were administered orally 30 minutes prior to the rats being briefly anaesthetised with isofluorane and injected with capsaicin (10μg in 10μl intraplantar) into the heel of the paw. For intrathecal injection, GW273629 or vehicle, were administered between L4 and L5, under isofluorane anaesthesia, immediately prior to injection of capsaicin. Rats were allowed 30 minutes to recover from the anaesthetic and for development of secondary hyperalgesia prior to testing.

Mechanical hyperalgesia was assessed by applying von Frey monofilaments of increasing intensity (10 applications per filament; range 1.4-100g) to the footpad and recording withdrawal frequencies.

For each rat, log transformed von Frey values were used to calculate an "EG_{50}" (grams required to produce 5 withdrawals in 10 applications). Data were analysed using a one-way ANOVA then the results were back transformed to obtain least squares (l.s.) mean EG_{50}s for each treatment group (n=5).

The mean EG_{50}s for capsaicin treated and contralateral paws were 5.1±0.6g and 31.4±3.2g respectively. Systemic GW274150 significantly inhibited capsaicin-induced secondary hyperalgesia (30mg.kg^{-1} p.o., l.s. mean EG_{50} 16.38g; with 95% c.i. (12.35-21.73); $P<0.001$). The peripherally restricted inhibitor, GW273629 was ineffective when administered systemically (100mg.kg^{-1} p.o., l.s. mean EG_{50} 5.84g with 95% c.i. (4.44-7.68)) but significantly inhibited secondary hyperalgesia following intrathecal injection (153μg in 10μl i.t., l.s. mean EG_{50} 22.31g with 95% c.i. (16.97-29.34); $P<0.001$).

In conclusion, the centrally penetrant iNOS inhibitor GW274150, significantly inhibited capsaicin-induced secondary hyperalgesia when dosed systemically, whilst the peripherally restrictive iNOS inhibitor GW273629, required intrathecal administration to exhibit efficacy. Therefore, inhibition of iNOS in the spinal cord may be of utility in the clinical treatment of centrally mediated pain states.

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254P NITROUS OXIDE EXERTS AGE-DEPENDENT ANTINOCICEPTIVE EFFECTS IN FISCHER RATS

Janet M. Stowell, Yoko Ohashi, Toshikazu Hashimoto, Laura E. Nelson, Mervyn Maze, Masahiko Fujinaga (Introduced by Faruq Noormohamed). Department of Anaesthetics and Intensive Care, Imperial College of Science, Technology and Medicine, University of London, London, UK

Nitrous oxide (N_2O) is commonly used for paediatric anaesthesia, based on the assumption that it produces a similar antinociceptive response in children and adults. In a recent study, however, we demonstrated that N_2O does not have antinociceptive effects in newborn rats using the tail flick test, which involves a spinal reflex (Fujinaga et al., 2000). This finding is consistent with the theory that N_2O transduces its antinociceptive effects via descending noradrenergic inhibitory pathways, which are known to be immature in the newborn. In the present study, we have further examined the age-dependence of N_2O-induced antinociceptive effects using the formalin test (inflammatory stimulation), which is more analogous to surgical-induced nociception.

Fischer rats of various ages (7, 15, 19, 23 and 29 day-old and adult) were exposed to either air or 75% N_2O, and were injected with either saline or 5% formalin into left hind paw. Behavioural responses were evaluated according to a predetermined set of nociceptive ratings; the antinociceptive effects of N_2O was expressed by % reduction in nociceptive scores (n=4 each), but statistical comparison was made between air/formalin and N_2O/formalin groups for each developmental stage by a repeated measures two-way analysis. In addition, immunohistochemical analysis of c-Fos expression in the spinal cord was performed (n=3 each); data were analysed using the unpaired two-tailed *t* test.

In adults, formalin caused nociceptive behaviour and increased the number of c-Fos positive cells in the superficial layer of the spinal cord dorsal horn. N_2O almost totally suppressed formalin-induced nociceptive behaviour (99.1%) and c-Fos expression ($p<0.05$ for both). In 7 day-old rats, nociceptive behaviour was not readily measurable because of the profound hypnotic effect of N_2O; formalin-induced c-Fos expression was unchanged by N_2O (not significant-NS). In 15 day-old rats, N_2O showed a weak hypnotic effect; formalin-induced nociceptive behaviour was slightly suppressed (38.8%; NS) and c-Fos expression was not decreased by N_2O. In 19 day-old rats, N_2O partially suppressed formalin-induced nociceptive behaviour (53.3%; NS) but did not change c-Fos expression. N_2O suppressed formalin-induced nociceptive behaviour in 23 day-old (88.1%) and 29 day-old (97.5%), and blocked formalin-induced c-Fos expression ($p<0.05$ for both).

These results indicate that N_2O imparts age-dependent suppression of formalin-induced nociceptive behaviour in Fischer rats. Adult-like antinociceptive responses to N_2O, both behaviourally and immunohistochemically, are only present in rats older than 3 weeks. These findings are consistent with our hypothesis that N_2O lacks antinociceptive effects in the newborn due to immaturation of the descending noradrenergic inhibitory neurones, which play a pivotal role in the antinociceptive mechanisms of N_2O.

Fujinaga M, Doone R, Davies MF, *et al.* Anesth Analg 91:6-10, 2000.

255P EVIDENCE FOR THE INVOLVEMENT OF SPINAL CORD ALPHA-1 ADRENOCEPTORS IN MEDIATION OF THE ANTINOCICEPTIVE EFFECTS OF NITROUS OXIDE IN FISCHER RATS

Ryo Orii, Yoko Ohashi, Toshikazu Hashimoto, Tianzhi Guo, Laura E. Nelson, Mervyn Maze & Masahiko Fujinaga (Introduced by Faruq Noormohamed) Department of Anaesthetics and Intensive Care, Imperial College of Science, Technology and Medicine, University of London, London, UK

Activation of descending noradrenergic inhibitory neurones plays a pivotal role in the antinociceptive action of nitrous oxide (N_2O) (Maze et al., 2000). We previously found that exposure to N_2O induces c-Fos (protein product of the immediate early gene, c-*fos*, and immunohistochemical marker of neuronal activation) expression in spinal cord GABAergic neurones, suggesting their involvement in the antinociceptive action of N_2O (Hashimoto et al., 2001). In this study, we sought evidence of $\alpha 1$ adrenoceptors (AR) involvement in the activation of spinal cord GABAergic neurones by N_2O.

Adult male Fischer rats were exposed to air or 75% N_2O for 90 min (n=4 each), then anaesthetised by intraperitoneal injection of pentobarbital, and transcardially perfused with phosphate buffer saline followed by 4% paraformaldehyde fixative. Spinal cords were dissected, frozen-sectioned, and double-stained for c-Fos and $\alpha 1$ AR immunoreactivity. In addition, the effects of prazosin ($\alpha 1$ AR antagonist, 1 mg/kg) or yohimbine ($\alpha 2$ AR antagonist, 1 mg/kg) pre-treatment (intraperitoneal injection; 15 min before N_2O exposure) were examined on N_2O-induced c-Fos expression (n=4 each) and on N_2O-induced antinociceptive effects using the plantar test (n=6 each; cut-off time=10 sec). Data were expressed as mean ± S.D., and were analysed by one-way analysis of variance with Bonferroni correction.

N_2O administration increased the number of c-Fos positive cells in the spinal cord, particularly in laminae III-IV (74.0±2.4 per section vs 29.0±3.7 in air/saline group). Analysis by double-staining methods indicated that N_2O-induced c-Fos expression was strongly co-localised with $\alpha 1$ AR (81/123= 65.9% vs 22/54=40.7% in air/saline group). Administration of prazosin significantly decreased N_2O-induced c-Fos expression (32.0±2.1), while yohimbine pre-treatment showed no effect (76.0±5.8). In the plantar test, N_2O showed the anti-nociceptive effect; % maximum possible effect = 36.8±8.3% vs -1.2±3.8% in air/saline group. N_2O-induced antinociceptive effect was blocked by either prazosin (-4.8±11.4%) or yohimbine (-4.7±6.0%).

These results indicate that both $\alpha 1$ and $\alpha 2$ AR transduce N_2O-induced antinociceptive action, but only the $\alpha 1$ AR is involved in a mechanism that mediates activation of GABAergic neurons. Assuming that $\alpha 2$ and $\alpha 1$ AR mediate inhibitory and excitatory activities, respectively, our previous and current findings suggest that there are at least two neuronal systems that may be involved in the antinociceptive action of N_2O at the spinal cord level. One is the direct presynaptic inhibition of the nociceptive primary afferent neurones and/or postsynaptic inhibition of the second order neurones through activation of the $\alpha 2$ AR. The second is the indirect activation of GABAergic inhibitory interneurones via $\alpha 1$ AR.

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Maze M, Fujinaga M. Anaesthesia 55:311-314, 2000.

256P ANANDAMIDE-EVOKED Ca^{2+} RESPONSES IN ADULT RAT DORSAL ROOT GANGLION NEURONES

P.J. Millns, V. Chapman & D.A. Kendall. School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH.

The endocannabinoid anandamide acts at both inhibitory CB_1 receptors and excitatory VR1 receptors, both of which are present on dorsal root ganglion (DRG) neurones (Di Marzo et al., 2001). CB_1 receptor activation inhibits capsaicin evoked VR1 mediated responses of DRG neurones (Millns et al., 2001). Here, effects of anandamide on Ca^{2+} responses in DRG cells were studied.

DRG were isolated from adult Wistar rats and neurones cultured as described by Lindsay (1988). Cells were grown on 19mm glass cover slips for 24 hours prior to incubation with Fura 2-AM (5μM, 30 min, 37°C). Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in individual neurones in fields of 20-40 cells were calculated as the ratios of peak fluorescence intensities (measured at 500 nm) at excitation wavelengths of 340 and 380 nm respectively. DRG neurones were superfused with 0.3 and 1μM anandamide, alone or in combination with either the cannabinoid receptor antagonist SR141716A (1μM, 60 seconds) or VR1 receptor antagonist capsazepine (3μM). Drug applications had a 30 minute wash-out period. Data are expressed as mean percentage of the 60mM KCl evoked $[Ca^{2+}]_I$ +/- SEM. Statistical analysis was performed using one way ANOVA and Newman-Keuls multiple comparison test.

Anandamide (0.3 and 1μM) evoked increases in $[Ca^{2+}]_i$ in DRG neurones (n = 52, Fig 1). 96% of cells studied responded to 1μM anandamide. Following second exposure of DRGs to 1μM anandamide, with a 45 min inter-treatment interval, the second response was 65±11 % of the first response (n = 52). In the presence of SR141716A (1μM), 1μM anandamide did not evoke an increase in $[Ca^{2+}]_i$ in DRG neurones (Fig 1), significant differences between the effect of anandamide alone and in the presence of SR141716A are reported (Fig 1). Capsazepine (3μM) significantly increased anandamide evoked $[Ca^{2+}]_i$ in DRG neurones (Fig 1). However, capsazepine (3μM) significantly inhibited capsaicin-evoked increases in $[Ca^{2+}]_i$ in DRG neurones (66±11% inhibition of capsaicin response, $p< 0.05$). Following second exposure of cells to 100 nM capsaicin, the second response is 85±6% of the first response (n = 26). SR141716A (1μM) did not alter capsaicin-evoked increases in $[Ca^{2+}]_i$ in DRG neurones (25±5% inhibition of capsaicin response).

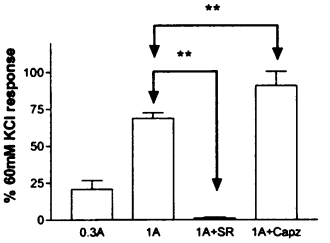

Figure 1. Anandamide (A) evoked increases in $[Ca^{2+}]_i$ in DRG neurones. SR141716A (SR, 1μM), but not capsazepine (3μM), blocked the anandamide evoked increases in $[Ca^{2+}]_i$ in DRG neurones. Data expressed as percentages of the response to 60mM KCl response, statistical analysis one way ANOVA.

Our data demonstrate anandamide evoked $[Ca^{2+}]_i$ increases in DRG neurones in a primary cell culture. This excitatory effect of anandamide does not appear to be mediated by VR1 receptors, but was blocked by a high concentration of SR141716A. The mechanism underlying this blockade remains to be elucidated.

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257P CHARACTERIZATION OF THE NOCICEPTIN RECEPTOR IN DOG BRAIN MEMBRANES

E.E. Johnson & D.G. Lambert University Dept. of Anaesthesia, Critical Care and Pain Management, Leicester Royal Infirmary, Leicester, LE1 5WW.

Nociceptin (NC) is the endogenous ligand for the nociceptin receptor, NOP (Calo' *et al.*, 2000) whose characteristics in the dog remain to be described. In this study we have characterised and compared NOP in the dog and rat.

Dog and rat brain membranes were prepared essentially as described (Okawa *et al.*, 1998; 1999). Assays were performed in 0.5ml 50mM Tris-HCl, 5mM $MgSO_4$, pH7.4 supplemented with 0.5% BSA and 10μM peptidase inhibitors (amastatin, bestatin, captopril and phosphoramidon). Non-specific binding was determined in the presence of 1μM unlabelled NC. In saturation studies 100μg and 200μg of rat and dog membranes were incubated with 0.01-2nM [*leucyl*-^{3}H]NC. In displacement studies 200μg of each membrane preparation was incubated with ~0.2nM [*leucyl*-^{3}H]NC and increasing concentrations of: NC(1-17)OH (NC), $NC(1-13)NH_2$ (NC13), dynorphin A (DA), nocistatin (NS), [Phe$^1\Psi$(CH_2-NH)Gly^2]$NC(1-13)NH_2$ ([F/G]), [Nphe^1]$NC(1-13)NH_2$ ([Nphe^1], antagonist), III-BTD (peptide antagonist), $AcRYYRWK-NH_2$ (CTD), J113397 (non-peptide antagonist, Ozaki *et al.*, 2000), and naloxone benzylhydrazone (NB). Data (mean±s.e.mean) were analysed as described in Okawa *et al.* (1998;1999).

[*leucyl*-^{3}H]NC binding was concentration-dependent and saturable (n=5) in both dog and rat. B_{max} (fmol mg^{-1} protein) and pK_d values for dog were 35.8±1.2 and 10.44±0.07. Values for the rat were 121.1±5.1 and 10.53±0.09 respectively.

[*leucyl*-^{3}H]NC was displaced by NOP ligands with pK_i values shown in *Table 1*. DA and NS were inactive.

	Dog	**Rat**
NC	11.07±0.23	10.99±0.11
NC13	12.16±0.32	12.34±0.44
[F/G]	9.89±0.24	11.05±0.34*
[Nphe^1]	8.79±0.18	8.90±0.23
III-BTD	8.28±0.06	8.28±0.10
CTD	10.79±0.23	10.75±0.19
J113397	9.21±0.07	9.67±0.04
NB	7.32±0.23	7.87±0.08

Table 1. pK_i values for NOP binding in dog and rat (n=3-15)
**P=0.025 compared with dog*

Data for the rat are consistent with our previous studies (Okawa *et al.*, 1998;1999) and we report a low density of NOP in the dog brain. As there was a strong positive correlation (r^2=0.93; p=0.0001) between pK_i values in both species, these data indicate pharmacological similarity of NOP receptors in the dog and rat.

We thank Dr R Guerrini and C De Risi (University of Ferrara) for providing peptides and J113397 used in this study. EE Johnson is in receipt of a Pfizer sponsored studentship.

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258P *IN VITRO* CHARACTERISATION OF [(pF)Phe^4]NOCICEPTIN(1-13)NH_2 A NOVEL AGONIST FOR THE NOP RECEPTOR

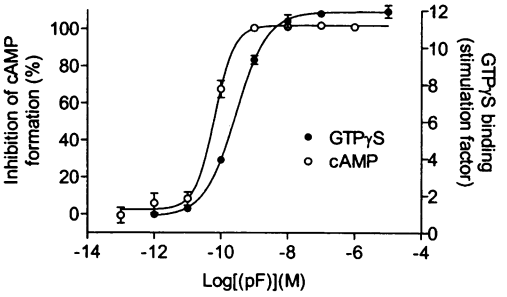
J. McDonald[1], T.A. Barnes[1], R. Guerrini[2], G. Calo'[2], D.J. Rowbotham[1] & D.G. Lambert[1]. [1]University Department of Anaesthesia, Critical Care and Pain Management, LRI, Leicester UK. [2]Department of Pharmacology, University of Ferrara , Italy.

Nociceptin (NC) is the endogenous ligand for the G_i-protein coupled NOP receptor and NC(1-13) is the smallest fragment to retain full biological activity (Calo' *et al.,* 2000). NC can be sub-divided into an N-terminal Phe^1-Gly^2-Gly^3-Phe^4 message and a basic C-terminal address domain with Phe^1 and Phe^4 representing the pharmacophores (Guerrini *et al.,* 2000). We have made several modifications of the Phe^4 pharmacophore (Guerrini *et al.,* 2001) and in this study we describe a novel peptide molecule, [(pF)Phe^4]NC(1-13)NH_2 (pF).

^{35}S-Guanylyl-5'-O-(γ–thio)-trisphosphate ([^{35}S]GTPγS) binding to membranes prepared from CHO cells expressing the human NOP receptor was measured in 0.5ml volumes of buffer (50mM Tris, 0.2mM EGTA, 1mM $MgCl_2$, 100mM NaCl, 1mg/ml BSA, 0.15mM Bacitracin, 10μM amastatin, bestatin, captropril and phosphoramidon) containing ~150pM [^{35}S]GTPγS as described previously (Hashiba *et al.,* 2001). cAMP was measured in whole CHO cells expressing the human NOP receptor in 0.3ml volumes of Krebs/HEPES buffer containing 1μM forskolin and 1mM IBMX as described previously (Hashiba *et al.,* 2001). J113397 and III-BTD (see Guerrini *et al.,* 2000) and (pF) were also included in various combinations. Data are mean±s.e.mean(n).

(pF) stimulated [^{35}S]GTPγS binding and inhibited cAMP formation with pEC_{50} values of 9.55±0.01(11) and 10.19±0.06(9), Figure 1. (pF) stimulation of [^{35}S]GTPγS was reversed by J113397 and III-BTD with pA_2 values (Schild) of 8.53±0.06(5) and 7.96±0.05(4) respectively. (pF) inhibition of cAMP formation was also reversed by J113397 and III-BTD with pK_B values (single concentration of antagonist) of 7.89±0.17(4) and 7.27±0.15(4) respectively.

Figure 1. (pF) stimulates [^{35}S]GTPγS binding and inhibits cAMP formation. [^{35}S]GTPγS data are expressed relative to basal specific binding (stimulation factor).



These data indicate that (pF) is a highly potent peptide agonist that will be of use in pharmacological studies of NOP.

Funded by a BJA/RCA project grant (DGL/DJR).

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259P DISTRIBUTION OF 5-HT RECEPTOR mRNA IN THE SUPERFICIAL DORSAL HORN OF THE RAT SPINAL CORD AND IN DORSAL ROOT GANGLIA.

R. Nicholson[1,2], J. Small[1], A. Dixon[1], R. Pinnock[1], D. Spanswick[2] & K. Lee[2]. [1]Pfizer Global Research and Development, Cambridge University Forvie Site, Cambridge, CB2 2QB, U.K. & [2]Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, U.K.

5-HT (serotonin) is believed to play an important role in nociceptive processing, at least in part by modulation of activity at the level of the spinal dorsal horn (Fields *et. al.,* 1991; Sufka *et. al.,* 1992). Despite the potential therapeutic importance of this system, the precise nature and distribution of 5-HT receptors expressed within the dorsal horn and dorsal root ganglia (DRG) remains to be established. Thus, we have performed a detailed investigation of the distribution of 5-HT receptor mRNA in these structures using in-situ hybridisation.

Male Wistar rats (200-400g) were killed using Home Office approved procedures and the lumbar spinal cord and L5/L6 DRGs rapidly dissected and snap frozen in isopentane. 10μm transverse sections were thaw-mounted onto sterile poly-d-lysine coated slides and fixed in 4% paraformaldehyde.

For each receptor, two oligonucleotide probes designed against different receptor regions were 3' labelled using [^{35}S]deoxy ATP (1000Ci/mmol; 5μl). Following overnight hybridisation at 42°C with ^{35}S labelled probe in hybridisation buffer, slides were washed and dehydrated. The slides were subsequently coated with LM-1 photographic emulsion and stored at 4°C for 8 weeks. Following development, slides were stained with cresyl violet, and analysed by measuring grain count density using the MCID (M4, Imageworks) imaging system. The signal from a given probe was considered positive when the grain count density was significantly greater than background counts obtained from the corresponding labelled sense probes (students *t* test; $P<0.01$). Probe specificity was confirmed by BLAST searching against all public databases.

Within spinal cord laminae I and II, all neurons were found to specifically express 5-HT2B, 3B, 4 and 7 mRNA. In contrast, only 41% of these neurons expressed mRNA for 5-HT2C. No mRNA expression was found for 5-HT1A, 1B, 1D, 1E, 2A, 5A, 5B or 6. Within DRGs, no expression of 5-HT1A, 1E, 2C, 5A, 5B, 6 or 7 mRNA was detected. In contrast, mRNA for the remaining 5-HT receptors was differentially expressed throughout small (<500μm^2), medium (500-1200μm^2) and large (>1200μm^2) diameter neurons as summarised in Table 1.

