1P ROLE OF BACTERIA AND ENDOTOXIN IN NITRIC OXIDE SYNTHASE INDUCTION AND RAT INTESTINAL INJURY PROVOKED BY NON-STEROID ANTI-INFLAMMATORY AGENTS

S. M. Evans & B.J.R. Whittle. Wellcome Foundation Ltd., Glaxo-Wellcome, Beckenham, Kent, BR3 3BS. U.K.

Bacteria located in the gut lumen have been implicated in the pathogenesis of chronic intestinal damage caused by non-steroidal anti-inflammatory drugs. Induction of a calcium-independent nitric oxide synthase (iNOS) has also recently been proposed to be involved in the chronic intestinal microvascular injury provoked by indomethacin *(Whittle et al, 1995)*. The role of local gut bacteria in the intestinal damage provoked by such agents has now been further explored by histology and by using the broad-spectrum antibiotic, ampicillin, metronidazole and polymixin B which binds to and inactivates endotoxin lipopolysaccharide.

Indomethacin (10 mg kg^{-1} s.c.) caused a time-dependent increase in macroscopic injury in the jejunum, with a score (1-5 scale) after 24h of 3.25±0.25 (n=10). After 15-18h, the epithelial barrier was disrupted, with histological evidence of bacterial invasion of the underlying tissue, predominantly with gram-negative staining organisms. Treatment with ampicillin (200 mg kg^{-1} day^{-1}) or metronidazole (200 mg kg^{-1} day^{-1}) abolished the appearance of macroscopic damage (score 0.2±0.2 and 0.1±0.1 respectively n=6). Administration of indomethacin (10 mg kg^{-1}, p.o.) also caused a time-dependent increase in leakage of radiolabelled human serum albumen and in iNOS activity in the jejunum, determined by the conversion of ^{14}C-L-arginine to citrulline in the presence of EGTA (1mM) commencing 18h after challenge. A single oral dose of indomethacin, diclofenac or flurbiprofen (10, 40 & 40 mg kg^{-1} respectively) increased jejunal plasma leakage by Δ258±15, 166±6 and 183±14µl g^{-1} tissue respectively (P<0.001; n=4), determined 24h later. Jejunal iNOS activity substantially increased from a basal value of 14±1 to 109±20, 90±10 and 83±1 pmol min^{-1} mg^{-1} protein respectively (P<0.01) 24h following these doses of indomethacin, diclofenac and flurbiprofen. The iNOS activity induced by indomethacin was inhibited (98±1 and 95±5%, n=5 for each, P<0.001) by pretreatment with ampicillin or metronidazole,which also abolished the plasma leakage (P<0.001 for each). Treatment with polymixin B (3 mg kg^{-1} s.c.) likewise abolished the macroscopic damage, the increase in plasma leakage and the expression of jejunal iNOS determined 24h after indomethacin (n=5 for each;P<0.001).

These findings confirm the involvement of gut bacteria, in the small-intestinal damage caused by these anti-inflammatory agents. Moreover, the associated expression of iNOS, which is implicated in microvascular injury, is likewise abolished by the anti-bacterial agents. The inhibitory effects of polymixin B further suggest that the local release of endotoxin, perhaps following translocation of the luminal bacterial into the mucosa, that leads to iNOS induction, are important events in the pathogenesis of such intestinal injury.

Whittle, B.J.R. Laszlo, F., Evans, S.M. & Moncada, S., (1995). *Br. J. Pharmacol.*, 116, 2286-2290.

2P PRETREATMENT WITH DEXAMETHASONE OR DELAYED TREATMENT WITH AMINOGUANIDINE AMELIORATES THE CIRCULATORY FAILURE AND ORGAN INJURY IN A RAT MODEL OF GRAM-POSITIVE SHOCK

M. Kengatharan, S.J. De Kimpe and C. Thiemermann. The William Harvey Research Institute, St Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ.