	% DRG neurons expressing 5 -HT receptor		
	SMALL	MEDIUM	LARGE
5-HT1B	52	70	63
5-HT1D	0	34	68
5-HT2A	23	16	5
5-HT2B	100	100	100
5-HT3B	29	20	41
5-HT4	48	82	14

Table 1: The percentage of DRG neurons expressing 5-HT receptor mRNA.

These results indicate that the nociceptive role of 5-HT within the spinal cord may be mediated through a variety of receptor subtypes located both pre- and postsynaptically.

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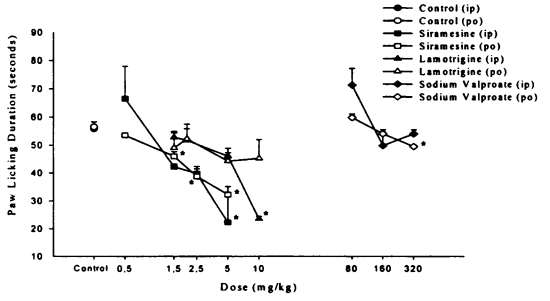
260P THE EFFECT OF THE SELECTIVE SIGMA 2 LIGAND, SIRAMESINE, IN A RODENT MODEL OF ACUTE NOCICEPTION

V. Vogel & C. Sánchez Neuropharmacology Department, H. Lundbeck A/S, Ottiliavej 9, 2500 Valby, Copenhagen, Denmark. (introduced by W.P.Watson)

Formalin induced behaviour in rats is a well-characterised model of tonic chemogenic pain. Formalin injection into the hindpaw produces a biphasic response; an acute phase occurs 0-5 minutes after injection, followed by a period of quiescence and a resumption of nociceptive behaviour (paw licking), 20-30 min after injection.

Groups of 8 male Sprague Dawley rats (200-250g), were used to examine the effects of the sigma$_2$ ligand, siramesine (Lu 28-179) (Perregaard et al., 1995), against paw licking induced by intraplantar injection of formalin (2.5%, 50µl) in the right hindpaw. Two antiepileptic drugs used clinically in the treatment of pain conditions, sodium valproate and lamotrigine were also studied.

Figure 1: Duration of paw licking (s) 0-5 min after formalin injection. Data are mean (± s.e.m) (*P<0.05 c.f. control group)

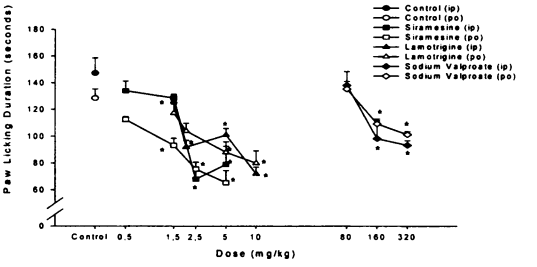


Drugs were given 30 min prior to formalin injection by either ip or po routes. The duration (s) of licking of the injured paw was assessed between 0-5 min and again 20-30 min after the initial injection. One way ANOVA followed by Dunnett's test was used to compare the effect of the drugs vs. control (saline).

Figures 1 and 2 show that siramesine, lamotrigine and sodium valproate all significantly suppressed paw licking behaviour 0-5 min and 20-30 min post formalin injection.

The results achieved with sodium valproate and lamotrigine agree with those found in the literature (Hunter & Loughead, 1999), The results observed with siramesine may indicate a beneficial effect of this compound in the treatment of nociceptive pain conditions.

Figure 2: Duration of paw licking (s) 20-30 min after formalin injection. Data are mean (± s.e.m) (* P<0.05 c.f. control group)



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261P LINK_TUMOR NECROSIS FACTOR STIMULATORY GENE-6 (LINK_TSG6) HAS NEUTROPHIL ANTI-MIGRATORY EFFECTS *IN-VIVO*

Thong Cao[1], Anthony J Day[2] & Mauro Perretti[1] [1]William Harvey Research Institute, Queen Mary College, University of London, UK. [2]Department of Biochemistry, University of Oxford, South Parks Road, Oxford.

TSG-6 (a product of Tumor Necrosis Factor-Stimulated Gene-6) is a 35kDa glyco-protein that contains a link module (LINK) that binds to hyaluronan (HA), an extracellular matrix component. In vivo, recombinant TSG-6 is able to reduce inflammation in animal models of carrageenan-induced inflammation and collagen induced arthritis (Wisniewski et al. 1996; Mindrescu *et al.* 2000).

An *in-vivo* intravital microscopy system was used to assess the effect of recombinant LINK (LINK_TSG6) on processes of neutrophil extravasation. Male Swiss albino mice (11-15g) were given an intraperitoneal injection (i.p.) of IL-β (5 ng in 0.5 ml of sterile PBS). LINK_TSG6 (1 µg and 5 µg i.p. or i.v.) was given 15 min prior to IL-1 β administration. After 2 h, animals were anaesthetized with diazepam (60 mg/kg subcutaneously) and Hypnorm (0.7 mg/kg fentanyl citrate and 20 mg/kg fluanisone intramuscularly). Cautery incisions were made along the abdominal region, and the mesenteric vascular bed was exteriorized and placed on a viewing Plexiglas stage. Leukocyte interaction with the endothelium was monitored in post-capillary venules and cell rolling velocity, adhesion and migration were measured. In another set of experiments, peritoneal cavities were lavaged with 2 ml of PBS containing 3 mM EDTA. Aliquots of the lavage fluid were then stained with Turks solution (0.01% crystal violet in 3% acetic acid) and differential cell counts were performed using a Neubauer haemocytometer and a light microscope (B061, Olympus, Melville, NY). Data (mean s.e.mean of n mice per group were analysed by ANOVA + Bonferroni test.

In the peritonitis experiments, IL-1 β induced a 4 h neutrophil influx (1.97 ± 0.3 x106 cells per cavity, n=6). LINK_TSG6 (5 µg given i.p. but not i.v.) significantly inhibited neutrophil recruitment (0.46 ± 0.12 x 106 cells per cavity, n=5, P<0.05). In the intravital microscopy experiments, IL-1 β reduced neutrophil rolling velocity (13.9 ± 2.4 (m s-1) compared to control values (vehicle given i.p. 50.4 ± 9.3 µm s^{-1}) (n=4, P<0.001). Treatment of mice with LINK_TSG6 did not significantly modulate IL-1 β-induced reduction in cell velocity. In contrast, LINK_TSG6 affected the number of adherent neutrophils promoted by IL-1 β. In the absence of cytokine a value of ~1-1.4 adherent cells per vessel was measured, and this was increased to 6.4 ± 0.2 cells after IL-1 β injection (n=6, P<0.05). In LINK_TSG6-treated mice values of 4.1 ± 0.4, 3.8 ± 0.6 and 2.2 ± 0.5 adherent cells were obtained for doses of 1 µg i.v., 1 µg i.p. and 5 µg i.v, respectively (P<0.05). IL-1 β-induced leukocyte extravasation was higher but not significantly different from control mice and this was not altered by LINK_TSG6 treatment.

The results show an effect of LINK_TSG6 in mediating a specific step of the neutrophil migration process, that is adhesion to the post-capillary endothelium. Focusing on this step may allow identification of LINK_TSG6 anti-migratory mechanism of action.

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262P EFFECTS OF SODIUM SALICYLATE ON THE EXPRESSION OF CYCLOOXYGENASE (COX) ISOFORMS IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

A. Heinemann, R. Amann, T. Egger *, R. Schuligoi & B.A. Peskar, Institute for Experimental & Clinical Pharmacology and * Institute for Medical Biochemistry & Medical Molecular Biology, University of Graz, A-8010 Graz, Austria.

The impact of salicylate on COX-2 induction is still unclear in view of conflicting results (Xu *et al.*, 1999; Fernandez de Arriba *et al.*, 1999). Therefore, we have compared directly the effects of salicylate on prostaglandin (PG) biosynthesis with its effect on the expression of COX isoenzymes using human peripheral blood mononuclear cells (PBMC).

PBMC were prepared from fresh whole blood by dextran sedimentation followed by centrifugation over a ficoll gradient and resuspended in SFM macrophage medium containing penicillin and streptomycin. Aliquots of PBMC (2×10^6) were incubated at 37° C in 5% CO2 : 95% air. Cell viability was assessed by Trypan blue staining. PGE_2 content in the supernatants was determined by radioimmunoassay (Jobke *et al.*, 1973), while expression of COX isoforms was investigated by Western blot and analysed by computerised densitometry. Data were expressed as means±s.e.m. and further analysed using Mann-Whitney U test or Kruskal-Wallis H test followed by Dunn's post test, as appropriate.

An 18 h incubation of PBMC with endotoxin (E.coli serotype 055:B5; 10 μg ml^{-1}) resulted in expression of COX-2, and increased immunoreactive PGE_2 in the supernatant (7098±638 pg ml^{-1}, n=6, $p<0.05$), as compared with vehicle treatment (below the detection limit of 147 pg ml^{-1}). The COX-2 selective inhibitor NS-398 [N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide] at 1 μM suppressed the endotoxin-induced increase of PGE_2 by 90.6±1.3 % (n=13, $p<0.01$) with respect to its vehicle, without significantly ($p>0.5$) affecting the expression of COX-1 or COX-2 protein (95±16 % and 108±17 % of vehicle control, respectively; n=3). In PBMC exposed to endotoxin for 18 h in the presence of 1 or 3 mM salicylate the PGE_2 content of the supernatant was reduced by 26.0±3.4 % and 44.3±4.3 % (each n=14, $p<0.05$), whereas COX-2 expression was enhanced by 148±51 % and 268±91 % (each n=6, $p<0.05$), respectively, as compared to the vehicle of salicylate. After a subsequent 2 h incubation of PBMC in drug-free medium, PGE_2 contents in samples that had been exposed to endotoxin together with 1 or 3 mM salicylate were increased by 114±19 % and 207±36 %, respectively (each n=16, $p<0.05$) relative to samples exposed to endotoxin alone.

In summary, salicylate can enhance the expression of COX-2 in endotoxin-exposed PBMC and at the same time reduce PGE_2 formation. After wash-out and removal of salicylate-induced COX inhibition, increased COX-2 expression results in enhanced PGE_2 formation. Theses results suggest that under certain conditions salicylate-induced stimulation of COX-2 expression might contribute to salicylate side effects such as Reye's syndrome.

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263P EFFECTS OF PHOSHATIDYLINOSITOL 3 (PI 3)-KINASE INHIBITORS ON ARGINASE ACTIVITY AND NO SYNTHESIS IN RAT NR8383 ALVEOLAR MACROPHAGES

V. Stumpf, F. Wenzel, M. Fuhrmann & K. Racké, Institute of Pharmacology & Toxicology, University of Bonn, Reuterstr. 2b, D-53113 Bonn, Germany.

L-Arginine is a substrate of NO synthase and arginase, important pathways in macrophages. Arginase can limit L-arginine supply for NO synthase (Hey *et al,*. 1997) and in this way can cause airway hyperreactivity (Meurs *et al.,* 2000). In the present experiments, the role of PI 3-kinase in the regulation of these L-arginine pathways in rat alveolar macrophages was studied by the use of two inhibitors, wortmannin and LY 294002.

Rat NR 8383 alveolar macrophages were maintained in Ham's F-12 medium containing 15% FCS. Freshly resuspended cells (10^6 cells $well^{-1}$) were cultured for 20 h in the absence or presence of lipopolysaccharides (LPS) and/or other test substances. Thereafter, arginase activity was measured. In addition, nitrite accumulated in the culture media was determined as a measure of NO synthesis (Klasen *et al.,* 2001; Mössner et *al.,* 2001).

After culture under control conditions nitrite concentration in the culture media was 25 ± 1 μM and arginase activity amounted to 94 ± 22 mU μg^{-1} (means ± s.e.mean, n=6 - 7). Similar to previous observations in primary alveolar macrophages (Hey *et al.,* 1995; 1997), LPS (0.001 – 10 μg ml^{-1}) caused a concentration dependent increase in nitrite accumulation (to maximally 128 ± 1 μM, EC_{50}: 9 ng ml^{-1}) and arginase activity (to 230 ± 33 mU μg^{-1}; EC_{50}: 7 ng ml^{-1}).

Whereas wortmannin (0.1 - 10 μM) had no effect on basal nitrite production, LY 294002 (1-30 μM) caused a reduction by maximally 47 ± 5%. LPS (0.1 μg ml^{-1}) stimulated nitrite accumulation was inhibited by LY 294002 (1 - 30 μM) by maximally 46 ± 6% and by wortmannin (0.1 - 10 μM) by maximally 41 ± 9% (each $p < 0.01$). LY 294002 and wortmannin caused a reduction in arginase activity by maximally 55 ± 5% and 25 ± 7%, respectively. The LPS (0.1 μg ml^{-1}) –induced increase in arginase activity was inhibited by LY 294002 (1-30 μM) by maximally 100% and by wortmannin (0.1-10 μM) by maximally 95 ± 4%.

In conclusion, the two PI 3-kinase inhibitors wortmannin and LY 294002 down-regulated iNOS and arginase activity in rat alveolar macrophages. Since in the present expriments LY 294002 was more effective and more potent than wortmannin, although wortmannin is known to inhibit PI 3-kinase more potently than LY 294002, the question arises whether the present effects are caused by inhibition of PI 3-kinase.

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264P EVIDENCE THAT BX471 IS AN INVERSE AGONIST AT THE HUMAN CCR1 RECEPTOR

D.J. Beer, C.M. Scaramellini Carter & S.J. Charlton. Novartis Respiratory Research Centre, Horsham, RH12 5AB.

BX471 (R-N-[5-chloro-2-[2-[4-fluorophenyl)methyl]-2-methyl-piperazinyl]-2-oxoethoxy]phenyl]urea hydrochloric acid salt) has been reported to be a potent CCR1 receptor antagonist that may be used for the treatment of chronic inflammatory diseases involving MIP-1α, RANTES and CCR1 (Liang *et al*, 2000). The present study extends the *in vitro* pharmacological characterisation of this compound.

For binding studies, membranes (5 μg) prepared from Chinese hamster ovary cells expressing human CCR1 (CHO-CCR1), [^{125}I]MIP-1α (20 pM), ligand and wheatgerm agglutinnin-poly (vinyltoluene) (WGA-PVT) Scintillation proximity assay (SPA) beads were incubated (2 hours, 22°C). For GTPγS binding studies, CHO-CCR1 membranes (10 μg), ligand(s), [^{35}S]GTPγS (300 pM), GDP (5 μM) and WGA-PVT SPA beads were incubated (45 min, 22°C). Both assays were terminated by centrifugation (377g, 10 min) and measured by scintillation counting. Multi-curve modelling of binding data was performed using GraphPad Prism, fitted to two equations (one and two-site competition model) and compared with a F-test ($P < 0.05$).

In [^{125}I]MIP1α competition binding studies, MIP1α and BX471 showed shallow binding curves with Hill slopes of -0.47 and -0.37 respectively. The data was best described by a two-site binding model when compared to one-site (F test, $p = 0.021$ and 0.017 for MIP1α and BX471 respectively) and yielded pK_H and pK_L values of 9.78 and 8.12 for MIP1α and 9.27 and 7.49 for BX471. The effect of uncoupling G proteins from CCR1 was examined by including GTP (100 μM) in the assay mix. The binding curve to MIP1α shifted to the right and was best described by a one-site binding model with a slope of -1.00 and pIC_{50} of 8.19. The binding curve for BX471 also became monophasic (slope of -0.89), but was shifted to the left (pIC_{50} of 8.79).

The data obtained experimentally was simulated in an Excel model of the extended ternary complex model (Samama *et al*, 1993) with two competing ligands. The data for BX471 could be predicted for a neutral antagonist or an inverse agonist.

The mode of action of BX471 was therefore examined using a functional GTPγS assay. MIP1α was an agonist with a pEC_{50} of 9.40. BX471 inhibited the MIP1α (1 nM) response with a pIC_{50} of 7.41. However, BX471 also inhibited the basal GTPγS binding with a pIC_{50} of 7.48 ± 0.19 ($n = 4$), demonstrating inverse agonism. This functional data was modelled using the same parameters as those used for the binding data, with BX471 being modelled as an inverse agonist. Similar data was predicted to that obtained experimentally, but with potencies shifted a log to the left.

We have demonstrated that in a recombinant system, the human CCR1 receptor shows some constitutive activity and that BX471, originally described as an antagonist, is an inverse agonist. In addition, we have shown how modelling of experimental data can assist in the interpretation and understanding of biological systems.

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265P *IN VITRO* AND *IN VIVO* CHARACTERISATION OF CAT-192 AND 1D11: NEUTRALISING ANTIBODIES TO TRANSFORMING GROWTH FACTOR BETA

A.M. Carruthers[1], D. Abraham[2], L. Conroy[1], M. McCourt[1] J. Thompson[1] & I.K. Anderson[1]. [1]Cambridge Antibody Technology, The Science Park, Melbourn, Cambridgeshire SG8 6JJ. [2]Royal Free Hospital Medical School, Hampstead, London NW3 2PF.

The mechanisms involved in the pathogenesis of fibrosis implicate a major role for transforming growth factor beta (TGF-β; Border & Noble 1994). The present study has investigated the effect of TGF-β-neutralising antibody, 1D11 (anti-TGF-β1, β2 and β3, mouse monoclonal IgG1) on fibrotic responses to TGF-β *in vitro* and in bleomycin-induced lung fibrosis *in vivo*. The effects of 1D11 were compared to the selective TGF-β1 neutralising antibody, CAT-192 (fully human monoclonal IgG4).

Competition binding assay: The ability of TGFβ1 , β2 and β3 (up to 100nM) to compete with [^{125}I]-TGF-β1 (50pM) binding to immobilised CAT-192 (0.3nM) was tested in a FlashPlate™ assay. Only TGF-β1 was shown to bind to CAT-192, demonstrating selectivity of this antibody for TGF-β1. Furthermore, 1D11 and CAT-192 fully inhibited binding of [^{125}I]-TGF-β1 (10pM) to A549 cells (pIC_{50}: 10.33 and 8.43, respectively, n=2) which contain TGF-β type II receptors.

Collagen production assay: TGF-β isoforms ± 1D11 were pre-incubated for 30min in DMEM (with 1 % (v/v) FBS, L-proline and ascorbate) and applied to NIH-3T3 fibroblasts ($1x10^6$ cells/well) for 48h at 37°C. The collagen was extracted from the cell monolayer (0.5M acetic acid, 30min) and was analysed using the Sircol dye-binding method (Biocolor, Belfast, U.K.). TGF-β1, β2 or β3 induced concentration-dependent increases in collagen production. 1D11 inhibited collagen production caused by TGFβ1, β2 or β3 (EC_{80} response) in a concentration-dependent manner (Table 1).

In-vivo: 8-12 week old double transgenic mice harbouring the collagen type I α2 chain promotor driving the beta galactosidase and luciferase reporter genes (Yutaka *et al.* 1998) were instilled with bleomycin 0.125U i.t. or saline, 50μl i.t., under avertin anaesthesia (125mg kg^{-1} i.p.).

Table 1. Potency of TGF-β isoforms and inhibition of collagen production by ID11 in NIH-3T3 fibroblasts (basal collagen 6±1μg).

Isoform	Collagen (μg)	pEC_{50} (M)	1D11 pIC_{50} (M)	n
TGF-β1	31 ± 2	10.5 ± 0.13	9.48 ± 0.23	6
TGF-β2	33 ± 3	11.4 ± 0.16	9.26 ± 0.29	5
TGF-β3	32 ± 1	10.8 ± 0.28	9.04 ± 0.15	3

Antibody treatments 1D11 (5mg kg^{-1}) or CAT-192 (0.5 mg kg^{-1}) or PBS vehicle were administered on days 0, 4 and 9 i.v. via the tail vein (10ml kg^{-1}). On day 14 post bleomycin, mice were killed by an overdose of CO_2, lungs removed, and assayed for luciferase. Lungs were homogenised and luciferase activity was measured with a luciferase assay kit (Promega). Collagen type I promotor activity was used as marker of fibrosis and was quantitated by luciferase activity (per wet weight of lung tissue). Collagen type I promotor activity was expressed as a percentage of the bleomycin & PBS treatment group (Table 2).

Table 2. Pulmonary collagen type I promotor activity

Treatment	mean ± s.e.m.	n
Saline & PBS	36.35±7.45	10
Bleomycin & PBS	100±17.19*	9
Bleomycin & CAT-192 0.5mg kg^{-1}	28.90±5.62#	8
Bleomycin & 1D11 5mg kg^{-1}	37.37±11.80#	12

*P<0.01 vs. Saline & PBS, # P<0.01 vs. Bleomycin & PBS using ANOVA and Dunnett's test.

CAT-192, 0.5mg kg^{-1} and 1D11 5mg kg^{-1} reduce collagen type I promotor activity induced by bleomycin. This study suggests that the pulmonary fibrosis caused by bleomycin is mediated by TGF-β1 presumably following activation of lung fibroblasts. The fully human antibody CAT-192 is currently undergoing clinical testing as a therapeutic for fibrotic diseases.

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266P DESENSITISATION OF $ß_2$-ADRENOCEPTOR-MEDIATED RESPONSES BY LONG-ACTING $ß_2$-AGONISTS IN HUMAN LUNG MAST CELLS

A. Scola, L.K. Chong & P.T. Peachell. Section of Molecular Pharmacology & Pharmacogenetics, Sheffield University, Royal Hallamshire Hospital (L-Floor), Sheffield S10 2JF.

$ß_2$ –adrenoceptor ($ß_2$-AR) agonists are effective inhibitors of the stimulated release of histamine from human lung mast cells (HLMC). However, long-term (24 h) treatment with the non-selective ß-AR agonist, isoprenaline (ISO), reduces the subsequent effectiveness of ISO to inhibit the IgE-mediated release of histamine from these cells (Chong *et al.*, 1997). The aim of the present study was to determine whether the long-acting $ß_2$-AR agonists, formoterol (FORM) and salmeterol (SALM), also cause a functional desensitisation to ß-AR agonists in HLMC.

Mast cells were obtained by physical and enzymatic disruption of human lung tissue and were further purified by countercurrent elutriation. For histamine release, HLMC were incubated with or without a ß-AR agonist for 10 min before challenge with a maximal releasing concentration of anti-human IgE (1:300) for 25 min. Histamine release was measured by an automated fluorometric technique. Total cell cyclic adenosine monophosphate (cAMP) was measured in HLMC after treatment with ß-AR agonists (10^{-5}M for 10 min) using commercially-available EIA kits. Results were analysed statistically using the SPSS (version 10.0.7) programme, running ANOVA tests. Results were taken as significant when $P < 0.05$.

The effects of ISO, FORM and SALM (all 10^{-10}-10^{-5} M) on the IgE-mediated release of histamine were evaluated (n=10). Relative to ISO, FORM was roughly equipotent (EC_{50},5 nM) and was close to being a full agonist whereas SALM was ineffective as an inhibitor of histamine release up to 10^{-6} M, inhibiting histamine release only at a high concentration (31±4% inhibition at 10^{-5} M).

This inhibitory effect of SALM at 10^{-5} M was probably due to the lipophilic tail (SALM being a hybrid of salbutamol and a lipophilic tail) because the tail (10^{-5} M) also inhibited histamine release by 31±3% (n=5). The full agonist nature of FORM and the relative ineffectiveness of SALM as inhibitors of histamine release was paralleled by effects of these agonists on cAMP. In HLMC (purity 91±6%), ISO, FORM and SALM elevated cAMP levels over basal by 361±91%, 321±90%, and 60±28%, respectively (n=4). The effects of long-term treatment of the ß-AR agonists (all 10^{-6} M) on the subsequent ability of ISO (10^{-10}-10^{-5} M) to inhibit histamine release were evaluated (n=7). Both SALM and FORM completely abrogated ($P<0.05$) the maximal inhibitory effects of ISO, whereas pretreatment (24 h) with ISO reduced the maximal inhibitory response to ISO by 65±6% ($P<0.05$). The functional desensitisation induced by SALM and FORM was concentration-dependent and FORM caused greater levels of desensitisation at lower concentrations (<10^{-6} M) than SALM (n=4).

These data show that long-term exposure of HLMC to both SALM and FORM causes a functional desensitisation to the ß-AR agonist ISO. Moreover, the data indicate that the efficacy order for the inhibition of histamine release and elevating cAMP of ISO=FORM>>SALM does not parallel the efficacy order for the extent of desensitisation which is FORM SALM>ISO.

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267P THE EFFECT OF VALSARTAN ON LUNG REMODELLING IN A MOUSE MODEL OF BLEOMYCIN-INDUCED PULMONARY FIBROSIS

D.Bayley, C.H.Battram, C.T.Poll J.Giddings & E.M. Campbell. (Introduced by K.Butler) Novartis Horsham Research Centre, RDTA, Wimblehurst Road, Horsham, West Sussex, RH12 5BW.

Bleomycin is an antineoplastic drug commonly used for the treatment of cancers including carcinomas and lymphomas. However, a toxic side effect is the induction of irreversible pulmonary fibrosis in lung tissue (Carrion *et al,* 1999). This effect of bleomycin allows it to be a useful tool in the development of animal models of fibrosis (Smith *et al,* 1996). Recently, angiotensin II has been demonstrated to induce proliferation of human lung fibroblasts through the AT_1 receptor (Marshal *et al,* 2000). We have therefore used a mouse model of bleomycin induced lung injury to investigate the effects of a specific AT_1 receptor antagonist, valsartan on airway fibrosis.

To induce pulmonary fibrosis, bleomycin (2mg/kg) was administered intra-tracheally to female balb/c mice (25g, n = 10 per group) under halothane anaesthesia on day 0. Mice were treated orally once daily with valsartan (3 or 30mg/kg), prednisolone (30mg/kg) or PBS on days –1 to +13 relative to bleomycin. On day 14 mice were terminally anaesthetised with pentobarbitone sodium (200mg/kg) i.p. After exsanguination, bronchoalveolar lavage (BAL) was performed with 3 x 0.4mL PBS. Total and differential cell counts were assessed using standard light microscopy and morphological techniques. Lung tissue was homogenised in PBS containing protease inhibitor and the whole lung homogenate (WLH) supernatant analysed for soluble collagen levels, a marker of fibrosis, using a colorimetric assay. BAL fluid and WLH fluids were also analysed for the profibrotic mediators (Zhang *et al,* 1994) Monocyte Chemotactic Protein – 1(MCP-1) and Transforming Growth Factor β –1(TGFβ-1) using specific ELISA.(R&D systems,UK)

Bleomycin induced severe inflammation within the airways as assessed by BAL, increases in macrophages (2.69±0.36 to 6.64±1.12 x 10^4/mL), lymphocytes(0.06±0.03 to 0.78±0.18 x 10^4/mL), eosinophils (0.01±0.01 to 0.34±0.1 x 10^4/mL) and neutrophils (0.04±0.03 to 1.02±0.17 x 10^4/mL) were significant ($p<0.05$). There were also significantly increased levels of soluble collagen in the lung tissue (see Table 1). Treatment of mice with valsartan did not significantly change this inflammation but did decrease significantly ($p<0.05$) the levels of collagen in the lung.

To investigate the mechanism of this effect in fibrosis, levels of profibrotic mediators were assessed. Both TGFβ-1 and MCP-1 protein levels were significantly ($p<0.05$) reduced following administration of valsartan. Prednisolone also significantly ($p<0.05$) reduced TGFβ-1 compared to vehicle but not MCP-1.

Table 1 : Effect of valsartan on cytokines 14 days after intra-tracheal bleomycin treatment

Groups	WLH Collagen µg/ml	BAL TGFβ-1 pg/ml	WLH MCP-1 pg/ml
Vehicle	5.44 ± 1.2	20.1 ± 3.7	12.03 ± 1.1
Vehicle/Bleomycin	23.67 ± 3.8†	166.5 ± 25.6†	35.85 ± 4.3†
Valsartan 30mg/kg	12.23 ± 2.1*	112.3 ± 21.3	21.99 ± 3.1
Valsartan 10mg/kg	12.7 ± 0.7*	80.2 ± 18.5*	22.5 ± 3.6
Valsartan 3mg/kg	13.49 ± 2.2*	101.3 ± 28.9	19.08 ± 3.2*
Prednisolone 30mg/kg	18.51 ± 6.6*	35.8 ± 7.5*	33.04 ± 4.8

Results represent mean ± s.e.m. * $p<0.05$, ANOVA one way with Dunnett's multiple t test compared to bleomycin control. and † $p<0.05$ compared to vehicle control

The data indicate that valsartan was effective in preventing the progresssion of airway fibrosis in a murine model of bleomycin-induced lung injury. This effect was not associated with a decrease in inflammation *per se* but rather with a reduction in the levels of mediators MCP-1 and TGFβ. This study provides the first data to demonstrate the effectiveness of the AT_1 receptor antagonist valsartan to protect against bleomycin-induced fibrosis *in-vivo.*

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268P REGULATION OF THE RELEASE OF COLONY-STIMULATING FACTORS FROM HUMAN AIRWAY SMOOTH MUSCLE CELLS BY ISOPROSTANES

D.L. Clarke, M.A. Giembycz, #M.H. Yacoub & #M.G. Belvisi. Thoracic Medicine, #Respiratory Pharmacology, Cardiothoracic Surgery, National Heart & Lung Institute, ICSM, London.

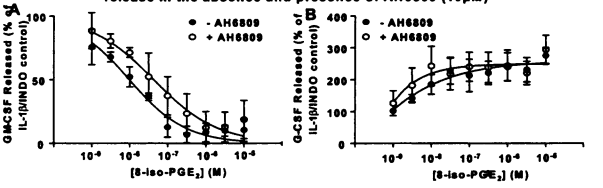
Isoprostanes are formed by the enzymatic and non-enzymatic peroxidation of arachidonic acid by free radicals and reactive oxygen species. 8-Isoprostanes are present in urine and plasma from normal subjects, but are increased in diseases such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis and interstitial lung disease and are now used as biomarkers for some of these diseases (Karatinov & Barnes, 2001).

We have previously found that PGE_2 modulates the release of the colony stimulating factors granulocyte/macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF), which are released from HASM cells in response to interleukin-1β (Clarke *et al.*, 2000). In this study we have investigated the effect of isoprostanes on the IL-β-induced release of CSFs from HASM and evaluated the receptor involved.

HASM cells from donor tracheae (2 male, 1 female, aged 32-52) were cultured in 96 well plates. At sub-confluence cells were growth-arrested for 24h before treatment with the drugs under investigation (diluted in DMEM containing 3% FCS). At 24h the supernatants were assayed for CSFs by ELISAs. None of the drugs affected cell viability as determined by mitochondria-dependent reduction of MTT to formazan. The vehicle for indomethacin, 8-iso PGE_2, AH6809 ($EP_{1/2}$/DP-receptor antagonist) and SQ29,548 (TP-receptor antagonist) (0.1% DMSO), had no effect on CSF release. Experiments were performed in the presence of 10^{-5}M indomethacin to inhibit endogenous prostanoid formation. 8-Iso-PGE_2 (10μM - 10μM) produced from a concentration-dependent inhibition of GM-CSF release (884.5 ± 153.7 to 70.6 ± 44.9 pg ml^{-1}, 92.4 % inhibition, n=9 determinations from 3 donors), and increase of G-CSF release (3234.1 ± 1214.2 to 8296.4±2723.86 pg ml^{-1} 275% increase n=9 determinations from 3 donors) from IL-1β-treated HASM cells. Neither 8-iso $PGF_{1\alpha}$ or 8-iso $PGF_{2\alpha}$ had any effect on GM-CSF or G-CSF release from HASM cells.

Pre-treatment of HASM cells with SQ29,548 (1μM) produced no effect on GM-CSF or G-CSF release from IL-1β-treated HASM cells. Pre-treatment of HASM cells with AH 6809 (10μM) produced a parallel rightwards shift of the concentration-response curve that described the inhibition of GM-CSF release by 8-iso PGE_2 (pA_2 = 5.6 ± 0.23, n=9 determinations from 3 donors), but had no effect on the release of G-CSF (Figure 1).

Figure 1 Effect of 8-iso-PGE_2 on IL-1β-induced GM-CSF (A) and G-CSF (B) release in the absence and presence of AH6809 (10μM)



This pA_2 value is indicative of blockade of the recombinant EP_2-receptor subtype expressed in other cells types (Woodward *et al.*, 1995). In conclusion this data suggests that 8-iso-PGE_2 acts via the prostanoid EP_2 receptor to regulate GM-CSF, but not G-CSF release from HASM cells. The data also suggest that the receptor(s) through which 8-iso-PGE_2 augments G-CSF release from HASM cells is unknown. This study illustrates, for the first time, the anti-inflammatory potential of certain isoprostanes in the airways.

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269P ATP PRIMING OF MACROPHAGE-DERIVED CHEMOKINE RESPONSES IN CELLS EXPRESSING THE CCR4 RECEPTOR

E.M. Rosethorne & S.J. Charlton. Novartis Horsham Research Centre, West Sussex, RH12 5AB.

Macrophage-derived chemokine (MDC) is an agonist at the CC chemokine receptor 4 (CCR4) (Imai et al, 1997). Previous work within our group demonstrated that MDC was unable to stimulate the release of intracellular calcium ($Ca^{2+}{}_i$) from CHO-CCR4 cells. In this study, the effect of priming with ATP prior to stimulation with MDC was investigated.

Chinese Hamster Ovary (CHO) cells transfected with the human CCR4 receptor (Euroscreen) were seeded into 96-well plates (30000 cells/well) and incubated for 30 minutes with Fluo-4-AM (0.1 μM) and brilliant black (10 μM). Agonist-induced changes in $[Ca^{2+}]_i$ were monitered after 30 minutes using FLIPR (Molecular Devices, UK).

Addition of ATP (10 μM) resulted in a prolonged $Ca^{2+}{}_i$ signal that returned to basal levels after approximately 4 minutes. MDC (100 nM), added 10 minutes after this initial ATP challenge, elicited a $Ca^{2+}{}_i$ response that was not observed without priming. The relationship between the concentration of ATP used to prime and the maximal MDC response elicited was investigated. ATP increased $[Ca^{2+}]_i$ with a pEC_{50} of 7.12 ± 0.16 (75 nM), n = 4. The response to MDC (100 nM) closely mirrored the ATP signal up to a concentration of 3 μM. Higher concentrations of ATP resulted in a reduced subsequent MDC signal.

The time dependency of the priming effect was examined between 5 and 30 minutes. The priming effect increased up to 10 minutes pre-incubation, after which the signal to MDC was stable. After ATP (1 μM) priming MDC elicited $Ca^{2+}{}_i$ signals with a pEC_{50} of 8.66 ± 0.16 (2.2 nM), n = 3.

The effect of $[Ca^{2+}]_i$ on subsequent MDC responses was investigated. The calcium ionophore ionomycin and the Ca^{2+}-ATPase inhibitor thapsigargin both increased $[Ca^{2+}]_i$ (pEC_{50} values of 7.59 ± 0.57 (25.8 nM) and 6.81 ± 0.31 (150 nM), respectively, each n = 3) but did not prime for the MDC response at any concentration.

Chemotaxis in response to CCR4 is mediated via pertussis toxin-sensitive G proteins (Inngjerdingen *et al*, 2000). Preincubation of the cells with pertussis toxin (200 μg/ml, 20 hours) did not affect the ATP signal but abolished the MDC response, demonstrating that priming does not alter the G protein coupling specificity of the CCR4 receptor.

We have demonstrated that priming CHO-CCR4 cells with ATP allows the CCR4 receptor to elicit $Ca^{2+}{}_i$ response. This effect is independent of increases in $[Ca^{2+}]_i$. G protein specificity is unaffected by priming. The priming of responses to CCR4 may be an important regulatory mechanism in physiological systems.

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Inngjerdingen M., Damaj B. & Maghazachi AA. (2000) *J. Immunol.* **164**(8), 4048

270P PULMONARY EFFECTS OF REPEATED ALLERGEN CHALLENGE IN ACTIVELY SENSITISED BROWN NORWAY RATS ASSESSED NON-INVASIVELY BY MAGNETIC RESONANCE IMAGING (MRI).

B. Tigani, N. Beckmann, M. Irouschek & J. R. Fozard. Research Department, Novartis Pharma AG, CH-4002 Basel, Switzerland.

Recently, magnetic resonance imaging (MRI) was applied to follow non-invasively the development of an oedematous signal in the lung induced by a single challenge with ovalbumin (OA) in the actively sensitised (AS) Brown Norway (BN) rat (Beckmann *et al.*, 2001). The same approach has now been used to follow the effects of repeated challenge with OA in the rat lung.

Ten male BN rats weighing 200-300 g were actively sensitised to OA (Beckmann *et al.*, 2001), and divided into 2 groups.The first group was challenged once with OA and killed with pentobarbital (250 mg kg^{-1} i.p.) 24 h after challenge for bronchoalveolar lavage fluid (BALF) analysis. Animals from the second group were challenged 4 times at 0, 96, 192 and 288 h and killed 24 h after the last challenge for BALF analysis. A gradient echo MRI sequence was used to define the oedematous signal in response to OA challenge in rats anaesthetised with 2 % Forene in a mixture of O_2/N_2O. Baseline images were acquired before the first challenge and at 6, 24, 48 and 96 h after each OA challenge. Total and differential leukocyte cell counts in BAL fluid were measured using an automated cell analysing system (Cobas Helios). Eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities, and protein concentrations in BAL fluid were determined using standard photometric assays.

The time course of signal development in the lung of AS BN rats after the first OA-challenge was qualitatively similar to that presented in Beckmann *et al.*, (2001), with a maximum signal detected 48 h after challenge and decreasing markedly at 96 h. After the second and subsequent OA-challenges, the shape of the time course was different; the maximum signal was at 6 h with a time-dependent decline over the remainder of the time course (Fig.1). Moreover, the magnitude of the signal at 24 h after the fourth challenge was reduced by 55 % compared to the signal measured 24 h after a single challenge.

Analysis of BAL fluid revealed that eosinophil and lymphocyte numbers were similar 24 h after a single, or the last of four OA-challenges. However, neutrophil numbers (-65 %), EPO (-55 %) and MPO (-26 %) activities and protein concentration (-37 %) were all significantly reduced after repeated challenge ($p < 0.05$).

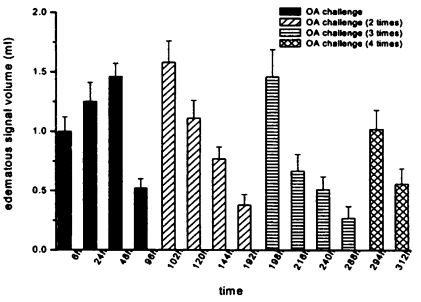

Figure 1: Oedematous signals in the lungs of actively sensitised Brown Norway rats as a function of time after repeated ovalbumin challenges at 0, 96, 192 and 288 h. Columns represent mean values (± s.e.mean) of data from 4 individual animals.

Our data provide further evidence that the increase in oedematous signal is a reflection of the inflammatory status of the lungs. Thus, the decline in response to OA, exemplified by changes in markers of inflammation in BAL fluid, is reflected by a decrease in the MRI signal. Our results also confirm that the inflammatory response to allergen challenge is reduced following repeated administration of OA (Elwood *et al*, 1991). The MRI technique provides a non-invasive means to demonstrate this resistance to OA.

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Elwood, W., Lötvall J. O., Barnes, P. J. *et al.*, (1991). *J. Allergy Clin. Immunol.*, **88**, 951-960.

271P AN AUTOMATED METHOD FOR EVALUATING COUGH NUMBERS IN GUINEA PIGS

Joanne L Hardstaff, M.J. Peck, J.L. Pype & F. Stiernet Allergo-Respiratory Pharmacology, UCB Pharma. S.A. B-1420, Braine l'Alleud, Belgium.

Aerosolised citric acid-induced cough is a frequently used model for testing the effects of anti-tussives. The procedure is, however, labour-intensive and subject to varying degrees of observer bias. We have developed an automated model in which up to eight animals at a time may be monitored.

Male Dunkin-Hartley guinea pigs (300-450g, Charles River, Germany) were used throughout the study. Animals were acclimatised for 1-2 weeks in temperature/humidity controlled rooms with free access to food and water.

Animals were placed into individual whole body Perspex plethysmographs (6L) through which air was constantly drawn at the rate of 1.5-2l min^{-1}. The box was fitted with a differential pressure transducer for the measurement of pressure changes within it and a microphone for recording sound. These were interfaced with a PC that monitored sound and pressure using specialist cough analysis software (EMKA Technologies, France). The software calculated the value Penh (enhanced pause) as a measure of pulmonary function. Cough was induced by exposing the animals to a 4 minute aerosol of 0.5M citric acid (generated by a small volume ultrasonic nebuliser (System Assistance Medical L. S., France)). Coughs were counted manually and automatically during the last three minutes of nebulisation and for 10 minutes after the aerosol was stopped.

The results were subjected to a one way ANOVA analysis and Dunnet's multiple comparison test using GraphPad Prism Software.

The model has been evaluated using SR 48968 (an NK_2 antagonist [Advenir & Edmond-Alt, 1996]) and 8-hydroxy-2-(di n-propylamino)tetraline (8-OH-DPAT, a 5-HT_{1A} agonist, Tocris). Both compounds were administered i.v. 5 min. before the exposure to citric acid. The number of coughs is expressed as the mean±s.e.m. of 4-10 animals (man=manual count, auto= automated count).

SR 48968

Control:	man = 17.0±6.4	auto = 12.7±4.9
1mg/kg:	man = 9.4±2.2*	auto = 8.0±2.5
5mg/kg:	man = 7.0±2.4*	auto = 5.0±1.0*

8-OH-DPAT

Control:	man = 26.6±3.6	auto = 23.3±4.8
1µg/kg:	man = 16.1±3.8*	auto = 12.9±2.1
5µg/kg:	man = 5.7±2.5*	auto = 5.2±2.5*

*$p<0.05$ vs control

In conclusion, we have developed a reliable and unbiased methodology for the measurement of cough in a guinea pig model.

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272P ADENYLYL AND GUANYLYL CYCLASE AND PDE ACTIVITIES TOWARD cAMP AND cGMP IN ISOLATED LUNGS DURING LPS-INDUCED AIRWAY REACTIVITY CHANGES.