The cell wall components of *Staphylococcus aureus*, lipoteichoic acid (LTA) and peptidoglycan (PepG), synergise to induce nitric oxide synthase (iNOS) and to cause multiple organ dysfunction syndrome (MODS) and circulatory failure in the rat (De Kimpe *et al*, 1995). Pretreatment of rats with aminoguanidine (AMG) prevents circulatory failure, MODS (except renal failure) and the expression of iNOS protein caused by LTA+PepG (Kengatharan *et al*, 1996). Here, we investigate whether prevention of iNOS expression (with dexamethasone) or inhibition of iNOS activity (with AMG) protects against MODS and circulatory failure elicited by LTA+PepG.

Male Wistar rats (250-350g) were anaesthetised with thiopento-barbitone sodium (120mgkg^{-1}, i.p.). The trachea was cannulated to facilitate respiration, the carotid artery for measuring mean arterial blood pressure (MAP), heart rate and arterial oxygen tension (PaO_2), and the jugular vein for administration of compounds. Dexamethasone (DEX, 3mgkg^{-1}, i.p.) was given 120 min before injection of LTA (3mgkg^{-1}, bolus) plus PepG (10mgkg^{-1}, injected over 30-45 min), whereas the iNOS-selective inhibitor AMG (5mgkg^{-1} i.v. bolus plus 10mg$kg^{-1}h^{-1}$ infusion) was given 120min after PepG+LTA. The pressor response to noradrenaline (NA, 1µgkg^{-1}) was assessed prior to and every 60 min after the injection of LTA+PepG. At 90 min, plasma levels of TNFα were measured using an ELISA kit and, at 360 min, plasma samples were taken for the determination of biochemical markers of organ injury and nitrite+nitrate (total plasma nitrite). At 360 min, the rats were killed and various tissues were removed to determine iNOS activity in homogenates via the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, and for Western blot analysis.

Treatment with DEX or AMG attenuated (i) the hypotension and the vascular hyporeactivity to NA, (ii) the fall in PaO_2, (iii) the increase in plasma levels of alanine aminotransferase (ALT), (iv) the increase in iNOS activity, and (v) the rise in total plasma nitrite induced by LTA+PepG (Table 1). The expression of iNOS protein and the increases in urea or creatinine were attenuated by DEX, but not by AMG. Furthermore, DEX reduced the increase in TNFα levels induced by PepG+LTA (TNFα, 39±4 $ngml^{-1}$ for PepG+LTA *vs* 10±2 $ngml^{-1}$ with DEX treatment, n=6-8, p<0.05).

Thus, enhanced formation of NO due to expression of iNOS contributes importantly to circulatory failure, respiratory failure and liver injury caused by LTA+PepG in anaesthetised rats.

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Table 1. Effect of DEX or AMG on the circulatory failure, MODS, iNOS activity and increase in total plasma nitrite elicited by LTA+PepG.

Experimental group	MAP (mmHg)	NA response (% control)	Lung iNOS activity (pmol L-citrulline $30min^{-1}$ $mg\ protein^{-1}$)	ALT (iuL^{-1})	Urea (mM)	Creatinine (µM)	PaO_2 (mmHg)	Total plasma nitrite (µM)
sham	113± 4	114± 10	9±3	63±6	6±1	35±4	81±3	44±3
LTA+PepG	77±5#	16±2#	660±120#	1000±500#	17±1#	65±4#	59±3#	365±33#
plus DEX	119±8*	117±8*	118±39*	78±48	8±1*	29±3*	75±2*	62±11*
plus AMG	108±3*	44±7*	172±41*	85±45	17±1	71±2	74±3*	162±16*

Values for 360 min after injection of bacterial components are given as mean ± s.e.mean (n=6-8); # $P<0.05$ compared to sham control and * $P<0.05$ compared to LTA+PepG by ANOVA (Bonferroni's test).