T.J. Toward & K.J. Broadley, Pharmacology Division, Welsh School of Pharmacy, Cardiff University, Cardiff CF10 3XF, UK.

Exposure of guinea-pigs to aerosolised lipopolysaccharide (LPS) causes airway hyperreactivity (AHR) and hyporeactivity (AHOR) to inhaled histamine (Toward and Broadley, 2000). During the AHR and AHOR, there was deficiency and over-production of airways nitric oxide (NO), respectively. NO increases intracellular cGMP, causing bronchodilation (Toward & Broadley, 2001). This study investigated whether a correlation exists between LPS-induced AHR and AHOR *in vivo* and lung cGMP and cAMP levels *ex-vivo*.

Specific airway conductance (sGaw) was measured in groups (n=6) of conscious Dunkin-Hartley guinea-pigs (male, 300-350g) by whole-body plethysmography (Griffiths-Johnson *et al.*, 1988). Baseline sGaw values were obtained and 30min later they received a nose-only exposure to either a threshold (1mM, 20s) or bronchoconstrictor (3mM, 20s) dose of nebulised (0.2ml.min^{-1}) histamine and sGaw was recorded at 0, 5 and 10min. 24h later, guinea-pigs were box-exposed (1h) to LPS (30μg.ml^{-1}), or vehicle (0.9% LPS-free saline). Airway function was measured at 0, 15, 30min and hourly thereafter. Airway reactivity to 1 or 3mM histamine was re-assessed at 1 or 48h later, respectively. Other guinea-pigs were overdosed with pentobarbitone sodium (60mg.kg^{-1} body weight, i.p.), at 1 or 48h after LPS or saline. Lungs were removed and aliquots (0.3mg. ml^{-1}) of sliced (<1mm) lung were incubated (pH 7.4, 37±4°C) in phosphate-free Krebs-bicarbonate solution and gassed (5% CO_2 / 95% O_2). In separate aliquots, the β-adrenoceptor agonist, isoprenaline (ISO: 10^{-9}-10^{-5}M), or the NO donor, S-nitroso-N-acetylpenicillamine (SNAP: 10^{-6}-10^{-3}M), was added, in the absence or presence of the non-selective PDE-inhibitor, 3-isobutyl-1-methylxanthine (IBMX: 10^{-4}M). At 0 or 20min, 0.5ml of aliquot was added to 0.5ml HCl (1N), homogenised (20,000r.p.m., 20s) and centrifuged (13,000r.p.m., 3min). Supernatant SNAP-mediated cGMP and ISO-mediated cAMP were determined using enzyme- linked immunosorbent assays (R & D Systems, Oxon, UK). Cyclic nucleotide levels were expressed as the mean±s.e.m. pmol.mg^{-1} of oven-dried lung pellet. Levels of cAMP and cGMP in the presence of IBMX indicated adenylyl and guanylyl *cyclase activity*, respectively. Difference between cAMP or cGMP levels, with and without IBMX, reflected relevant *PDE activity*.

After LPS, guinea-pigs displayed AHR at 1h and AHOR at 48h, to inhaled (20s) histamine (1 or 3mM, respectively). During AHR, *PDE activity* towards cAMP (ISO:10^{-5}M) or cGMP (SNAP:10^{-3}M) increased (35.7±4.0 pmol.mg^{-1}, P<0.05 and 34.7±9.6 pmol.mg^{-1}, P<0.01, respectively) compared with 1h after vehicle (21.4±4.6 and 9.2±8.8 pmol.mg^{-1}, respectively). Adenylyl and guanylyl *cyclase activity* also increased during AHR (ISO(10^{-5}M): 46.4±3.3 pmol.mg^{-1}, P<0.05 and SNAP(10^{-3}M): 62.7±8.3 pmol.mg^{-1}, P<0.001), compared with 1h after vehicle (31.5±4.5 and 20.2±7.4 pmol.mg^{-1}). *Net* cAMP levels were unaltered, whereas *net* SNAP-mediated (10^{-3}M) cGMP levels were increased (27.9±3.6 pmol.mg^{-1}, P<0.05), compared with 1h after vehicle exposure. (11.0±1.8 pmol.mg^{-1}). During AHOR, *net* levels of cAMP (ISO(10^{-5}M): 243%) and cGMP (SNAP(10^{-3}M): 268%) increased (P<0.01) and adenylyl and guanylyl *cyclase activity* (35.2±7.2 and 40.4±13.2 pmol.mg^{-1}, respectively) also rose (P<0.05), compared with 48h after vehicle (29.5±3.9 and 21.2±5.0 pmol.mg^{-1}, respectively). In contrast, PDE activity towards ISO-mediated cAMP (10^{-5}M: 3.9±4.3 pmol.mg^{-1}, P<0.05) and SNAP-mediated cGMP (10^{-3} M: 2.6±9.8 pmol.mg^{-1}) was reduced compared with 48h after vehicle (18.3±4.1 and 8.2±9.0 pmol.mg^{-1}, respectively).

Thus, while *net* cAMP and cGMP levels appeared raised during AHR and AHOR, *PDE activity* increased during AHR and decreased during AHOR. This study supports the use of PDE-inhibitors for AHR.

The authors gratefully acknowledge the assistance of Dr A.T. Nials and a GlaxoSmithKline studentship awarded to TJT.

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273P EFFECTS OF INHALED CORTICOSTEROID ADMINISTERED TO OVALBUMIN-CHALLENGED CONSCIOUS GUINEA-PIGS

B.J. Nevin & K.J. Broadley, Pharmacology Division, Welsh School of Pharmacy, Cardiff University, Cathays Park, Cardiff CF1 3XF

Inhaled corticosteroids do not reduce the early asthmatic reaction (EAR) but reduces the late asthmatic reaction (LAR) (Pepys *et al*, 1974) bronchoalveolar lavage (BAL) leukocytes (Duddridge *et al,* 1993) and airway hyperreactivity (AHR) to histamine in humans (Burke *et al*, 1992). This study determined whether inhaled corticosteroid produces similar effects in an animal model of asthma.

Male Dunkin-Hartley guinea-pigs (250-300g) were sensitised with ovalbumin (OA) (10μg OA and 100mg Al(OH)$_3$ in 1ml). 14-21 days later they received inhaled OA (100μg.ml^{-1} in saline) or saline for 1h (Spruntulis & Broadley, 1999). Airway function was measured as changes in specific airway conductance (sGaw) at intervals up to10h and at 24h by whole body plethysmography (Spruntulis & Broadley, 1999). Airway reactivity to inhaled histamine (1mM nose only for 20s) was determined 24h before and 24h after OA. 24h following OA challenge, guinea-pigs were overdosed (pentobarbitone sodium 200mg.kg^{-1}) and lungs lavaged (saline 1ml.100g^{-1}, twice) to determine leukocyte numbers. Further groups (n=6-12) received inhaled budesonide (0.448 or 1.35mg.ml^{-1}) or its vehicle (DMSO 30%, ethanol 30%, saline 40%) for 15min at 24h and 0.5h before OA challenge. Statistical analysis was by analysis of variance followed by paired or unpaired Student's *t*-test (P<0.05).

OA caused an immediate EAR (-55.6±9.5% reduction in sGaw) recovering by 5h and followed by a LAR between 6 and 10h (-27±4.5%). 24h after OA, histamine caused significant bronchoconstriction (–20.6±4.2%) compared with no response before OA, indicating AHR. There were significant increases in total cells (10.25±1.5×10^6.ml^{-1}), macrophages (4.9±0.8×10^6.ml^{-1}) and eosinophils (5.4 ± 0.7 × 10^6.ml^{-1}) compared with saline treated guinea-pigs (2.7±0.3, 2.4±0.2 and 0.3±0.07×10^6.ml^{-1}). In vehicle-treated OA challenged animals, there was no significant reduction in LAR (-23.5±3%), cell counts (15.5±2.8, 8.7±2.0 and 6.9 ± 1.3 × 10^6.ml^{-1}) or AHR to histamine (–19.2±3.4%). However, the EAR was significantly attenuated (-23.1±6.3%). After budesonide (0.448 and 1.35mg.ml^{-1}) there was no further reduction of the EAR (-21.0±6.3% and -19±7.8%). The LAR was not affected by the low dose budesonide (-21.0%±5.4%) but was reduced by high dose budesonide (-4.7±4.3%). Budesonide (0.448 and 1.35mg.ml^{-1}) prevented the AHR to histamine (3.6± 2.5%, -0.4±2.8%) and attenuated the increases in eosinophils (2.5±0.4, 1.9±0.2×10^6.ml^{-1}).

Thus in spite of the effects of vehicle on the EAR, our asthma model displays the effects of inhaled corticosteroids seen in human asthma.

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274P EFFECTS OF *IN-VIVO* AND *IN-VITRO* CHALLENGE WITH OVALBUMEN ON BRONCHOCONSTRICTION OF GUINEA-PIG ISOLATED LUNG TO ADENOSINE

Timothy J. Martin & Kenneth J. Broadley. Department of Pharmacology, Welsh School of Pharmacy, Cardiff University, CF10 3XF.

Many studies have investigated the *in-vitro* (Thorne and Broadley, 1992; Lewis and Broadley, 1995) and *in-vivo* (Pretolani *et al.*, 1994) response of sensitized guinea-pig airways after an *in-vivo* ovalbumen (OA) challenge. This approach is often used as a model for studying the mechanisms involved in an allergic response. The early phase occurs within minutes and results from the release of preformed mediators, such as histamine and leukotrienes (Barnes *et al.*, 1998), from pulmonary mast cells (Busse *et al.*, 1993). This study sought to investigate the response of isolated lungs to adenosine and OA after either an *in-vitro* or *in-vivo* OA challenge. Secondly, to investigate the subtype of mast cell involved, responses to OA and adenosine were examined after *in-vitro* exposure to the selective degranulator of rat connective tissue mast cells, compound 48/80 (Metcalfe *et al.*, 1997).

Male Dunkin-Hartley guinea-pigs (250-300g) were sensitized to OA (1mg i.p.) or not sensitized. ***In-vivo* challenge:** After OA inhalation (300 µg/ml, 7.5mins), the guinea-pigs were killed 1, 24 or 48 hours later. The lung halves were removed, suspended in a heated jacket, (37°C), and perfused with pre-warmed (37°C) and gassed (5%CO_2 in O_2) Krebs-solution at 5ml.min^{-1}. After equilibration, boluses of adenosine (1mM), histamine (10µM) and OA (10µg) were added. ***In-vitro* challenge:** Lungs were set-up as described above and after equilibration were exposed to repeated OA (10µg) or compound 48/80 (1mg) challenges until the lungs showed a substantial loss of response. Adenosine, histamine and OA were then administered. Increases in perfusion pressure (mmHg) were measured and mean ± s.e.m. compared using a paired Student's *t*-test. P values of 0.05 were taken to indicate significance.

The *in-vitro* constrictor responses of the lungs to adenosine (4±1mmHg) and OA (19±6) were significantly reduced 1 hour after an *in-vivo* OA challenge, when compared to the sensitized non-challenged control (11±2, 51±6). Adenosine (9±2) and OA (50±1) responses returned to original levels within 24 hours. The *in-vitro* response to adenosine (2±1) and OA (4±2) was reduced after two *in-vitro* OA challenges, compared to the paired non-challenged tissues (9±3, 55±9). Four challenges with compound 48/80 had no effect on the adenosine (6±2) or OA (69±11) response, compared to the paired non-challenged tissues (6±1, 70±3). Histamine responses were not affected by these *in-vitro* or *in-vivo* challenges.

An *in-vivo* challenge of sensitized guinea-pigs with OA results in the degranulation of mast cells and the release of mediators. One hour later the *in-vitro* responses to adenosine and OA were reduced. This suggests that adenosine is acting through a similar mechanism to OA and that it takes 24 hours for mast cell numbers to recover. *In-vitro* challenges with ovalbumen also reduced the responses to adenosine and OA. The lack of effect of exposure to the selective depletor of rat connective tissue mast cells implies that mucosal tissue mast cells are the main mast cells involved in the contractions of sensitized lungs to adenosine and OA.

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275P CHARACTERISATION OF AN ACUTE MODEL OF COPD IN THE RAT

M.A. Birrell, K. McCluskie, S. Wong, M.H. Yacoub & M.G. Belvisi. Respiratory Pharmacology, Cardiothoracic Surgery, National Heart and Lung Institute, Faculty of Medicine, Imperial College, London SW3 6LY.

It is believed that neutrophil recruitment into the airway tissue may play a role in the development of COPD (Barnes, 2001). We have previously described an acute model of LPS-induced airway neutrophilia (Haddad *et al.*, 2001). In this study we performed further characterisation of this model to include measurement of neutrophil infiltration, increases in neutrophil-related cytokine protein expression and changes in matrix metalloproteinases (MMP) 2 and 9 activity in the lungs.

Male Wistar rats (200 gm, n = 6) were challenged with aerosolised saline or LPS (0.3 mg ml^{-1}, 30 min.) and culled at increasing times (1, 2, 4, 6, 8, 12 and 24 hours) after challenge with an overdose of pentobarbitone (200 mg kg^{-1}, i.p.). The lungs were lavaged with RPMI (2 ml twice and pooled) and the lungs removed, perfused with RPMI to remove any blood and finely chopped. The amount and type of white blood cells (WBC) in the lung lavage and tissue was determined using a method described by Underwood *et al* (1997). The remaining lung was flash frozen in liquid nitrogen and stored at -80°C. MMP-2 and 9 activity levels were determined in the lavage supernatant by Zymography according to the manufacturers (Novex) instructions. TNFα, MIP-2 and MCP-1 levels were determined in lung tissue homogenates by ELISA kits from R&D and Biosource according to the manufacturers instructions. Statistical analysis was by one-way analysis of variance with an appropriate post test. Values are expressed as mean ± s. e. mean.

LPS challenge induced a significant ($p<0.05$) increase in neutrophils in the lung lavage and tissue (Figure 1). TNFα, IL-1β and MIP-2 levels in the tissue were significantly ($p<0.05$) increased compared to controls (Figure 2). MMP-9 but not MMP-2 activity levels was significantly ($p<0.05$) increased (Figure 3).

*Figure 1 Effect of LPS on lavage (left) and lung tissue (right) neutrophil numbers in the rat * = $p<0.05$.*

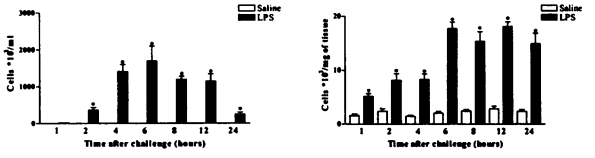


*Figure 2 Effect of LPS on tissue levels of TNFα (left), MIP-2 (centre) and IL-1β (right) in the rat * = $p<0.05$.*

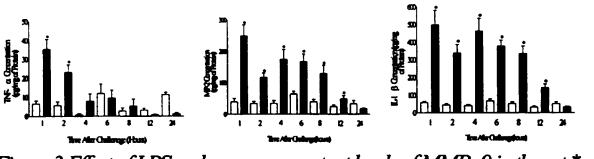
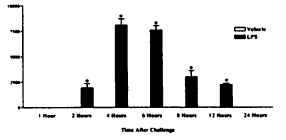


*Figure 3 Effect of LPS on lavage supernatant levels of MMP- 9 in the rat * = $p<0.05$.*



In conclusion, this model demonstrates many aspects (cellular inflammation and molecular biomarkers) believed to be important in COPD. Furthermore, it may be useful as an acute model for testing possible anti-COPD agents before moving on to more long term models of lung damage and emphysema.

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276P THE ROLE OF NITRIC OXIDE SYNTHASE IN ANTIGEN-INDUCED AIRWAY EOSINOPHILIA IN THE RAT

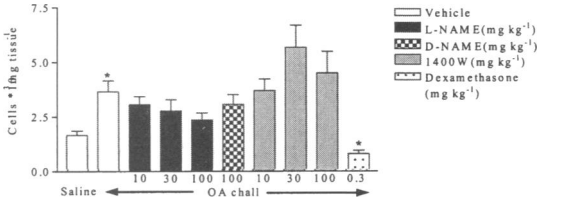
K. McCluskie, M.A. Birrell, M.H. Yacoub, J.A. Mitchell & M.G. Belvisi. Respiratory Pharmacology, Cardiothoracic Surgery, National Heart and Lung Institute, Faculty of Medicine, Imperial College, London SW3 6LY.

Excessive production of nitric oxide (NO) associated with increased inducible NO synthase (iNOS) expression may play a role in the eosinophilic inflammatory response characteristic of diseases such as asthma (Barnes & Belvisi, 1993). However, we have shown that iNOS does not appear to mediate airway eosinophilia in a commonly used, non-allergen, model of airway eosinophilia (Sephadex-treated rats; Birrell *et al*, 2000). The aim of this work was to determine the apparent role of iNOS in airway eosinophilia using an allergen model.

Male Brown Norway rats (200 g) were sensitised on days 0, 14 and 21 with ovalbumin (OA; 100 µg, i.p.) and aluminium hydroxide (100 mg, i.p.). Rats were challenged on day 28 with aerosolised saline or OA (10 g L^{-1}, 30 min.). Vehicle (saline, i.p.) or compound was administered 2 hours before and 4 and 12 hours after challenge (NOS inhibitors) or 24 and 1 hour before challenge (dexamethasone). 24 hours after challenge the animals were culled with a lethal dose of pentobarbitone (200 mg kg^{-1}, i.p.), lungs removed, perfused, chopped and white blood cells (WBC) analysed as described previously (Underwood *et al*, 1997) . In separate animals, sensitised and challenged as above and after 0.5, 1, 2, 4, 6, 8, 12 or 24 hours, lungs were removed for analysis of NOS mRNA using RT-PCR as described previously (Feder *et al*., 1997) and expressed as a ratio of GAPDH.

Antigen challenge significantly increased eosinophils in lung tissue ($p<0.05$). The inhibition by L-NAME (65 %, 100 mg kg^{-1}) did not reach statistical significance. D-NAME and 1400W showed no inhibition at doses tested. Dexamethasone significantly reduced lung tissue eosinophilia ($p<0.05$, figure 1) as expected. RT-PCR analysis of the lung tissue at different time points showed that iNOS mRNA expression was induced at 4 hours and was undetectable at other time points. There was no increase in eNOS or nNOS expression at any time point.

*Figure 1. Effect of L-NAME, D-NAME,1400W and Dexamethasone on antigen-induced airway eosinophilia in the rat. Data is represented as mean ± s.e.m. of n=6-8 animals and analysed by one-way ANOVA and appropriate post test * = $p<0.05$.*



In conclusion it appears that iNOS does not have a role in antigen-induced airway eosinophilia despite evidence of increased gene expression. In addition, whilst statistical significance was not achieved, our data with L-NAME cannot rule out a role of NO derived from constitutive forms of NOS in this model. These data are in keeping with our previous work using a Sephadex model of airway eosinophilia. These data, using rats and a pharmacological approach, are in contrast to others using knockout mice where iNOS appeared to mediate the eosinophilic inflammation associated with allergen sensitisation and challenge (Xiong *et al*, 1999).

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277P THE ROLE OF NITRIC OXIDE SYNTHASE IN AN ACUTE MODEL OF LPS-INDUCED AIRWAY INFLAMMATION IN THE RAT

M. Birrell, K. McCluskie, M.H. Yacoub & M.G. Belvisi. Respiratory Pharmacology, Cardiothoracic Surgery, National Heart and Lung Institute, Faculty of Medicine, Imperial College, London SW3 6LY.

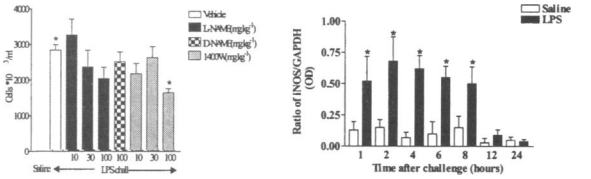
Production of nitric oxide (NO) due to the increased expression/activation of inducible NO synthase (iNOS) may play a role in airway inflammatory diseases such as Chronic obstructive pulmonary disease (COPD) (Barnes & Belvisi, 1993). It is believed that neutrophil recruitment into the airway tissue may play a role in the development of COPD (Barnes, 2001). We have previously shown a non-selective NOS inhibitor, L-NAME, but not its inactive isomer D-NAME or a selective iNOS inhibitor, 1400W, to inhibit 'asthma-like' airway eosinophilia in the rat (Birrell *et al*, 2000). In this study the aim was to determine if there was a role for NO, and the NOS isoform responsible for its production, in LPS-induced airway neutrophilia. This model represents certain aspects of the cellular pathology associated with COPD.

Male Wistar rats (200 g, n = 6 - 8) were challenged with aerosolised saline or LPS (0.3 mg ml^{-1}, 30 min.). Vehicle (saline, i.p.) or compound (10, 30 or 100 mg kg^{-1}, i.p.) was administered 2 hours prior and 1 hour after challenge. Dexamethasone (1 mg kg^{-1}, p.o.) was included as a positive standard and administered 24 and 1 h before challenge. Six h after challenge the animals were culled with an overdose of pentobarbitone (200 mg kg^{-1}, i.p.) and the lungs lavaged with cell culture medium (2 ml twice and pooled). The amount and type of white blood cells (WBC) in the lung lavage was determined using a method described by Underwood (1997). A separate group of rats were challenged with saline or LPS as above and culled at different times (1, 2, 4, 6, 8, 12 and 24 hours) after challenge. The lungs were removed, cleaned, flash frozen in liquid nitrogen and stored at -80°C. These tissues were analysed for iNOS, eNOS and nNOS gene expression by RT-PCR and expressed as a ratio of the housekeeping gene GAPDH. Statistical analysis was by one-way analysis of variance with a correction for multiple comparison using an appropriate post test. Values are expressed as mean ± s. e. mean.