3P EXPRESSION OF LOW DENSITY LIPOPROTEIN SCAVENGER RECEPTORS IN DIFFERENTIATED THP-1 MACROPHAGES

S.J. De Kimpe, M.J. Carrier and E.E. Änggård, William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ

Scavenger receptors mediate the uptake and degradation of chemically modified proteins, such as oxidised and acetylated low-density lipoproteins (LDL). Degradation of modified LDL in macrophages results in elevated cellular cholesterol levels. This in turn leads to the subendothelial formation of lipid-loaded foam cells from macrophages which is a critical early event in the pathogenesis of atherosclerosis. The expression of the scavenger receptor is low in monocytes, but is substantially increased upon their activation and subsequent differentiation into macrophages. We have investigated which intracellular events can modulate the uptake of acetylated LDL (AcLDL) in the human monocyte/macrophage cell line THP-1 following activation by the phorbol ester phorbol myristate acetate (PMA).

THP-1 cells were grown in suspension in RPMI-1640 medium containing 5×10^{-5}M β-mercaptoethanol and 10% foetal bovine serum. Cells were seeded onto 6-well plates at 1.5×10^{6} cells per well. PMA, 10^{-7}M, was added to differentiate THP-1 cells into macrophage -like cells. Drugs were added 30 min prior to the addition of PMA. After 48 h cells which had adhered to the bottom of the well, were washed, lifted from the dish and transferred to microcentrifuge tubes. The cells were incubated at 37°C for 2.5 h with 50 μg ml^{-1} Ac-LDL labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). Subsequently, cells were fixed in 1% paraformaldehyde and analysed by flow cytometry. For every sample, the median of the fluorescence per cell was determined and expressed as percentage of cells treated with PMA alone.

Differentiation of THP-1 with PMA increased the uptake of DiI-AcLDL. This was inhibited by cycloheximide indicating that the expression of macrophage scavenger receptors is upregulated. The tyrosine kinase inhibitor typhostin and the hydroxyl radical scavengers dimethylthiourea and sodium benzoate decrease the enhanced DiI-AcLDL uptake. Furthermore, reactive oxygen species (ROS), formed by leakage of electrons in the mitochondria between respiratory chain complex I (inhibited by rotenone) and III (inhibited by antimycin A), appear to play a role in the control of scavenger receptor activity. Pyrrolidine dithiocarbamate (PDTC), an inhibitor of the activation of NFκB did not affect DiI-AcLDL uptake.

Table 1. Uptake of DiI-AcLDL by THP-1 macrophages.

Treatment	Fluorescence (% PMA)
Control (no PMA)	18 ± 2 *
PMA	100 ± 7
+ cycloheximide (1 μg ml^{-1})	9 ± 4 *
+ tyrphostin AG126 (10 μM)	41 ± 5 *
+ dimethylthiourea (10 mM)	70 ± 4 *
+ sodium benzoate (50 mM)	13 ± 2 *
+ rotenone (3 μM)	44 ± 4 *
+ antimycin A (1 μM)	90 ± 13
+ PDTC (25 μM)	88 ± 7

Values are given as mean ± s.e.mean (n=4-6); * $P < 0.05$ compared to PMA by ANOVA (Dunnett's test).

These results indicate that tyrosine kinase activity and ROS, but not NFκB, modulate the uptake of modified LDL through the scavenger receptor, in PMA-stimulated THP-1 cells.

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4P INHIBITION BY INTERLEUKIN-13 OF THE EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN MACROPHAGES AND VASCULAR SMOOTH MUSCLE

H. Ruetten & C. Thiemermann, The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1 6BQ.

A distinct isoform of NOS (inducible NOS, iNOS) can be induced by proinflammatory agents such as endotoxin (*E. coli* lipopolysaccharide, LPS), interleukin-1β, interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α) in a variety of cells including macrophages and smooth muscle cells (Xie and Nathan, 1994). Interleukin (IL)-13 is generated by activated lymphocytes, and inhibits the formation of pro-inflammatory cytokines by monocytes (Minty et al., 1993). Here, we compare the effects of IL-13 on the LPS-induced expression of iNOS protein and activity in cultured macrophages and rat aortic smooth muscle cells (RASM), and the production of TNF-α in macrophages.