LPS challenge induced a significant ($p<0.05$) increase in neutrophils in the lung lavage and 1400W administration caused a significant ($p<0.05$) inhibition of this response, L and D-NAME showed no significant inhibition at the dose tested (100 mg/kg) (Figure 1, left).

The positive standard, dexamethasone significantly ($p<0.05$) reduced lung lavage neutrophilia (from 2828 ± 152 to 459 ± 98 cell ml^{-1}). RT-PCR analysis of the lung tissue at different time points after challenge showed a significant ($p<0.05$) increase in iNOS expression when compared to saline controls (Figure 1, right). There was no increase in eNOS expression over time or between saline and LPS challenged groups. We failed to measure any nNOS in any tissue except the positive control, naïve rat brain tissue.

*Figure 1 (left) Effect of L-NAME, D-NAME or 1400W on LPS-induced airway neutrophilia in the rat, (right) iNOS gene expression in lung tissue after saline or LPS challenge in rats measured by RT-PCR. * = $p<0.05$.*



It appears that iNOS does have a role in LPS-induced airway neutrophilia; further evidence of this is the increased expression of iNOS after LPS challenge. Therefore, it seems that different isoforms of NOS are important in models of airway inflammation with constitutive NOS being more important in our "asthma like" model, whereas iNOS is more important in our "COPD like" model. In conclusion, these data suggests that iNOS inhibitors may be of some benefit in the treatment of COPD.

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278P THE ROLE OF PDE ISOFORMS IN OXAZALONE-INDUCED PROLIFERATION AND ADOPTIVELY TRANSFERRED CONTACT SENSITIVITY IN MICE.

S. Shaw Celltech R & D, 216 Bath Road, Slough, UK. (Introduced by R. Foulkes)

Previous studies *in vitro* have demonstrated the ability of PDE 4 inhibitors to inhibit mitogen-induced proliferation and cytokine production in murine (Souness *et al.*, 1997) and human peripheral blood mononuclear cultures. In contrast, the PDE 3 inhibitor SK&F 95654 exerted no inhibitory effect on T-cell proliferation, but potentiated the ability of the PDE 4 inhibitor Rolipram to suppress T-cell proliferation (Giembycz *et al.*, 1996). Present studies have investigated the ability of a novel selective PDE 4 inhibitor CT5357, N-(4-{4-[1-(3,4-Bis-difluromethoxy-phenyl)-2-(1-oxy-pyridin-4-yl)-ethyl]-phenoxy}-phenyl)-bis[methylsulphonyl]amine (enzyme IC_{50} 0.1nM) alone or in combination with the PDE 3 inhibitor, milrinone, to inhibit Oxazalone (OX)-induced proliferation and adoptively transferred contact sensitivity.

Male BALB/c mice (16-20g) received 25µl of either vehicle (4:1 acetone:olive oil) or 0.25% w/v OX on the dorsum of both ears daily for 3 consecutive days under light gaseous halothane anesthesia. On day 3, mice were sacrificed and the auricular draining lymph nodes excised and lymph node cultures (LNC) prepared as a single cell suspension in tissue culture media at a concentration of $1x10^7$cells/ml. Proliferation was assessed by seeding LNC into a 96 flat-bottomed plate (200µl) and pulsing with 2µCi [^{3}H] methylthymidine for 24 hours prior to harvesting. The OX-induced contact sensitivity response was assessed by transferring intravenously in 0.1ml either $1x10^7$ vehicle-sensitised or OX-sensitised LNC into naive recipient mice. Simultaneously, mice were topically challenged with 25µL of a 1%w/v OX solution to the dorsum of the right ear. Elicitation of the contact sensitivity response was assessed by change in ear thickness 24 hours post challenge relative to pre-challenge values. Data are expressed as mean ± s.e. mean, differences are assessed by ANOVA.

Pre-treatment with CT5357 orally, twice daily, at 10mg/kg during OX sensitisation caused a 45%± 7.7 ($p<0.05$; n=6) reduction in proliferation whilst 1mg/kg had no inhibitory effect. Transfer of CT5357 (10mg/kg) treated OX sensitised LNC into recipient mice and immediate OX challenge resulted in a 70%± 5.6 ($p<0.01$; n=10) reduction in ear swelling response whist 1mg/kg CT5357 caused a 43% ± 6.7 (ns) reduction. In contrast, using an identical dosing regimen, pre-treatment with milrinone failed to suppress OX-induced proliferation or OX induced ear swelling following transfer of cells. However, combination treatment with CT5357 and milrinone, (both 1mg/kg) caused a 60% ± 6.7 ($p<0.01$; n=10) reduction of the ear swelling response. In a subsequent experiment, the effect of CT5357 (0.1mg/ear) and milrinone (0.5mg/ear) given topically twice daily during OX sensitisation were investigated. Milrinone pre-treatment failed to inhibit either OX-induced proliferation or OX-induced ear swelling response in recipient mice. CT5357 pre-treatment failed to inhibit OX-induced proliferation, but did inhibit OX-induced ear swelling response by 66%± 10.7 ($p<0.05$; n=10). In contrast, combination treatment during OX sensitisation resulted in a small but significant 30% ± 2.9 ($p<0.05$; n=6) reduction in OX-induced proliferation and a substantial 92%± 6.1 ($p<0.001$; n=10) reduction in the ear swelling response.

These *in vivo* data demonstrate that selective PDE 4 inhibitors are capable of suppressing T-cell proliferation and T-cell effector function, which are potentiated by a PDE 3 inhibitor.

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279P THE INDUCTION OF A HYPERSECRETORY PHENOTYPE BY INTERLEUKIN 13 (IL-13) IN HUMAN BRONCHIAL EPITHELIAL CELLS (HBECs) - A ROLE FOR A CALCIUM ACTIVATED CHLORIDE CONDUCTANCE.

H. Atherton, C.T. Poll & H. Danahay. Novartis Respiratory Research Centre, Horsham. UK.

IL-13 is a CD4+ cytokine which has been implicated in respiratory conditions associated with a hypersecretory phenotype (Huang *et al.* 1995; Pawankar *et al.* 1995; Hamilos *et al.* 1996). To date the effects of CD4+ cytokines on the ion transport characteristics of the human airway epithelium have not been studied.

HBECs (Clonetics) were cultured on Snapwell permeable supports for 21 days, the final 14 days at an apical air interface. These conditions provided a differentiated transporting epithelial structure. HBECs were then treated with either vehicle or IL-13 ($10ngml^{-1}$) added to the basolateral media for 48h. Epithelia were then placed in Ussing chambers bathed in Ringer solution containing (mM): 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.8 K2HPO4, 1.2 CaCl2, 1.2 MgCl2, 10 glucose (37°C, 5%CO_2:O_2) and voltage clamped at 0mV. The basal characteristics of the cells in addition to the amiloride (10µM)-sensitive short circuit current (ISC) were determined before the addition of either UTP (30µM) or ionomycin (1µM). In a subsequent study the effect of DIDS (300µM) on the ionomycin stimulated ISC was determined. All compounds were added to the apical surface of the cells with exception of ionomycin which was also added basolaterally. Data are expressed as absolute changes in ISC (mean±s.e.mean) and significance assumed when $P<0.05$ (paired Student t-test).

Additionally, the effects of IL-13 on the basal and stimulated levels of intracellular Ca^{2+} $[Ca^{2+}]_i$ were studied. 24h after seeding onto plastic 96 well plates, HBECs were treated with IL-13 ($10ngml^{-1}$). After a 48h treatment with IL-13, cells were loaded with Fluo-4 and changes in $[Ca^{2+}]_i$ were monitored using FLIPR (FLuorescence Imaging Plate Reader). The peak increase in $[Ca^{2+}]_i$ was used to construct concentration response curves to UTP (0.1-100µM) and ionomycin (0.01-3µM) in both control and IL-13 treated cells.

IL-13 treatment attenuated the basal ISC from 20.2±0.8µAcm^{-2} (n=4) to 5.8±0.6µAcm^{-2} ($P<10^{-5}$; n=4) and the amiloride-sensitive ISC from 7.8±0.4µAcm^{-2} (n=4) to 0.9±0.5µAcm^{-2} ($P<0.0001$; n=4). In the presence of amiloride, UTP induced an increase in ISC of 11.8±0.6µAcm^{-2} (n=4) that was increased in the IL-13 treated cells to 99.4±6.0µAcm^{-2} ($P<0.0001$; n=4). In a separate study, IL-13 induced a similar effect on the basal and amiloride-sensitive currents and the subsequent ISC response to ionomycin was enhanced from 5.6±0.9µAcm^{-2} (n=6) in control to 76.8±11.5µAcm^{-2} ($P<0.0001$; n=6) in IL-13 treated cells. DIDS limited the IL-13 enhanced ionomycin-stimulated ISC response to an increase of 28.2±3.6µAcm^{-2} ($P<0.003$; n=6).

There were no differences in the resting levels of $[Ca^{2+}]_i$ between control and IL-13 treated HBECs (data not shown). UTP induced an increase in $[Ca^{2+}]_i$ with EC50 values of 0.67±0.20µM and 0.45±0.02µM with and without IL-13 pre-treatment respectively (n=3). Ionomycin induced changes in $[Ca^{2+}]_i$ with EC50 values of 43±6nM and 52±24nM with and without IL-13 treatment respectively (n=3).

This study indicates that IL-13 is able to convert the human bronchial epithelium from an absorptive to secretory phenotype. This is achieved through both the attenuation of amiloride-sensitive Na^+ absorption and the enhancement of a DIDS-sensitive Ca^{2+}-activated secretory current. IL-13 did not affect the changes in $[Ca^{2+}]_i$ induced by either UTP or ionomycin suggesting that IL-13 enhances the Ca^{2+} activated secretory current through direct modulation of the ion conductances involved.

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280P MUCIN GENE EXPRESSION IN HUMAN RESPIRATORY EPITHELIAL CELL LINES: ACTIVATION BY PHORBOL ESTERS AND EFFECT OF A CORTICOSTEROID AND CYCLIC AMP ACTIVATING AGENTS

W. de Graaf, A.K. Smith, P.J. Barnes & D.F. Rogers, Thoracic Medicine, National Heart & Lung Institute, Faculty of Medicine, Imperial College, London SW3 6LY

Airways mucus hypersecretion is a feature of a number of severe respiratory diseases, including asthma and chronic obstructive pulmonary disease (COPD). The gene products of MUC5AC and MUC5B predominate in respiratory secretions from normal individuals as well as in patients with asthma and COPD. Understanding regulation of these genes may aid understanding of the pathophysiology of airway mucus hypersecretion. The scarcity of human tissue has prompted investigation of cell lines. The aims of the present study were to determine: 1) baseline expression of MUC5AC and MUC5B in two human respiratory epithelial cell lines: A549 cells (pulmonary carcinoma-derived) and BEAS 2B cells (immortalised bronchial cells); 2) whether activation of PKC (by the phorbol esters PMA and PDBu) increased MUC gene expression; 3) whether increased expression could be inhibited by a corticosteroid (dexamethasone) or by cAMP-elevating agents (salbutamol or 8-bromo-cAMP); both treatments used in management of asthma and COPD. MUC gene expression was determined as steady state mRNA levels by semi-quantitative RT-PCR, with product density expressed as a ratio to GAPDH (as an internal standard; in arbitrary units, AU). Data are mean and s.e.mean. The significance of differences between experimental groups was assessed using Student's *t*-test, with n the number of experiments.

A549 cells expressed both MUC5AC and MUC5B, whereas BEAS 2B cells did not (n = 5 each). PMA (0.01-10 μM) increased MUC5AC expression in A549 cells in a time-dependent (maximal at 6h out of 2, 4 and 8h) and concentration-dependent manner with an increase of 114% above controls at 10 μM at 6 h (0.42 ± 0.06 AU *vs* 0.91 ± 0.04 AU, P<0.01, n=5). PDBu also increased MUC5AC expression (183% above controls at 10μM). In contrast, neither PMA nor PDBu altered MUC5B expression in A549 cells. PMA did not affect MUC5AC or MUC5B levels in BEAS 2B cells. The PMA (1 μM)-induced increase in MUC5AC expression in A549 cells was inhibited by the PKC inhibitor Ro318220 (3-[1-3-(Amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide) in a concentration-dependent manner, with complete inhibition at 10 μM (n=5). PMA-induced MUC5AC in A549 cells was also inhibited by dexamethasone (0.1 and 1 μM), with complete inhibition at 1 μM (Fig. 1). In contrast, salbutamol (1 and 10 μM) did not significantly inhibit PMA-induced MUC5AC expression. Similarly, 8-bromo-cAMP (0.1 and 1 mM) did not inhibit PMA-induced MUC5AC expression.

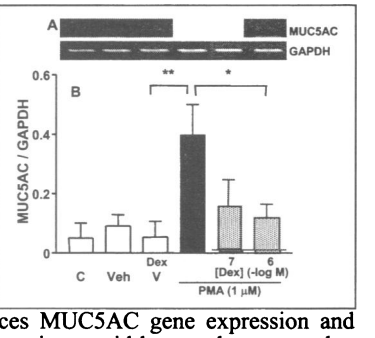

Fig.1. Effect of dexamethasone (1h pre-treatment) on levels of MUC5AC induced by PMA (1 μM) in A549 cells. A) Representative PCR; B) Mean data (bar = s.e.m.). *P<0.05, **P<0.01.

We conclude that activation of PKC pathways in A549 cells induces MUC5AC gene expression and that this is inhibited by a corticosteroid but not by agents that elevate cAMP. In addition, the A549 cell line is preferable to the BEAS 2B cell line for study of these phenomena, although neither cell line shows induction of MUC5B gene expression.

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281P EXPRESSION OF EDG RECEPTORS IN HUMAN BRONCHIAL EPITHELIAL CELLS, AS PUTATIVE TARGETS OF THE GROWTH STIMULATOR LYSOPHOSPHATIDIC ACID

F. Wenzel, M. Fuhrmann & K. Racké Institute of Pharmacology & Toxicology, University of Bonn, Reuterstr. 2b, D-53113 Bonn, Germany.

Recently, the EDG (endothelial differentiation gene) receptors were identified as a family of G-protein coupled receptors for which lysophospholipids, either lysophosphatidic acid (LPA) or sphingosine 1-phosphate (S1P) are specific agonists (for review see Racké *et al.,* 2000). There is evidence that levels of extracellular lysophospholipids may be elevated during inflammatory reactions (see Racké *et al.,* 2000). Moreover, we showed that human and rat alveolar macrophages express mRNA for multiple EDG receptors and that both LPA and S1P mediated an activation of superoxide anions (O_2^-) production (Hornuß *et al.,* 2001). In the present study possible effects of LPA and S1P and the expression of EDG receptors in the human bronchial epithelial cell line A549 were investigated.

Freshly disseminated A549 cells (0.5 * 10^6 cell $well^{-1}$) were cultured for up to 48 hr in RPMI 1640 medium in the absence or presence of fetal calf serum (FCS, 10%) and/or LPA or S1P followed by 6 hr culture with the additional presence of [^{3}H]-thymidine (10 μM, 1 μCi). In parallel experiments total RNA was isolated and used for RT-PCR with primers specific for eight human EDG receptors or protein was extracted for use in immunoblotting.

As summarized in Table 1, A549 cells express multiple EDG receptors. The signal of the amplification product of mRNA for EDG2 receptors was the most prominent, but a clear signal was also detected for mRNA of EDG3, EDG4 and EDG8 receptors.

Table 1. Expression of mRNA for EDG receptors in A549 cells as detected by RT-PCR.

EDG1	EDG2	EDG3	EDG4	EDG5	EDG6	EDG7	EDG8
+	+++	++	++	-	+	-	++

In A549 cells cultured in the presence of FCS, the incorporated [^{3}H]-thymidine amounted to 733,202 ± 12,063 DPM $well^{-1}$ (mean ± s.e.mean, n=15). In cells cultured in the absence of FCS the incorporated [^{3}H]-thymidine amounted to 346,502 ± 20,956 DPM $well^{-1}$ (n=18) ($p < 0.001$, Student's t-test). In A549 cells cultured in the presence of FCS neither LPA nor S1P significantly affected the [^{3}H]-thymidine incorporation. However, in cells cultured in the absence of FCS, LPA (0.1-10 μM) caused an increase of the [^{3}H]-thymidine incorporation by 20.5 ± 3.5 % (n=12, $p < 0.001$, paired t-test), whereas S1P (0.1-10 μM) had no significant effect.

In A549 cells cultured in the absence of FCS, LPA 10 μM caused a rapid (10 min) activation of the MAP kinase pathway as detected by an increase in phosphorylated p44/42 using a "phospho-specific" antibody.

In conclusion, in A549 cells LPA, but not S1P, can partially substitute the growth stimulating activity of FCS. This correlates with the observation that A549 cells express mRNA of EDG receptors which are known to prefer LPA as agonist.

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282P U46619 INDUCED CONTRACTIONS: ROLE OF PROSTANOIDS AND NITRIC OXIDE IN HUMAN PULMONARY VESSELS

X. Norel, L. Walch & C. Brink CNRS ESA 8078, Centre Chirurgical Marie Lannelongue, 133 av. de la Résistance, 92350 le Plessis-Robinson, France.

Prostaglandins and thromboxanes are known to regulate human pulmonary vascular smooth muscle tone. In human pulmonary arteries (HPA), the prostanoid receptors involved in the vasoconstriction have been identified as: TP- (Lumley *et al.*, 1989; Norel *et al.*, 1991) and EP_3- (Qian *et al.*, 1994) receptors. In human pulmonary venous preparations (HPV) the activation of TP- and EP_1- receptors induced contraction (Walch *et al*, 2001; Norel *et al*, 2000). While different prostanoid receptors have been described, no information is available concerning the local regulation of these vasoconstrictions.

The aim of this study was to determine the role of the cyclooxygenase and nitric oxide (NO) synthase activities during the vasoconstriction induced by the thromboxane analogue (U46619).

HPA and HPV were cut as rings and set up in organ baths with Tyrode's solution. The endothelium in some pulmonary vascular rings was mechanically removed. Changes in force were recorded using isometric transducers and physiographs. Cumulative concentration response curves (0.1nM – 10μM) were performed with U46619 (TP-agonist). These curves were produced in presence or absence of indomethacin (1.7μM) and L-NOARG (1mM).

The data (Table 1) show that HPV and not HPA were significantly more sensitive to U46619 in presence of indomethacin and L-NOARG. The same increase in sensitivity was observed in HPV in absence of endothelium. Maximal contractions induced induced by U46619 were unchanged subsequent to these treatments.

These data indirectly suggest the release of prostanoids (relaxant) and/or NO in HPV but not in HPA during TP-receptor activation.

Table 1

	pEC_{50} values	
Treatment	HPA	HPV
Control	7.96±0.18 (9)	7.90±0.11 (14)
Indomethacin + L-NOARG	8.15±0.19 (7)	8.61±0.10* (18)
Absence of endothelium	NP	8.31±0.16* (7)

Results are means±s.e.mean derived from n lung samples indicated in parenthesis. NP not performed. * indicates $P<0.05$ versus appropriate control (Student's *t* test).

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283P MECHANISM OF HUMAN UROTENSIN-II-INDUCED VASODILATION IN HUMAN SMALL PULMONARY ARTERIES

M.R. MacLean, S.A. Douglas[1] & A. Stirrat. Division of Neuroscience, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ. [1]GlaxoSmithKline, King of Prussia, USA.

We have recently shown that the novel peptide human urotensin-II (hUT-II) is a potent vasodilator of human isolated pulmonary and abdominal resistance arteries (Stirrat *et al.*, 2001). Currently the mechanism of hUT-II-induced vasodilation in human vessels is unknown although other studies, on rat mesenteric and coronary arteries, suggest that hUT-II-mediated vasodilation is endothelium-dependent although not entirely nitric oxide-mediated (Bottrill *et al.*, 2000; Katano *et al.*, 2000). Here we investigated the mechanism of vasodilation to hUT-II in human small muscular pulmonary arteries.

Human small pulmonary arteries (150-190μm i.d.) were dissected from lung tissue removed during bronchial carcinoma excision. Vessels were mounted on wire myographs and tension applied to give a transmural pressure equivalent to 12-16mmHg. Vessels were bathed in Krebs at 37°C with a constant supply of $16\%O_2/5\%CO_2$ (balance N_2). Responses to 50mM KCl were determined, vessels then preconstricted with 0.1μM 5-HT and 1μM acetylcholine (ACh) added to test for endothelial integrity. Any vessels not vasodilating to ACh were excluded. Vessels were then preconstricted with either 3nM ET-1 or 50mM KCl. Cumulative concentration-response curves were constructed to hUT-II or porcine UT-II (pUT-II) in the presence or absence of the nitric oxide synthase inhibitor L-NAME (100μM), indomethacin (1μM) or 100nM of both apamin and charybdotoxin, inhibitors of endothelium-derived hyperpolarising factor (EDHF)-induced vasodilation. From each lung, adjacent vessels were set up and preconstricted but no vasodilator added to serve as time controls. Results are summarised in Figure 1. Figure 1 shows that L-NAME had no effect, indomethacin partially reduced, whilst apamin plus charybdotoxin completely abolished, hUT-II-induced vasodilation. pUT-II induced a vasodilation equivalent to that of hUT-II.