Murine macrophages (J774.2) were cultured in DMEM and RASM in RPMI containing L-glutamine (3.5 mM) and 10% foetal calf serum. To induce iNOS in macrophages or RASM, fresh culture medium containing LPS (1 μg ml^{-1}, serotyp: 0127:B8) or LPS (10 μg ml^{-1}) and IFN-γ (10 U ml^{-1}) was added. Fifteen min prior to LPS, cells were treated with vehicle (saline) or interleukins (1, 10 and 100 ng ml^{-1}, n=9). After 24 h, nitrite accumulation in the cell culture medium was measured by the Griess reaction and expression of iNOS by Western blot analysis following SDS-PAGE electrophoresis (7.5% polyacrylamide). The concentration of TNF-α in the cell culture medium of macrophages treated with LPS (1 μg ml^{-1}) and interleukins (100 ng ml^{-1}, n=5) was measured by ELISA.

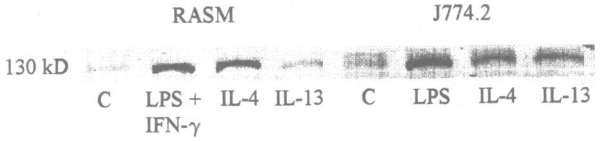
IL-13 and IL-4 caused concentration-dependent inhibitions of the increase in nitrite production elicited by LPS in macrophages, and by LPS and IFN-γ in RASM, respectively (Table 1). In contrast, IL-10 had no effect on nitrite production in macrophages or RASM activated with immunostimulants (Table 1). LPS and IFN-γ also resulted in the expression of iNOS protein in macrophages and RASM, which was attenuated by pretreatment of cells with IL-4 (macrophages) and IL-13 (macrophages and RASM) (Fig. 1). In addition, LPS caused an increase in the concentration of TNF-α, which was inhibited by IL-10, IL-4 and IL-13 (Table 1).

Table 1.

Treatment	J774.2 Nitrite (μM)	RASM Nitrite (μM)	TNF-α (ng/ml)
vehicle (saline)	1.7 ± 0.4	1.9 ± 0.6	0
LPS (IFN-γ, RASM)	56 ± 5	20 ± 0.8	4.4 ± 0.1
+IL-13 (100 ng/ml)	30 ± 3*	5 ± 0.5*	2.7 ± 0.2*
+IL-4 (100 ng/ml)	28 ± 4*	15 ± 0.4*	3.2 ± 0.1*
+IL-10 (100 ng/ml)	49 ± 4	19 ±0.6	2.2 ± 0.1*

mean ± s.e.mean. *p<0.05 vs. LPS (IFN-γ), unpaired Student's *t* test.

Fig 1.



Thus, IL-13 inhibits the expression of iNOS protein and activity in macrophages and RASM, as well as the rise in TNF-α production in macrophages activated with immunostimulants.

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5P A PEPTIDOGLYCAN FRAGMENT SYNERGISES WITH LIPOTEICHOIC ACID TO INDUCE NITRITE FORMATION IN MACROPHAGES

M. Kengatharan, S.J. De Kimpe, C. Thiemermann and J.R. Vane. The William Harvey Research Institute, St Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ.

Peptidoglycan (PepG), a cell wall component of Gram-positive bacteria, is a polymer with repeating units consisting of the sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), and a peptide subunit (or stem peptide) consisting of alternating L- and D-amino acids, linked to the next monomer by a pentaglycine bridge (Figure 1). PepG synergises with lipoteichoic acid (LTA) to induce nitric oxide synthase (iNOS) in macrophages (De Kimpe *et al*, 1995). We show here that a specific moiety of PepG, NAG-NAM-L-ala-D-isoglutamine, is mainly, if not totally, responsible for synergising with LTA as well as interferon-γ (IFNγ) to induce iNOS activity.

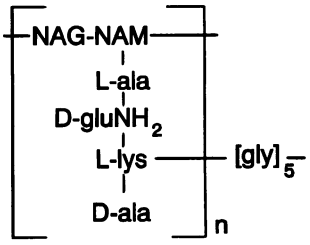

Figure 1. The monomer unit of peptidoglycan from *S.aureus*.

J774.2 cells were cultured in 96-well plates with 200 µl of culture medium (DMEM) containing foetal calf serum (10%) and glutamine (4 mM) until cells reached confluence. PepG (from *Staphylococcus aureus*) or various components of PepG, alone or in combination with LTA (1µgml^{-1}) or IFNγ (10iuml^{-1}) was added to the cells. In one experiment, PepG+IFNγ were incubated in DMEM for 1h at 37°C with *S. aureus* lytic enzyme (SALE, Timmerman, *et al*, 1993) before being transferred to the cells for a further incubation period of 24h. Nitrite accumulation, an indicator of NO formation, was measured 24h later in the supernatant of J774.2 cells by the Griess method.

Activation of J774.2 macrophages with LTA (1µgml^{-1}) or IFNγ (10iuml^{-1}) caused increases in nitrite concentration in the medium from 2±0.1µM (baseline) to 14±3 µM (n=12, $p<0.01$) and 6±2 µM (n=12). PepG and its specific moiety, NAG-NAM-L-ala-D-isoglutamine, synergised with LTA as well as IFNγ to induce iNOS activity (Table 1). In contrast, this fragment alone did not (up to a concentration of 100µgml^{-1}) have a significant effect on nitrite formation. The components NAM, NAG, stem peptide, pentaglycine or tetraglycine did not (up to a concentration of 100µgml^{-1}) synergise with LTA or IFNγ to induce iNOS activity.

Table 1. Various PepG components which do not alone induce nitrite formation synergise with LTA to induce iNOS activity.

Component	Conc. (µgml^{-1})	Nitrite level in the presence of LTA (% LTA control)
Peptidoglycan polymer	1	105±4
Peptidoglycan polymer	30	256±10*
NAG-NAM-L-ala-D-isoglutamine	1	300±6*
NAM-L-ala-**D**-isoglutamine	1	230±10*
NAM-**L**-ala-**L**-isoglutamine	1	170±5*
NAM-**D**-ala-**D**-isoglutamine	100	103±7

Value are given as mean ± s.e.mean (n=9). *$P<0.05$ compared to LTA (1µgml^{-1}) control by ANOVA (Dunnett's test).

Synergism with LTA was reduced in the absence of the NAG residue from NAG-NAM-L-ala-D-isoglutamine. Similarly, substitution of L-ala with D-ala or D-isoglutamine with L-isoglutamine on the NAM-L-ala-D-isoglutamine also reduced the synergism with LTA (Table 1). Furthermore, hydrolysis with the amidase, SALE, which breaks the linkage between the NAM and L-ala, abolished the synergism with IFNγ to induce iNOS.

Thus, NAG-NAM-L-ala-D-isoglutamine is the moiety of PepG which accounts for the ability of PepG to synergise with LTA or IFNγ to induce iNOS activity in macrophages.

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6P LPS INHIBITS DNA SYNTHESIS IN MACROPHAGES AND VASCULAR SMOOTH MUSCLE CELLS BY A MECHANISM INDEPENDENT OF NOS INDUCTION

A. Paul, C. E. Bryant[1], M. F. Lawson[2], E. R. Chilvers[2] & R. Plevin. Department of Physiology & Pharmacology, University of Strathclyde, Glasgow. G1 1XW [1]The William Harvey Research Institute, Department of Biochemical Pharmacology, Medical College of St Bartholomew's Hospital, University of London, London. EC1M 68Q [2]Respiratory Medicine Unit, Department of Medicine, University of Edinburgh, Medical School, Lauriston Place, Edinburgh. EH8 9AG

The active component of endotoxin, lipopolysaccharide (LPS), stimulates the induction of nitric oxide synthase (NOS) in both macrophages and vascular smooth muscle cells (Paul *et al.*, 1995; Paul & Plevin, 1995). LPS and cytokines such as IFNγ and TNF have also been shown to inhibit both DNA synthesis and induce cell apoptosis (Albina *et al.*, 1993; Nunojawa & Tanak, 1992), however, the role of nitric oxide in this process remains controversial. In this study we have examined the ability of LPS to inhibit DNA synthesis relative to the effects upon NOS induction.