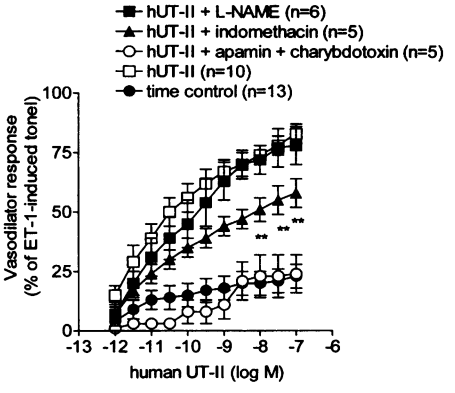

Figure 1. Effect of inhibitors on hUT-II-induced vasodilation in human pulmonary arteries. $^{**}P<0.01$ cf. hUT-II. ANOVA followed by Tukeys multi-comparison test.

No hUT-II-induced vasodilation was observed when vessels were pre-constricted with KCl (n=3) which would abolish EDHF-induced responses. Degrees of pre-constriction and ACh-induced dilations were comparable in all groups. pIC_{50} values were: hUT-II control: 10.8±0.3 (n=10); pUT-II: 9.9±0.6 (n=4); hUT-II plus L-NAME: 10.5±0.4 (n=6); hUT-II plus indomethacin: 10.7±0.5 (n=5).

The results show, for the first time in human vessels, that hUT-II-induced vasodilation is nitric oxide independent and may be mediated by an EDHF. Vasodilator prostanoids may also play a role.

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284P TWIN-PORE DOMAIN POTASSIUM CHANNELS IN RAT PULMONARY ARTERY - POTENTIAL CANDIDATES OF HYPOXIC PULMONARY VASOCONSTRICTION

I.T. Johnson, M.J. Gardener, G. Richards, M. Burnham, C.D. Glen, G. Edwards & A.H. Weston. School of Biological Sciences, University of Manchester, G38 Stopford Building, Oxford Road, Manchester, M13 9PT.

In contrast to the systemic circulation, pulmonary arteries constrict in the presence of reduced oxygen tension. Several lines of evidence implicate the inhibition of voltage-dependent potassium currents, with subsequent membrane depolarisation, as the initiator of hypoxic pulmonary vasoconstriction (HPV) (Patel and Honoré, 2001). However, many of the candidate channels would be inactive at resting membrane potentials. The recent discovery of a group of K^+ channels thought to be major contributors to the resting membrane potential, the twin-pore domain K^+ channels (2P-domain K^+ channels), may be more likely candidates as initiators of HPV (*see* Lesage and Lazdunski, 2000; Goldstein et al., 2001). Currently the molecular identities of seventeen such channels, eleven of which appear functional, are available. Reports of these channels in the pulmonary circulation are limited to TWIK-2 (Patel *et al.*, 2000). The presence of a number of these channels within the pulmonary circulation is reported here for the first time.

Male Sprague-Dawley rats (200-225g) were killed by stunning and cervical dislocation. *RT-PCR*: - Rat main pulmonary arteries were dissected on ice and homogenised. Total RNA was isolated using RNeasy mini kit (Qiagen) and samples were reverse transcribed subsequent to DNase treatment. Gene-specific PCR was performed using cDNA from each sample with primers designed to amplify the various 2P-domain K^+-channel sequences.

Immunohistochemistry: - Rat main and intrapulmonary arteries were fixed in a periodate-lysine-paraformaldehyde solution and embedded in OCT®. Sections (5μm) were stained with anti-2P-domain K^+-channel antibodies (Alomone) and visualised using Texas Red-conjugated secondary antibodies, while nuclei were labelled blue with DAPI.

Gene products matching the sequences of TASK-1, TASK-2, TREK-2, TWIK-2 and THIK-1 were present in pulmonary artery samples. In contrast, products matching the sequences of TASK-3, TREK-1, TWIK-1 and TRAAK were absent in pulmonary artery samples, despite the fact that these primers amplified products in the relevant control (brain or kidney) rat cDNA samples.

Immunohistochemical staining indicated, in confirmation with the PCR data, the presence of TASK-1 and TASK-2, while TREK-1 was absent. Both TASK-1 and TASK-2 were evident at low levels in smooth muscle and endothelium.

Both *PCR* and *immunohistochemistry* confirmed the differential expression of 2P-domain K^+ channels in the rat pulmonary artery. The data presented suggests that of the nine channels so far studied, five 2P-domain K^+ channels can be considered as candidates for further investigation into their possible role in HPV.

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285P CP55,940, A CANNABINOID RECEPTOR AGONIST, ATTENUATES SENSORY NEUROTRANSMISSION VIA CB_1-LIKE RECEPTORS IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

M. Duncan, D.A. Kendall & V. Ralevic. School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH.

Capsaicin-sensitive sensory nerves are widely distributed in the cardiovascular system (Maggi & Meli, 1988). In the mesenteric arterial bed calcitonin gene-related peptide (CGRP) is released upon activation of sensory nerves producing vasodilatation (Kawasaki *et al.*, 1988). Our preliminary data indicate that a cannabinoid receptor can modulate the efferent function of sensory nerves as the synthetic cannabinoids WIN55,212 and HU210 inhibit neurogenic vasorelaxation to electric field stimulation (EFS) in the rat isolated mesenteric arterial bed (Duncan *et al.*, 2001). The aim of the present study was to further characterise the receptor(s) involved using CP55,940, an agonist with high affinity and selectivity for both CB_1 and CB_2 receptors.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused with oxygenated Krebs' solution containing guanethidine (5μM) to block sympathetic neurotransmission (Ralevic & Kendall, 2001). After 30 min equilibration, preparations were preconstricted with methoxamine (10-100μM) and three consecutive frequency response curves to EFS (1-12Hz, 60V, 0.1ms, 30s) (EFS control, EFSI and EFSII) were constructed in each preparation. CP55,940 or vehicle (ethanol; 0.01%) was added after EFS control, 15 min before EFSI. LY320135 was added at the start of the equilibration period. In separate preparations, a dose response curve was constructed to CGRP (0.05 pmol – 0.5 nmol) in the presence of CP55,940 (0.1μM) and ethanol (0.01%). Data are expressed as mean±s.e.m. and analysed by ANOVA with Tukey's post hoc test or by Students unpaired t test.

EFS produced frequency-dependent relaxation (1-12Hz) of the rat mesenteric bed. CP55,940 (0.01, 0.1 and 1μM) attenuated sensory neurogenic relaxation evoked during EFSI and EFSII compared with EFS control in a concentration-dependent manner. In the presence of 1μM CP55,940 the response at a submaximal frequency of 8Hz was reduced from 41.26±4.29%, EFS control, to 19.24±3.08%, EFSII ($n=7$, $P<0.01$).

The selective CB_1 receptor antagonist LY320135 (1μM) partially blocked inhibition of the relaxation response by CP55,940; at 8Hz EFS control, 40.94±6.89% and EFSII, 27.07±5.71% ($n=6$). The CB_2 receptor antagonist SR144528 had no effect on CP55,940-mediated inhibition; at 8Hz EFS control was 53.93±7.8% and EFSII, 23.78±5.81% ($n=6$). There was no significant difference between EFS control, EFSI and EFSII generated in the presence of 0.01% ethanol, the vehicle for CP55,940 and LY320135. CP55,940 failed to affect the maximal relaxation to CGRP, but produced a small increase of the potency (pEC_{50} =11.2±0.1 and 10.5±0.1 in the presence and absence respectively of 0.1μM CP55,940; $P<0.05$, unpaired t test).

These data show that CP55,940 attenuates sensory neurogenic relaxation in the rat isolated arterial mesenteric bed. The ability of the CB_1 but not the CB_2 receptor antagonist to block these actions suggest the involvement of a CB_1 or CB_1-like receptor. CP55,940 was found to have no inhibitory actions on vasorelaxation produced by exogenous CGRP indicating that the likely site of action is prejunctional.

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286P ACTIVATION OF P2X RECEPTORS BY α,β-METHYLENE ATP EVOKES A BIPHASIC RESPONSE (CONTRACTION AND PROLONGED RELAXATION) IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

V. Ralevic, School of Biomedical Sciences, Medical School, Queen's Medical Centre, Nottingham NG7 2UH.

ATP and adenine and guanine dinucleotides evoke a multiphasic response including contraction followed by prolonged relaxation in the rat isolated mesenteric arterial bed (Ralevic, 2001; Ralevic *et al.*, 2001; Stanford *et al.*, 2001) and mesenteric resistance arteries (Juul *et al.*, 1993). The contraction is mediated principally by activation of vascular smooth muscle $P2X_1$-like receptors (Ralevic, 2001; Ralevic *et al.*, 2001). The mechanism of prolonged relaxation, however, is unclear, although it can be modulated by a variety of pharmacological agents (Ralevic, 2001; Stanford *et al.*, 2001). The present study used α,β-methylene ATP (α,β-meATP), a selective P2X receptor agonist and desensitizing agent, to investigate the possible role of P2X receptors in mediating prolonged relaxation to purines in the mesenteric arterial bed.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric arterial beds were isolated and perfused via the superior mesenteric artery with oxygenated Krebs' solution (37°C) at 5 ml min^{-1} (Ralevic, 2001). After 30 min equilibration, preparations were preconstricted (to about 40-80 mmHg) with methoxamine (4-50μM) and responses to bolus doses (50μl injections) of α,β-meATP (50 nmol) were investigated. The endothelium was removed by 10 min perfusion with distilled water (checked with acetylcholine; Ralevic *et al.*, 2001). Data reported are means ± s.e.m. and were compared by Student's paired t test. The time for the relaxation response to recover by half of its amplitude is denoted by $t_{1/2}$.

In preparations precontracted with methoxamine, injection of a dose of α,β-meATP elicited contraction (68±6 mmHg), followed by prolonged relaxation (82±3%; $t_{1/2}$ 14.6±1.5 min) (n=6). The response was reproducible: contraction 67±5 mmHg; relaxation 85±4%, $t_{1/2}$ 14.7 ± 1.9 min (n=6). The biphasic response was mimicked by bolus injection of KCl (50 μmol); contraction 58±12 mmHg; vasorelaxation 70±6%; $t_{1/2}$ 7.7 ± 1.9 min (n=4). Perfusion with α,β-meATP (10 μM; 15min) (which also elicited a biphasic response) in order to cause desensitization of P2X receptors, abolished both contraction and prolonged relaxation to bolus injection of α,β-meATP (50 nmol), revealing a rapid relaxation to α,β-meATP (32±7%; $t_{1/2}$ 32±2 s), which was abolished by endothelium removal (n=6).

Using a P2X selective agonist, α,β-meATP, the present study has shown that activation of P2X receptors evokes vasoconstriction followed by prolonged vasorelaxation in the methoxamine-preconstricted rat isolated mesenteric arterial bed. Moreover, desensitization of P2X receptors abolishes the biphasic response, as described previously for ATP (Ralevic, 2001). After P2X receptor desensitization, a rapid endothelium-dependent vaso-relaxation to α,β-meATP is revealed. These data indicate that activation of smooth muscle P2X receptors is involved in prolonged relaxation mediated by purine nucleotides in the rat isolated mesenteric arterial bed.

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287P EFFECTS OF SODIUM DEOXYCHOLATE AND DISTILLED WATER TREATMENT ON THE BIPHASIC RESPONSE TO α,β-METHYLENE ATP IN THE RAT ISOLATED MESENTERIC ARTERIAL BED

V. Ralevic & M.D. Randall, School of Biomedical Sciences, Medical School, Queen's Medical Centre, Nottingham NG7 2UH.

Distilled water and sodium deoxycholate have been widely used in order to remove the endothelium in pharmacological assays. Using the two different methods, however, opposite conclusions were recently drawn about the endothelial dependence of prolonged vasorelaxation to ATP in the rat isolated mesenteric arterial bed; distilled water had no effect on prolonged relaxation (Ralevic, 2001), whilst sodium deoxycholate treatment abolished it (Stanford *et al.*, 2001). The present study investigated the effects of the two treatments on the biphasic response to α,β-methylene ATP (α,β-meATP) (contraction followed by prolonged relaxation), which mimics the biphasic response evoked by ATP (Ralevic, 2001a,b).

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric arterial beds were isolated and perfused via the superior mesenteric artery with oxygenated Krebs' solution (37°C) at 5 ml min^{-1} (Ralevic, 2001). After 30 min equilibration, preparations were preconstricted (to about 40-80 mmHg) with methoxamine (2-50μM) and responses to bolus doses of α,β-meATP (5 pmol-0.5 μmol), KCl (5-200 μmol) and sodium nitroprusside (SNP; 0.5 pmol–50 nmol) were investigated. During equilibration the endothelium was removed by either 10 min perfusion with distilled water (Ralevic, 2001a) or injection with 4 ml of sodium deoxycholate (2 mg ml^{-1} in distilled water). Endothelial integrity was assessed with acetylcholine (ACh, 50 nmol; this dose elicits about 80% relaxation in endothelium-intact mesenteric arterial preparations). Data reported are means ± s.e.m. and were compared by ANOVA with Tukey's post hoc test.

Doses of α,β-meATP elicited a biphasic response of contraction followed by prolonged relaxation. Contractile and relaxant dose-response curves were constructed (R_{max} contraction = 76±9 mmHg; R_{max} relaxation = 78±11%; n=4). The biphasic response was mimicked by KCl (R_{max} contraction = 73±8 mmHg; R_{max} relaxation = 70±6%; n=4). Sodium deoxycholate treatment blocked contractile and relaxant responses to both α,β-meATP (R_{max} contraction = 12±5 mmHg; R_{max} relaxation = 43±12%; n=4; $P<0.05$) and KCl (R_{max} contraction = 3±2 mmHg; R_{max} relaxation = 12±12%; $P<0.01$; n=4), and the relaxant response to 50 nmol ACh (17±9%; n=4). Treatment with distilled water had no significant effect on the biphasic response to α,β-meATP (R_{max} contraction = 70±4 mmHg; R_{max} relaxation = 84±3%) and KCl (R_{max} contraction = 48±10 mmHg; R_{max} relaxation = 60±4%), but the response to ACh was reduced, to 28±5% (n=5).

The present study has shown that sodium deoxycholate and distilled water have different effects on the biphasic response to α,β-meATP. Distilled water treatment had no significant effect on the biphasic response to α,β-meATP, although endothelium-dependent relaxation to ACh was blocked. In contrast, sodium deoxycholate treatment blocked both components of the biphasic response to α,β-meATP, as well as the biphasic response to KCl, indicating likely damage to the vascular smooth muscle. These data indicate that the prolonged relaxation response to α,β-meATP, and likely to ATP, is endothelium-independent. Caution should be applied when using sodium deoxycholate to remove the endothelium because of possible damage caused by the detergent to receptors and/or the vascular smooth muscle.

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288P CAPSAICIN-SENSITIVE PERIVASCULAR NEUROTRANSMISSION IN ISOLATED PERFUSED MESENTERIC ARTERIAL BEDS FROM ENDOTOXAEMIC RATS

M.R. Farmer, V. Ralevic & S.M. Gardiner, School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH

Capsaicin-sensitive sensory nerves are known play an important afferent role in response to noxious stimuli (for review see Maggi & Melli, 1988). Upon stimulation, they release calcitonin gene-related peptide (CGRP), which is a potent vasodilator in the rat mesenteric arterial bed (Kawasaki *et al.*, 1988). Bolus injection of lipopolysaccharide (LPS) elicits raised plasma levels of CGRP in the rat (Wang *et al.*, 1992), and it has been suggested that CGRP may play a role in LPS-induced vasodilation (Hüttemeier *et al.*, 1993). The aim of the present study was to examine some aspects of capsaicin-sensitive perivascular nerve function following chronic infusion of LPS (Waller *et al.*, 1994). Rats were infused with LPS for 2h or 24h and measurements of vasorelaxations to electrical field stimulation (EFS) of capsaicin-sensitive perivascular nerves and bolus doses of CGRP were made in isolated mesenteric arterial beds.

Under anaesthesia (fentanyl and meditomidine; 300μg kg^{-1} of each i.p.) male Sprague-Dawley rats (300-419g) had i.v. catheters implanted, 24h before i.v. infusion, into the conscious animal, of saline (0.4ml h^{-1}, Sal) or LPS (150μg kg^{-1} h^{-1}; *E. Coli* serotype 0127:B8) (Waller *et al.*, 1994). After either 2h or 24h infusion animals were anaesthetised with sodium pentobarbitone (<200mg kg^{-1} i.v.) and killed by decapitation. Mesenteries were removed and prepared as described previously (Ralevic & Burnstock, 1988). The preparations were perfused with Krebs' solution, to which was added guanethidine (5μM) to block sympathetic neurotransmission and methoxamine (4-40μM) to raise perfusion pressure by 50-70 mmHg. Frequency-response relationships to EFS (1–12 Hz, 30 s, 0.1 ms, 60V) and dose-response relationships to CGRP were determined. Relaxations were calculated as the percentage change of methoxamine-induced tone, and compared by ANOVA, with Tukey's multiple comparison post-hoc test. Data are presented as means ± s.e.mean. There was frequency-dependent relaxation to EFS in all groups ($P<0.0001$), and no significant differences between them. Maximal relaxations, at 12 Hz, were 47.8±7.0%, 26.5±4.1%, 39.4±8.6% and 31.2±3.7%, for 2h Sal (n = 5), 24h Sal (n = 6), 2h LPS (n = 10) and 24h LPS (n = 7) respectively.

All four groups demonstrated dose-dependent relaxation to CGRP ($P<0.0001$). The maximum relaxations were 54.1±16.6%, 73.1±18.0%, 66.5±19.2% and 93.2±3.9% for 2h Sal, 24h Sal, 2h LPS and 24h LPS respectively (n = 5, 5, 6 and 8 respectively). There were no significant differences between the LPS groups and their respective time-control (Sal) groups.

One interpretation of these findings is that capsaicin-sensitive perivascular nerves are not activated in this endotoxaemic model, consistent with results obtained *in vivo* where a clear vasodilator role for CGRP could not be demonstrated (Gardiner *et al.*, 1999). Alternatively, it is possible that chronic activation of capsaicin-sensitive perivascular nerves does not result in impaired function assessed *in vitro*.

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289P CONTRACTILE RESPONSES TO METHOXAMINE OF AORTAE AND TAIL ARTERIES FROM ENDOTOXAEMIC RATS

M.R. Farmer, S.M. Gardiner & V. Ralevic, School of Biomedical Sciences, Medical School, Queen's Medical Centre, Nottingham NG7 2UH.

After continuous infusion of lipopolysaccharide (LPS) in conscious rats there are complex temporal and regional changes in haemodynamic responses to different vasoconstrictors *in vivo* (Waller *et al.*, 1994). Previously, impaired vasoconstriction to methoxamine (ME) in perfused mesenteric arterial beds isolated from rats following 24h, but not 2h, LPS infusion has been reported (Farmer *et al.*, 2001). In the present study contractile responsiveness to ME of both resistance (tail arteries) and conduit (aorta) vessels isolated 2h and 24h following the start of LPS infusion in rats was investigated.

Under anaesthesia (fentanyl and meditomine; 300 μg kg^{-1} of each i.p.) male Sprague-Dawley rats (300-419g) had i.v. catheters implanted, 24 h before i.v. infusion of saline (0.4 ml h^{-1}, Sal) or LPS (150 μg kg^{-1} h^{-1}; *E. Coli* serotype 0127:B8) (Waller *et al.*, 1994). After either 2h or 24h infusion animals were anaesthetised with sodium pentobarbitone (<200 mg kg^{-1} i.v.) and killed by decapitation. Thoracic aortae were removed and mounted in organ baths with 1 g resting tension applied. Following 1h of equilibration cumulative concentrations of ME (100 nM-1 mM) were applied. Tail arteries were removed and cannulated for perfusion with Krebs' solution at 2 ml min^{-1}. After 30 min equilibration contractile dose-response curves to α,β-methylene ATP (α,β-meATP, 50 pmol-0.5 μmol; P2X receptor agonist) and cumulative concentration-response curves to ME (0.1-100 μM) and KCl (10-300 mM) were constructed. Responses were measured as increase in tone above baseline (tail) or increase in tension (aortae), and analysed by ANOVA, with Tukey's post-hoc test. Data are presented as means ± s.e.mean of 4-7 experiments in each group.

In tail arteries, concentration-response curves to ME did not reach a maximum, but there was no significant difference in the maximal response attained between the groups, at 1 mM ME (207±12mmHg, 176±18mmHg, 147±19mmHg and 187±25mmHg after 2h Sal, 24h Sal, 2h LPS and 24h LPS, respectively). The maximal attained response to α,β-meATP (at 50 nmol) was greater after 24h LPS infusion (97±7 mmHg) than in the 2h Sal, 24h Sal and 2h LPS groups (52±3 mmHg, 41±3 mmHg, 49±8 mmHg, respectively) ($P<0.001$). The maximal attained response to KCl (at 0.1 M) was also greater 24h after LPS infusion (133±7 mmHg) compared to responses after 2 h Sal, 24h Sal and 2h LPS (77±2 mmHg; 55±4 mmHg; 67±12 mmHg, respectively) ($P<0.001$).

In aortae, the maximum response to ME was reduced 2 h and 24 h after LPS (0.44±0.05 g and 0.49±0.10 g, respectively) compared to the responses in the corresponding saline groups (0.94±0.09 g and 0.88±0.10 g) ($P<0.001$) but the EC_{50} values were not different (1.21±0.21μM, 5.35±3.43μM, 5.73±1.63μM and 2.40±0.65μM, respectively).

The present study has demonstrated that there is no change in the maximal achieved contractile response to ME in the tail artery isolated from endotoxaemic rats. This is in contrast with the hypocontractility to ME in the isolated aorta and, as reported previously, mesenteric arterial bed in this model, indicating that there is heterogeneity in changes occurring in different blood vessels in endotoxaemia. Moreover, increased responses to α,β-meATP and KCl indicates that endotoxaemia induces different changes in responses to different vasoconstrictors in the tail artery.

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290P EFFECTS OF ORGAN CULTURE ON THE CONTRACTILITY OF RAT MESENTERIC ARTERIES

L.S. Harrington, A.D Hughes & J.S. Lymn. Clinical Pharmacology, NHLI, Imperial College, London, W2 1NY

This study examined the effect of media composition in organ culture on the contractile responses to various agonists in rat resistance arteries.

Mesenteric small arteries (internal diameter ~250μm) from male Wistar rats (~316g ±20) were mounted on steel wires and cultured in serum-free culture medium (NCTC), or medium containing 10% foetal calf serum (FCS) or 10% dialysed foetal calf serum (dS) for up to 4 days. After culture arteries were mounted at a normalised resting tension in a wire myograph (Mulvany & Halpern, 1977) containing modified Krebs buffer at 37°C and bubbled with 95% O_2, 5% CO_2. Isometric contraction was assessed in response to high potassium solution (K), noradrenaline (NA; 10μM), 5-hydroxytryptamine (5HT; 10μM) and NA + K. Relaxation to the endothelium-dependent agents, acetylcholine (ACH; 10μM) and histamine (H; 10μM) was measured following contraction with NA. Contractile responses were calculated as effective active pressure (EAP; kPa) based on Laplace's law, relaxation was calculated as % reduction of contracted tone. Data were analysed by MANOVA and Dunnett's post-hoc tests; $p<0.05$ was considered significant.

Contraction in response to all stimuli reduced progressively with time in culture (Table 1). Contraction to NA and 5HT was also significantly further reduced by 10% FCS.

Relaxation to ACH and H was also impaired following culture (Table 1). Culture in NCTC caused significantly less impairment of H-induced relaxation than when serum was present.

Table1. Effect of organ culture on responses to agonists.

	Day 0	Day 5		
		NCTC	dS	FCS
K	21±1 (9)	9±2 (11)*	10±2 (5)*	4±2 (8)*
NA	18±2 (9)	10±2 (11)*	10±2 (5)*	2±1 (8)*†
5HT	17±2 (9)	11±2 (11)*	11±2 (5)*	3±2 (8)*†
NA+K	25±2 (9)	13±2 (11)*	12±2 (5)*	6±2 (8)*
ACH	44±6 (9)	11±5 (11)*	0 (5)*	0 (8)*
H	31±6 (9)	21±8 (11)*†	0 (5)*	0 (8)*

Data are EAP (kPa) or relaxation (%). * = $p<0.05$ compared with Day 0, † = $p < 0.05$ compared with other culture media.

Organ culture reduced contractile responses to a range of agonists. It is possible that this is a consequence of smooth muscle cell de-differentiation. Inclusion of undialysed FCS in the culture medium has an additional inhibitory effect on responses to NA and 5HT. Relaxation in response to ACH and H also decline following organ culture, whether this relates to endothelial cell loss or functional changes in the endothelium remains to be established.

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291P CONTRACTILE EFFECTS ON EQUINE DIGITAL ARTERIES AND VEINS OF MONOAMINES FORMED IN THE CAECUM

Y. Berhane, S.R. Bailey & J. Elliott, Dept of Veterinary Basic Sciences, Royal Veterinary College, London NW1 0TU

Equine laminitis is a painful ischaemia and reperfusion injury of the foot. Although the vasoactive mediator(s) triggering this disease still remain unknown, increased post capillary resistance as a result of digital venocontriction is thought to be a primary event (Allen *et al*, 1990). Previous work in our laboratory has demonstrated 5-hydroxytryptamine (5-HT) to be a potent vasoconstrictor of equine digital vessels (Bailey and Elliott, 1998). Amines such as tyramine (TYR), tryptamine (TYP) and phenylethylamine (PEA) are formed in the equine caecum (Bailey *et al*, 2000), and may mimic the effects endogenous amines if absorbed into the circulation. The aim of the present study was to examine the effects of these amines on isolated equine digital arteries and veins.

Rings (2 to 4 mm in length) of equine digital arteries (EDA) and veins (EDV) were obtained from adult mixed breed horses (n=4 to 6) killed at an abattoir. They were prepared for isometric tension recording in organ baths containing Krebs-Hensleit solution. Cumulative concentration response curves (CRCs) were constructed to TYR, TYP and PEA and the responses obtained in EDA and EDVs compared using a paired Student's *t*-test. Further experiments were undertaken to study the mode of action of TYP. Vessels were pretreated with the irreversible α-adrenoceptor antagonist, benextramine (0.1 mM for 30 min) and CRC to TYP were obtained in the presence and absence of ketanserin (0.1μM).

TYP was the most potent vasocontrictor of EDAs and EDVs and both TYP and TYR were more potent constrictors of EDV than EDA. Benextramine had no significant effect on the TYP CRC. However, benextramine-treated vessels examined in the presence of ketanserin, TYP gave a biphasic CRC with the 1st phase pEC_{50} 4.86 (4.64 – 5.07) and 6.33 (5.47 – 7.19) for EDAs and EDVs, respectively, and the 2nd phase pEC_{50} 3.42 (3.16 – 3.68) and 3.93 (3.63 – 4.23) for EDAs and EDVs, respectively.

These data show that monoamines derived from the caecum are more potent vasoconstrictors of EDVs. TYP's effects were mediated by both 5-HT_2 and 5-$HT_{1B/D}$ receptors (Bailey & Elliott 1998) and appeared to be selective for the venous side of the circulation. As such, it should be considered as a potential trigger factor for laminitis.

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Table 1. Contractile effect of amines on equine digital arteries and veins.

*P<0.05, **P<0.005 vs. EDA compared by paired *t*-test (n=6)

Amine	pEC_{50} (Geometric mean (95%CI))		Maximum response (% DKS)		Hill Slope	
	EDA	EDV	EDA	EDV	EDA	EDV
TYR	3.92 (3.81 - 4.02)	4.23 (4.0 - 4.45)*	92.4 ± 11.5	110.7 ± 11.0	0.9 ± 0.06	1.3 ± 0.3
TYP	4.42 (3.40 - 5.44)	5.97 (5.64 - 6.29)**	194.2 ± 12.3	197.2 ± 12.9	0.6 ± 0.04	0.9 ± 0.1
PEA	3.88 (3.78 - 3.97)	4.05 (3.81 - 4.28)	100.4 ± 3.5	166.6 ± 14.9**	1.5 ± 0.22	1.2 ± 0.1

292P SIGNAL TRANSDUCTION UNDERLYING α_1-ADRENOCEPTOR-MEDIATED VASOCONSTRICTION IN RAT MESENTERIC MICROVESSELS

V. Steenpaß, C. Altmann, C. Fetscher, P. Czyborra & M.C. Michel, Depts. of Medicine and Pharmacology, Univ. Essen, 45122 Essen, Germany

We have recently demonstrated that noradrenaline contracts rat mesenteric microvessels via α_1-adrenoceptors and that this involves influx of extracellular Ca^{2+} and possibly an activation of tyrosine kinases and of mitogen-activated protein kinases of the ERK family (Fetscher et al., 2001). Using identical methods, we have now investigated the possible involvement of additional signalling pathways.

Cumulative concentration-response curves for noradrenaline were generated in mesenteric microvessels from Wistar rats of either gender (males 300-450 g, females 200-350 g) before and after addition of the indicated inhibitors, their negative controls or their vehicles. Force of contraction after their addition was expressed as % of maximum noradrenaline responses within the same vessel before their addition (20.3±0.3 mN, n=219). Data are means ± SEM of n experiments. Statistical significance of differences between groups was assessed by unpaired t-tests or ANOVA followed by Dunnett's multiple comparison tests with a $p < 0.05$ considered as significant.

Relative to its vehicle, the phospholipase C inhibitor U 73,122 (1-(6-[([17ß]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-1H-pyrrole-2,5-dione, 10 µM) reduced maximum noradrenaline responses from 90±3% to 30±6 % of control with pEC_{50} being lowered from 6.69±0.05 to 6.05±0.25 (n=8, $p<0.05$). In contrast, its negative control U 73,344 (1-(6-[-([17ß]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-2,5-pyrrolidinedione, 10 µM) did not significantly affect maximum responses and lowered pEC_{50} from 6.78±0.07 to 6.20±0.06 (n=8, $p<0.05$). SK&F 96,365 (1-[ß-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H- imidazole HCl, 10 µM), an inhibitor of receptor-operated Ca^{2+} channels relative to its vehicle, or butan-1-ol, an inhibitor of phospholipase D, relative to its negative control butan-2-ol (0.3% each) had only little if any effects on noradrenaline-induced contraction (n=6 each). Experiments on a possible role of PI-3-kinase remained inconclusive: While neither wortmannin, nor LY 294,002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, 10 µM) or its negative control LY 303,511 (2-peperazinyl-8-phenyl-4H-1-benzopyran-4-one) significantly affected maximum noradrenaline responses relative to vehicle (10 µM and n = 5-7 each), its pEC_{50} was markedly lowered by LY 294,002, somewhat less by LY 303,511 and not at all by wortmannin (from 7.11±0.04 to 5.25±0.07, 6.21±0.04 and 6.88±0.09, respectively; $p<0.05$ vs. vehicle for the LY compounds). Y 27,632 (trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide, 10 µM), an inhibitor of rho-associated protein kinase, did not affect maximum noradrenaline responses but lowered pEC_{50} from 7.19 ±0.05 to 6.26±0.03 (n= 6-8, $p<0.05$).

We conclude that noradrenaline-induced contraction of rat mesenteric microvessels may involve activation of a phospholipase C and rho-associated protein kinase, whereas receptor-operated Ca^{2+} channels and phospholipase D are not involved; a possible role of PI-3-kinase cannot conclusively be determined from these experiments.

Fetscher, C. et al. (2001) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 363: 57-65

293P COMPARISON OF HISTAMINE- AND ACETYLCHOLINE-INDUCED RELAXATION OF RAT ISOLATED MESENTERIC SMALL ARTERIES

S. Fexby, R. Wimalasundera & A.D. Hughes. Clinical Pharmacology, NHLI, Imperial College, London W2 1NY.

Endothelium releases many vasodilators including NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF). This study compared the contributions of NO and EDHF to relaxation induced by acetylcholine (ACH) and histamine (H) in rat mesenteric small arteries.

Second order mesenteric small arteries (length ~2mm, internal diameter ~350µm) were isolated from male Wistar Kyoto rats (~200g) and mounted in a wire myograph (Mulvany & Halpern, 1977) containing modified Krebs buffer (PSS) at 37°C and bubbled with 95% O_2, 5% CO_2. Vessels were mounted at a normalised tension on the basis of their passive length-tension characteristics and Laplace's relationship (Mulvany & Halpern, 1977), and allowed to equilibrate for 1hr prior to the experiment. Arteries were contracted with 10µM phenylephrine (PE) or PSS containing an equimolar substitution of NaCl with 80mM KCl (K_{80}) and cumulative concentration-response relationships constructed to ACH (10^{-9} – 10^{-5}M), H (10^{-9} – $3x10^{-4}$M), and sodium nitroprusside (SNP; 10^{-9} – 10^{-5}M) alone, or in the presence of L-NAME (10^{-5}M), L-NMMA (10^{-4}M) to inhibit NO synthase and indomethacin (IND; $3x10^{-6}$M) to inhibit cyclooxygenase. Relaxation was calculated as % reduction of induced tone and data were fitted to a logistic equation by non-linear regression to determine EC_{50}. Data were compared using a paired Student's t-test.

ACH, H and SNP caused concentration-dependent relaxation. Maximum relaxation (R_{max}) in response to ACH and H was reduced in arteries contracted by K_{80} (Table 1). Inhibition of NO synthase and cyclooxygenase significantly reduced responses to ACH and H in PE– and K_{80}–contracted arteries. R_{max} to H was almost abolished in K_{80}–contracted arteries in the presence of L-NAME, L-NMMA and IND.

Table 1. Log EC_{50} and R_{max} values for relaxants in PE and K_{80} contracted arteries. Data are means ± s. e. mean of (n) observations. * = $p<0.05$, ** = $p<0.01$ compared with control. † = $p<0.05$ compared with PE.

	Control		L-NAME/L-NMMA/IND	
PE	log EC_{50}	R_{max}	log EC_{50}	R_{max}
ACH	-7.0±0.1	96±3 (7)	-6.9±0.1	80±10 (7)*
H	-5.6±0.1	90±4 (7)	-5.3±0.1	53±10 (7)*
SNP	-7.7±0.5	89±11 (7)	-7.9±0.5	84±11 (7)
K_{80}				
ACH	-7.0±0.3	72±8 (6)†	-6.5±0.2	44±7 (6)** †
H	ND	28±4 (6)†	ND	9±1 (6)* †
SNP	-7.2±0.1	76±3 (6)	-7.2±0.1	82±5 (6)

These findings suggest that NO and EDHF make different contributions to ACH and H responses, with relaxation to H being more dependent on release of EDHF. Relaxation to ACH is not abolished by a combination of depolarization, L-NAME L-NMMA and IND. Whether this is due to residual NO or some other mediator remains to be established.

Mulvany, M. J. & Halpern W. (1977) Circ. Res., **41**, 19-26.

294P VASORELAXATION TO 17β-OESTRADIOL IN AORTIC RINGS FROM MALE AND FEMALE RATS

P. Tep-areenan, D.A. Kendall & M.D. Randall, School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH

The mechanisms of vasorelaxation to 17β-oestradiol are at present unclear. Collins *et al.* (1994) demonstrated, in coronary arteries from oestrogen-treated oophorectomized rabbits, that 17β-oestradiol induced vasorelaxation by stimulating nitric oxide (NO) release. However, Freay *et al.* (1997) showed, in aortic rings from male and female rats, that vasorelaxation to 17β-oestradiol was unaffected by a NO synthase inhibitor, but was inhibited by high extracellular K^+. The aim of this study was to investigate the roles of NO and K^+ channels in vasorelaxation to 17β-oestradiol in aortic rings from male and female rats.

Aged-matched male (250-350g) and female (180-240g) Wistar rats were anaesthetized with sodium pentobarbitone (60mg kg^{-1}, i.p.) and exsanguinated. The thoracic aortae were dissected from the rats and cut into 5mm lengths. Each ring was bathed with oxygenated Krebs-Henseleit solution. The aortic rings were stretched to optimal passive tension of about 1g. Following a 1-hour equilibration period, methoxamine (80-140μM) was added to increase tension by 0.50-1.00g. 17β-oestradiol was added cumulatively to the perfusion fluid (30pM-1mM). The vasorelaxant effects of 17β-oestradiol were assessed in the presence of 10μM indomethacin, and either 300μM N^G-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor or 100nM charybdotoxin (ChTx), an inhibitor of large conductance calcium-activated K^+ channels. To investigate the effects of high extracellular K^+ on responses to 17β-oestradiol, 60mM KCl was added to increase tension (McCulloch *et al.*, 1997). Maximal responses (R_{max}) are expressed as mean with s.e.mean and pEC_{50} values are expressed as mean with 95% confidence intervals (CI). Data were modelled using GraphPad Prism and compared by the Student's *t*-test for unpaired values or ANOVA.

In aortic rings from male rats, 17β-oestradiol (30pM-1mM) induced concentration-related relaxations and the data were best-fitted (r^2=0.999) to a 2-site model ($pEC_{50\text{-}1}$ = 8.87(8.17-9.57, 95%CI), $pEC_{50\text{-}2}$ = 4.99(4.75-5.23), R_{max} = 80.2±1.7%, n=15). In female rat aortae, 17β-oestradiol (30pM-1mM) caused concentration-dependent relaxations and the data were best-fitted (r^2=0.999) to a 1-site model (pEC_{50} = 4.55(4.41-4.69), R_{max} =80.4±1.7%, n=15), and pEC_{50} was significantly ($p<0.01$) less than the pEC_{50} at the low potency site in males. However, the maximal relaxation to 17β-oestradiol in aortic rings from female rats was not significantly different from that in males. In aortic rings from male rats, L-NAME (300μM) abolished the actions of 17β-oestradiol at the high potency site and the data were now best-fitted to a 1-site model. Vasorelaxation to 17β-oestradiol of aortic rings from females was not affected by the addition of L-NAME. In male rat aortae, neither 60mM KCl nor ChTx (100nM) had inhibitory effects on the potency and maximal relaxation to 17β-oestradiol, but there was significant ($p<0.05$) augmentation of maximal relaxation (control: R_{max} = 80.2±1.7%, n=15; KCl: R_{max} = 143±5%, n=4; ChTx: R_{max} = 97.0±11.8%, n=4).

This study has demonstrated that 17β-oestradiol causes acute and potent vasorelaxations in aortic rings from both male and female rats, with the potency of 17β-oestradiol being greater in males than in females. In aortic rings from male rats, the acute vasorelaxant effects of 17β-oestradiol are partly mediated via activation of NO release, but not via BK_{Ca} channels. In contrast, in female aortae, NO does not account for vasorelaxation induced by 17β-oestradiol.

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McCulloch, A.I., *et al.* (1997) *Br. J. Pharmacol.*, **120**, 1431-1438.

295P EVIDENCE THAT CENTRAL AT_2 AS WELL AS AT_1 RECEPTORS PLAY A ROLE IN THE CARDIOVASCULAR EFFECTS EVOKED BY ACTIVATION OF CENTRAL 5-HT_{2A} RECEPTORS WITH QUIPAZINE IN ANAESTHETIZED RATS

I.D. Knowles, D Centurión*, C.M. Villalón* & A.G. Ramage, Departments of Pharmacology, UCL. London NW3 2PF & *CINVESTAV-IPN, C.P. 14330, México D.F. México

Activation of central 5-HT_{2A} receptors with quipazine causes an initial rise in blood pressure due to vasopressin release and reflex renal sympathoinhibition (Knowles & Ramage, 1999). This release of vasopressin is caused by activation of a central angiotensinergic pathway as it is blocked by losartan (Saydoff *et al.*, 1996). Therefore the present experiments were carried out to determine the role of central AT_1, using losartan, and AT_2 receptors, using PD123 319 (see Dudley *et al.*, 1990) in the cardiovascular effects evoked by i.c.v. quipazine.

Experiments were carried out as previously described (Knowles & Ramage, 1999). Anaesthesia was induced in male Sprague-Dawley rats (250-375 g) with isoflurane and maintained with α-chloralose (80 mg kg^{-1}). Simultaneous recordings were made of blood pressure (BP), heart rate and renal (RNA) and phrenic nerve activities. Drugs were given i.c.v. in a volume of 5 μl over 15s. Changes were compared with vehicle control, by two-way ANOVA and the least significant difference test was used to compare the means. All values are means ± s.e.mean.

Quipazine (2 μmol kg^{-1}; n=5) in the presence of losartan (1 & 4 μg kg^{-1};n = 5) caused no change in BP (-2±2 & +3±3 mmHg) after 3 min, although there was a significant ($P < 0.05$) rise in RNA of 33±11 & 34±9. However, quipazine (n=8) alone caused an increase in BP of 15±4 mmHg and a decrease in RNA of -21±11% (Knowles & Ramage, 1999). Quipazine in the presence of PD123 319 (1 and 4 μg kg^{-1} n=5) evoked a significantly larger increase in BP (36±9 & 19±7 mmHg) and this rise in BP was not associated with inhibition but a large rise in RNA (128±15 & 63±15%). In the presence of both AT antagonists (4 μg kg^{-1}; n=5) quipazine, after 3 min, had no significant effect on BP (9±5 mmHg) or RNA (24±11%).

These data imply that both AT_1 and AT_2 receptors are involved in the cardiovascular effects evoked by activation of central 5-HT_2 receptors. Further, if the peripheral effects of vasopressin release are blocked with a V_1 receptor antagonist, then quipazine alone evokes a rise in BP (20 mmHg) associated with an increase in RNA (52%; Knowles & Ramage, 1999). As losartan blocks the release of vasopressin it would be expected that the action of losartan on the effects of quipazine would be similar to that in the presence of the V_1 receptor antagonist. However, the rise in BP was attenuated in the presence of losartan. This would suggest that the rise in BP caused by quipazine, in the presence of a V_1 receptor antagonist, is due to sympathoexcitation, which is also mediated by activation of central AT_1 receptors. As PD123 319 blocks the expected sympathoinhibition caused by quipazine alone this suggests that this sympathoinhibition involves the activation of central AT_2 receptors.