RAW 264.7 macrophages and rat aortic smooth muscle cells (RASMC) were cultured in serum free medium for 12 or 48 h respectively before DNA synthesis was assayed by measuring [^{3}H]thymidine incorporation into cells over a 24 h period. NOS induction was estimated by either Western blotting or measuring the extracellular accumulation of nitrite.

In RAW 264.7 macrophages, low concentrations of LPS stimulated a small increase in DNA synthesis, however, at higher concentrations, an inhibitory effect was observed (IC_{50}±s.e.mean: 0.05±0.03µgml^{-1}, n=3). In RASMC, LPS only inhibited DNA synthesis (IC_{50}±s.e.mean: 9.7±0.4 µgml^{-1}, n=3). This effect was not associated with cellular apoptosis in either cell type. In RAW 264.7 macrophages, LPS stimulated a concentration-dependent increase in the expression of NOS over a similar concentration range which inhibited DNA synthesis. However, in RASMC LPS alone did not induce NOS and required the additional presence of forskolin. In both cell types, DNA synthesis and NOS induction could be dissociated by serum. In RAW 264.7 macrophages, removal of serum prevented the induction of NOS but had little effect on the ability of LPS to inhibit DNA synthesis (IC_{50}±s.e.mean: LPS-serum=0.05±0.03µgml^{-1}; LPS+serum=1.4±0.85ngml^{-1}; n=3). In RASMC, however, serum abolished LPS-mediated NOS induction but did not dramatically affect inhibition of DNA synthesis (IC_{50}±s.e.mean: LPS+serum=57.3±7.8µgml^{-1}; LPS-serum=9.7±0.4µgml^{-1}; n=3). In both cell types incubation with inhibitors of NOS, either L-NAME or L-canavanine, abolished both LPS-stimulated NOS activity *in vitro* and extracellular nitrite accumulation (IC_{50}±s.e.mean: 46±15µM and 90±45µM respectively; n=3) but were without effect upon DNA synthesis. These results show that LPS-mediated effects upon cellular DNA synthesis are not dependent upon the ability to stimulate NOS expression and subsequent nitric oxide production. Other cellular events may contribute to the deleterious effects of LPS upon cell growth and survival.

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7P DISSOCIATION OF LPS-STIMULATED MAP KINASE ACTIVATION AND NITRIC OXIDE SYNTHASE INDUCTION IN RAW 264.7 MACROPHAGES

A. Paul, A. Cuenda[1], C. E. Bryant[2], G. W. Gould[3], P. Cohen[1] & R. Plevin. Department of Physiology & Pharmacology, University of Strathclyde, Glasgow. G11XW [1]MRC Protein Phosphorylation Unit, The University, Dundee. DD1 4HN [2]The William Harvey Research Institute, Department of Biochemical Pharmacology, Medical College of St Bartholomew's Hospital, University of London, London. EC1M 68Q [3]Division of Biochemistry & Molecular Biology, Institute of Biomedical & Life Sciences, University of Glasgow, Glasgow. G12 8QQ