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296P EFFECTS OF THE NITRIC OXIDE SYNTHASE INHIBITOR, S-METHYL L-THIOCITRULLINE, (SMTC), ON THE REGIONAL HAEMODYNAMIC RESPONSES TO ACETLYCHOLINE IN CONSCIOUS SPRAGUE DAWLEY RATS

[1,2]I. D. Wakefield, [1]S.M. Gardiner, [2]J.-P. Valentin and [1]T. Bennett. [1]School of Biomedical Sciences, Queen's Medical Centre, Nottingham NG7 2UH and [2]AstraZeneca R&D UK, Macclesfield, Cheshire SK10 4TG

Bolus doses of the putative nNOS inhibitor, SMTC, or the non-selective NOS inhibitor, L-NAME, have qualitatively similar haemodynamic effects in conscious rats, consistent with both acting non-selectively (Wakefield *et al.*, 2002). Now we assessed responses to infusions SMTC or L-NAME on resting haemodynamics, and on the renal vasodilator effect of acetylcholine (ACh). Under anaesthesia (fentanyl and meditomidine, 300 μg kg^{-1} of each i.p., reversed with nalbuphine and atipamezole, 1 mg kg^{-1} of each s.c.), male, Sprague-Dawley rats (350-450g) were chronically implanted with renal (R), mesenteric (M), and hindquarters (H) Doppler flow probes, and, at least 14 days later, intravascular catheters for recording blood pressure (BP) and heart rate (HR), and for i.v. drug administrations. The following day, animals were infused with saline (vehicle) for 90 min (1 ml kg^{-1} h^{-1}), before infusion of ACh (10 μg kg^{-1} min^{-1} for 3 min). Two days later, animals received SMTC (n = 10) or L-NAME (n = 10) at 3 mg kg^{-1} h^{-1} for 90 min, before ACh. There were no effects of vehicle (data not shown). SMTC and L-NAME both caused rises in BP and falls in R, M and H vascular conductances (VC) (Table 1). The effects of L-NAME were greater (Kruskal-Wallis test) than those of SMTC, and the former also caused bradycardia. The renal vasodilatation caused by ACh was markedly attenuated by L-NAME, but not by SMTC (Table 2). The inhibitory effect of L-NAME on ACh-mediated renal vasodilatation is consistent with the latter being, at least in part, due to NO (Gardiner *et al.*, 1991). The lack of effect of SMTC on the response to ACh indicates the compound is not acting non-selectively. Thus, it is possible the influence of SMTC on resting haemodynamics are due to an involvement of nNOS in cardiovascular regulation (Komers *et al.*, 2000).

Table 1. Cardiovascular variables (mean ± s.e.mean; n=10 in each group) before and during infusion with SMTC or L-NAME. Units for VC are (kHz $mmHg^{-1})10^3$. * $P < 0.05$ vs baseline (Friedman's test).

	SMTC		L-NAME	
	0 min	90 min	0 min	90 min
HR (beats min^{-1})	321±19	325±15	336±13	292±12*
BP (mmHg)	100±3	110±4*	100±2	132±4*
RVC (units)	100±7	88±7*	82±10	62±9*
MVC (units)	96±10	80±8*	99±10	56±7*
HVC (units)	40±3	34±3*	40±3	23±3*

Table 2. Integrated (mean ± s.e.mean, 0-3 min areas under or over curves (AUC, AOC)) changes in HR (beats), BP (mmHg min) and RVC ([kHz $mmHg^{-1}]10^3$ min) in response to ACh in the presence of Vehicle, SMTC or L-NAME. # $P < 0.05$ vs corresponding vehicle (Wilcoxon's test).

	Vehicle	SMTC	Vehicle	L-NAME
HR (AUC)	225±22	200±19	201±26	171±25
BP (AOC)	-13±4	-14±4	-13±4	-20±7
RVC (AUC)	106±13	107±9	87±17	26±6#

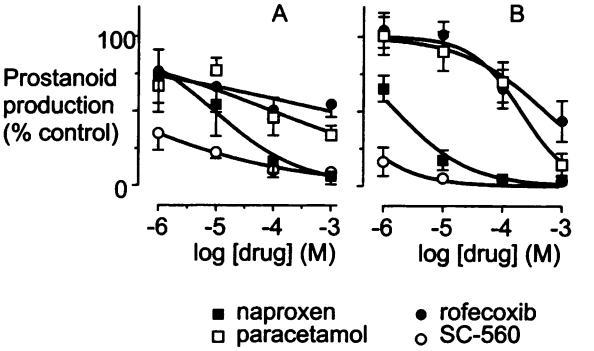
Gardiner, S.M. *et al* (1991) *Br. J. Pharmacol.*, 103, 1725-1732.
Komers, R. *et al* (2000) *Hypertension*, 35, 655-661.
Wakefield, I.D. *et al* (2002) *Br. J. Pharmacol.*, (in press).

297P PROSTACYCLIN PRODUCTION FROM INTACT RAT BLOOD VESSELS IS VIA THE ACTIVITY OF CYCLOOXYGENASE-1 AS DEFINED BY NAPROXEN, PARACETAMOL, ROFECOXIB AND SC560.

Francesco Giuliano, Ivana Vojnovic & Timothy D. Warner. Department of Cardiac, Vascular and Inflammation Research, The William Harvey Research Institute, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ.

Nonsteroid anti-inflammatory drugs (NSAIDs) inhibit the activity of cyclooxygenase (COX) and the formation of prostanoids. The anti-inflammatory effects of NSAIDs appear associated with the inhibition of COX-2, the inducible isoform of COX. Inhibition of COX within the vasculature may also explain some of the other actions of NSAIDs. For example, inhibition by aspirin of COX-1 within platelets underlies this drug's anti-thrombotic effects. COX-2 selective drugs have been developed because of the notion that they should be as efficacious as NSAIDs but produce fewer side effects. However, it has recently been suggested that COX-2-selective agents may reduce selectively prostaglandin (PG) I_2 production from the blood vessel wall while leaving COX-1 and thromboxane (Tx) A_2 production within platelets unaffected, so promoting thrombosis FitzGerald & Patrono, 2001). Here we have compared the abilities of drugs to inhibit prostanoid production from intact blood vessels and platelets.

Male Wistar rats (220-250 g) were anaesthetised with Inactin™ (120 mg kg^{-1}, i.p.) and blood removed via a cannula placed into the carotid artery. Some blood was centrifuged to produce plasma and the rest aliquoted into 96-well plates. Animals were then killed and the aortae removed. The aortae were quickly cleaned of connective tissue, cut into rings and placed into 100 μl incubates of rat plasma. The test compounds, naproxen, paracetamol, rofecoxib or the COX-1-selective inhibitor SC560 (all 10^{-6} to 10^{-3} M) were then added and incubations continued for 60 min. The plasma was then removed from the aortic rings and frozen, while calcium ionophore (A23187, 5 x 10^{-5} M) was added to the whole blood incubates to stimulate platelet COX-1 activity. 15 min later these blood samples were centrifuged and the plasma frozen. The prostanoid levels in the frozen plasma samples from the aortic ring and platelet incubates were then assayed for their contents of, respectively, 6 keto-$PGF_{1\alpha}$ (as a marker of PGI_2) and thromboxane B_2 (as a marker of TxA_2 production).



The four compounds tested (n=6 for each, mean ± s.e.m.) inhibited both vascular PGI_2 (panel A) and platelet TxA_2 (panel B) formation with the same order of potency; that is SC560 << naproxen < paracetamol < rofecoxib. This is in accordance with both production of PGI_2 from the blood vessel wall and production of TxA_2 from platelets being via the activity of COX-1, with no evidence for an involvement of COX-2.

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FitzGerald GA, Patrono C. The coxibs, selective inhibitors of cyclooxygenase-2. N Engl J Med. 2001; 345: 433-42.

298P INHIBITION OF PHOSPHODIESTERASES CAUSES UP-REGULATE NO SYNTHESIS AND ARGINASE ACTIVITY IN RAT ALVEOLAR MACROPHAGES

S. Koschorreck, F. Wenzel, C. Stichnote & K. Racké, Institute of Pharmacology & Toxicology, University of Bonn, Reuterstr. 2b, D-53113 Bonn, Germany.

L-Arginine is the substrate of NO synthase (NOS) and arginase, important pathways in alveolar macrophages and macrophages in general. Both pathways appear to play a significant role in the development of inflammatory and obstructive airway diseases (e.g. Barnes & Liew, 1995; Meurs *et al.*, 2000). Moreover, there is evidence for multiple interactions between both pathways (e.g. Hecker *et al.*, 1995; Hey *et al.*, 1997). Since phosphodiesterase (PDE) inhibitors play an increasing role in the treatment of inflammatory and obstructive airway diseases, the effects of PDE inhibitors on NO synthesis and arginase activity in rat alveolar macrophages were studied.

Rat alveolar macrophages were cultured for 20h in the absence or presence of 1 μg ml^{-1} lipopolysaccharides (LPS) and/or PDE inhibitors as described previously (Hey *et al.*, 1995). Thereafter, nitrite accumulated in the culture media and arginase activity at the end of the culture period were determined (Klasen *et al.*, 2001; Mössner *et al.*, 2001).

In culture media of control alveolar macrophages nitrite concentration amounted to 25±3 μM (mean±s.e.mean, n=9). Presence of LPS caused an increase to 109±13 μM. After culture in presence of IBMX (1-30 μM) nitrite concentration was increased to maximally 62±5 μM. IBMX, present in addition to LPS, caused only a small, additional increase in nitrite accumulation. Nitrite accumulation was increased maximally to 57±9 μM by the PDE3/4 inhibitor benzafendrine (1-30 μM), to 45±6 μM by the PDE4 selective inhibitor rolipram (0.01-30 μM) and to 32±3 μM by the PDE3 selective inhibitor siguadozan (0.01 - 30 μM). Using RT-PCR it was observed that IBMX caused an up-regulation of the iNOS mRNA expression.

Arginase activity in rat alveolar macrophages cultured in the absence of LPS was 261 ± 63 mU mg^{-1} protein. LPS caused an increase by about 150%. IBMX caused an increase by maximally about 50%, and the effects of LPS and IBMX tended to be additive.

In conclusion, inhibition of PDEs in rat alveolar macrophages caused a marked up-regulation of NO synthesis and a moderate increase in arginase activity. The pharmacological profile indicates that both, PDE4 and to a lesser extend PDE3 appear be involved in the control of NO synthesis in rat alveolar macrophages. Since in rabbit alveolar macrophages PDE inhibition resulted only in an increase in arginase activity without effects on NO synthesis (Hammermann *et al.*, 2000), important species differences with regard to the effect of PDE inhibitors on iNOS expression appear to be of significance.

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299P NO DOWN-REGULATES EXPRESSION OF ITS RECEPTOR, SOLUBLE GUANYLYL CYCLASE, IN A cGMP-INDEPENDENT MANNER IN VASCULAR ENDOTHELIAL CELLS

Ibarra, C.[1,4], Müller, H.[1], Nedvetsky, P.I.[1], Lohmann, S.[2], Feil, R.[3], Hofmann, F.[3], Schmidt, H.H.H.W.[1] [1]Rudolf-Buchheim-Institute for Pharmacology, Justus-Liebig-University, Giessen, [2]Department of Clinical Biochemistry and Pathobiochemistry, Medical University Clinic, Würzburg, [3]Technical University Munich, Institute for Pharmacology und Toxicology, Munich, Germany and [4]Faculty of Chemistry, University of Querétaro, México

Endothelium-derived nitric oxide (NO) feeds back on its generator cell to regulate endothelial cell growth, migration and angiogenesis, as well as permeability (Hölschermann *et al.*, 1997). The best established molecular target of NO is soluble guanylyl cyclase (sGC), an α/β heterodimeric heme-protein catalyzing the formation of cGMP. Besides this activity regulation, the expressional regulation of sGC by NO in endothelial cells has not been studied.

Using Western blot analysis, we determined that upon treatment of primary cultures of porcine pulmonary artery endothelial cells (PPAEC) with the NO-donor DETA-NO (0-200 μM for 0-96 h), sGC expression was down-regulated in a time- and concentration-dependent manner. Maximal NO effects were observed with 100 μM DETA-NO at 72 h (56.7±7% of control for $sGC\alpha_1$ and 44.4±8% of control for $sGC\beta_1$, $p<0.05$, n=6). Under these conditions, maximal sGC activity induced by 100 μM DEA-NO decreased from 616±54 (control) to 406±54 $pmol.mg^{-1}min^{-1}$ ($p>0.05$, n=4), as determined by a cGMP immunoassay system.

However, the cGMP mimetics 8-Br-cGMP (0-250 μM) or the NO-independent sGC activator YC-1 (0-25 μM), had no effect or at high concentrations, even increased sGC expression (with 500 μM 8-Br-cGMP to 123±2% of control for $sGC\alpha_1$ and 128±6% of control for $sGC\beta_1$, $p<0.05$ or with 50 μM YC-1 to 126±7% of control for $sGC\alpha_1$ and 137±13% of control for $sGC\beta_1$, $p<0.05$, n=6). Rp-8-Br-PET-cGMPS (0-10 μM), a cGMP-dependent protein kinase inhibitor and zaprinast (0-200 μM), a phosphodiesterase inhibitor, had no effect. In contrast to sGC expression, cGMP-dependent protein kinase I (cGK-I) expression was down-regulated by DETA-NO (100 μM, 34±6% of control, $p<0.05$, n=6), YC-1 (50 μM, 13±4 of control, $p<0.05$, n=6) or 8-Br-cGMP (19±5% of control, $p<0.05$, n=6).

sGC protein levels were increased about two-fold in aortic rings from NOS3 knockout ($NOS3^{-/-}$, genetic background C57BL/6) versus wild-type (WT) mice (205±30% of control for $sGC\alpha_1$ and 271±21% of control for $sGC\beta_1$, n=4) and not significantly altered in cGK knockout ($cGK^{-/-}$, genetic background 129/sv) versus WT mice (n=4).

Taken together, these findings suggest that NO down-regulates sGC expression in a cGMP-independent manner both *in vitro* and *in vivo*. Thus, NO donors and NO-independent activators of sGC, such as YC-1, will differently affect sGC and cGK-I expression levels and thus cGMP signaling in vascular cells.

Hölschermann H. *et al.* (1997) *Am J Physiol* 272, H91-H98

300P UPREGULATION OF NAD(P)H OXIDASES NOX1 AND NOX4 BY THE RENIN-ANGIOTENSIN-SYSTEM SUGGESTS A ROLE IN OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION

Wingler, K.[1], Wünsch, S.[1], Kreutz, R[2,3]., Paul, M.[2], Schmidt, H.H.H.W.[1] [1]Rudolf-Buchheim-Institute for Pharmacology, Justus-Liebig-University, Giessen, [2]Department of Clinical Pharmacology and Toxicology, [3]Department of Medicine IV Nephrology, Freie Universität Berlin, Benjamin Franklin Hospital, Berlin, Germany

In different cardiovascular disease states oxidative stress decreases the bioavailability of endothelial NO resulting in endothelial dysfunction. An important source of reactive oxygen species (ROS) is the enzyme family of NAD(P)H oxidases (Nox), homologs of the neutrophil $gp91^{phox}$/Nox2. Here we provide evidence that the vascular Nox-isoforms Nox1 and Nox4 appear to be involved in vascular oxidative stress in response to risk factors like angiotensin II (Ang II), LDL as well as in transgenic hypertensive rats overexpressing the *Ren2* gene [TGR(mRen2)27]. Nox mRNA and protein levels were quantified by real time RT-PCR (TaqMan®) and Western blotting, respectively.

We showed that in addition to Nox1, the isoform cloned from vascular smooth muscle cells (VSMC), Nox4, originally cloned from the kidney, was expressed in the VSMC line A7r5 and aortas of rats. Upon exposure of A7r5 cells to Ang II (1 µM, 4h), mean ± mean Nox-1 and Nox-4 mRNA levels were increased to 645±39% and 408±51% of the mRNA levels in control cells, respectively. This effect was mediated by the Ang II receptor type 1 since incubation of the cells with AngII in combination with losartan (1 µM) did not increase Nox1 and nNox4 mRNA levels in these cells (133.6±17.1% and 128.9±12.5% respectively). In contrast to Ang II native LDL (20 µg/ml) and oxidized LDL (5-20 µg/ml) only transiently increased Nox1 mRNA levels (288±9.5% and 62.5±13.5% at 4h and 24h native LDL, respectively, 410±14.7% and 50.7±7.8% at 4h and 24h 20 µg/ml oxLDL, respectively), while Nox4 mRNA was not affected (e.g. 89±3.2% at 4h 20 µl/ml ox LDL).

Neither the vasoconstrictor endothelin 1 (up to 500 nM, 1-24h) nor LPS (up to 10 µg/ml, 1-24h) had any effect on Nox1 and Nox4 expression in these cells (e.g. 3h 500nM ET1 63.7% and 68.3% and 4h 10 µg/ml LPS 132.9% and 85.9%, respectively). Consistent with these in vitro observations, aortas and kidneys of [TGR(mRen2)27] rats had significantly higher Nox1 and Nox4 mRNA (kidney 211.6±3.1% and 181.5±2.3% respectively) and Nox4 protein levels (aorta 197.2±32.9%, kidney 166±36.8%) as compared to wild type animals. Nox1 protein levels were too low for a quantification. Importantly, in all samples analyzed, Nox4 was more abundant than Nox1 which became obvious from the threshold cycle in case of the mRNA and the signals in Western blots in case of the protein.

In conclusion, Nox4 and even more so Nox1 are upregulated by the renin-angiotensin system and Nox1 also by lipoproteins. Increased superoxide production by upregulated vascular Nox isoforms may diminish the effectiveness of NO and thus contribute to the development of vascular diseases. Therefore, Nox1 and Nox4 are putative therapeutic targets to reduce vascular ROS production to increase the bioavailability of NO.

301P FUNCTIONAL POTENCY OF ENDOTHELIN RECEPTOR ANTAGONISTS IN HUMAN UMBILICAL VEIN CORRELATES WITH FUNCTIONAL POTENCY IN RAT AORTA AND SPA RADIOLIGAND BINDING AFFINITY

H.T. Parker, N.S. Crouch, A.J. Chuck, D.A. Flynn, S. Ballard & M.A. Tones, Discovery Biology, Pfizer Global R&D, Sandwich, Kent CT13 9NJ, UK

Understanding the relationship between the binding affinity of endothelin receptor antagonists (ETRAs) and their functional efficacy in man is the key to the drug development process. While the rat aorta has traditionally been used to assess functional efficacy, it is clearly preferable to be able to use a human vascular smooth muscle preparation. While the human umbilical vein is readily available (Maguire *et. al.* 1997) the response of this preparation to ETRAs has not been characterised. The purpose of this study was to clearly define the relationship between binding affinity and functional efficacy of ETRAs in the rat aorta and human umbilical vein (HUV).

Binding affinity of ETRAs were determined by competition experiments in SPA radioligand binding using human recombinant ET_A receptors expressed on CHO cell membranes and the ET_A selective peptide [^{125}I]PD-151242 as radioligand (Maguire *et. al.* 1997).

Aortae were obtained from 350-400g male Sprague Dawley rats (Charles River). Human umbilical cords were obtained from caesarean section (K&C Hospital) with written consent.

Rings were dissected from rat aortae and HUV, mechanically denuded of endothelium and suspended under 1g tension in Krebs-Ringer buffer at 37°C and gassed with 95% O_2 / 5% CO_2. Cumulative concentration-response curves were constructed to endothelin-1 (ET-1) in the presence of ETRA or vehicle. Data were expressed as a percentage of the contraction to 60mM KCl, from which the EC_{50} was obtained.

These values were used to calculate pK_B's using the Gaddum equation, with the assumption that the antagonists were acting competitively.

ETRA	Binding pK_i	Functional (pK_B) Rat aorta	HUV
Sitaxsentan	7.81 ±0.07	7.06 ±0.07	6.38 ±0.07
Darusentan	8.24 ±0.10	7.92 ±0.17	6.38 ±0.14
Bosentan	8.25 ±0.04	8.02 ±0.27	6.34 ±0.07
BMS-193884	9.25 ±0.04	8.14 ±0.09	7.29 ±0.10
SB-209670	9.38 ±0.15	8.64 ±0.10	8.14 ±0.08
TA-0201	9.85 ±0.10	8.80 ±0.18	8.81 ±0.22
A-127722	9.90 ±0.13	8.35 ±0.19	8.84 ±0.17

Table 1: All data expressed as mean ± s.e.m. of minimum of 3 experiments.

The pEC_{50} of ET-1 in HUV was 8.07 ± 0.08 compared to 8.92 ± 0.05 in rat aorta. The pK_B's obtained for a range of ETRAs in rat and human vessels are shown in table 1. A plot of HUV pK_B against binding pK_i reveals a good correlation with a slope of unity. A 43-fold translational difference from SPA ligand binding to human umbilical vein exists. A similar plot of rat pK_B versus human pK_B resulted in a 4-fold translational difference in potency from rat to human.

This study illustrates the relationship between ligand binding and functional assays for this system, and also the differences between rat and human vessel preparations. While functional potency varies, rat aorta may predict rank order of efficacy in human vascular smooth muscle.

Maguire, J.J. et al. (1997) *Brit. J. Pharmacol.* 122, 1647-1654