The active component of endotoxin, lipopolysaccharide (LPS), stimulates the induction of nitric oxide synthase (NOS) and cyclo-oxygenase-2 (COX-2) in macrophages (Paul *et al.*, 1995; Chanmugam *et al.*, 1995). However, the intracellular mechanisms by which these processes are mediated remains unclear. In macrophages, LPS stimulates the activation of both the classical isoforms of mitogen-activated protein (MAP) kinase and also the recently described stress-activated protein kinases which includes p38 MAP kinase-2 (Rouse *et al.*, 1994). In this study we have examined the effects of an inhibitor of the activation of the upstream activator of MAP kinase, MAP kinase kinase (MEK), termed PD098059 (Dudley *et al.*, 1995), and an inhibitor of p38 MAP kinase-2, SB203580 (Cuenda *et al.*, 1995), upon induction of NOS and COX-2.
RAW 264.7 macrophages were cultured in 10% FCS before stimulation. MAP kinase content and the induction of iNOS and COX-2 were assessed by Western blotting. Activation of MAP kinase, MEK and the immediate downstream target of p38 MAP kinase 2, MAPKinase-activated protein kinase-2 (MAPKAP-kinase-2) were also examined by *in vitro* kinase assay following immunoprecipitation. Results are expressed as mean±s.d. (for n=3) and statistical analysis performed using an unpaired *t*-test. In RAW 264.7 macrophages LPS (1μgml^{-1}) stimulated a time-dependent increase in both MAP kinase and MEK activity. Preincubation of the cells with 50μM PD098059 (PD) abolished MEK activity (fold stimulation: LPS 1.7; LPS+PD=0.75, n=2) and substantially reduced MAP kinase activity (cpm±s.d.: cont=3396±361; cont+PD=4226±25; LPS=8074±98; LPS+PD=4164±158, $p<0.05$, n=3). However, PD098059 failed to inhibit LPS-mediated induction of NOS although it reduced the stimulated expression of COX-2 by approximately 80% (Scanning density units (SDU):cont=0; cont+PD=0; LPS=1; LPS+PD=0.18). LPS also stimulated the activation of MAPKAP kinase-2 in RAW 264.7 macrophages and this effect was abolished by 20μM SB203580 (SB) (mUml^{-1}±s.d.: cont=80±10; LPS=750±35; LPS+SB=20±2; $p<0.05$, n=3). Under these conditions the induction of NOS mediated by LPS was not affected, however; induction of COX-2 was reduced by approximately 20% (SDU: cont=0; cont+SB=0; LPS=1; LPS+SB=0.78).
These studies argue against a role for both the classical p42 and p44 and p38 MAP kinases in the induction of NOS but indicate a possible involvement of these pathways in the regulation of stimulated COX-2 expression.

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8P THE PRO-APOPTOTIC EFFECT OF TNFα IN HUMAN NEUTROPHILS IS MEDIATED VIA THE TNF CD120b (p75) RECEPTOR

J. Murray, E. Kitchen, & E. R. Chilvers. Respiratory Medicine Unit, University of Edinburgh, Rayne Laboratory, Medical School, Edinburgh EH8 9AG.

Neutrophil (PMN) apoptosis is an important mechanism underlying the removal of redundant PMNs from inflamed sites. The ability of a number of pro-inflammatory agents to impede PMN apoptosis suggests that such agents act not only in a secretagogue capacity but also increase neutrophil longevity by delaying apoptosis (Lee *et al.*, 1993). We have recently shown that TNFα has the unique ability to promote PMN apoptosis at early time points (<8 h) despite causing inhibition at later times (>12 h) (Murray *et al.*, 1996). The concentration-dependence of the early pro-apoptotic effect (EC_{50} 2.8 ng ml^{-1}) is similar to that observed for TNFα mediated priming. This study sought to assess which TNF receptor (p55, CD120a or p75, CD120b) is involved in these responses.

Human PMNs were purified from peripheral blood (Haslett *et al.*, 1985) and resuspended in serum-supplemented Iscoves MDM. PMNs (5 x 10^6 ml^{-1}) were incubated at 37°C with 28 µg ml^{-1} rat IgG2b anti-human CD120b mAb (Genzyme) or control isotyped matched IL-2 receptor mAb (Genzyme) prior to addition of TNFα (12.5 ng ml^{-1}) or buffer. The mAb concentration was determined using a flow cytometry Ab titration assay. PMNs (6.75 x 10^5) were cultured (150 µl) at 37°C in a humidified 5% CO_2 atmosphere and harvested at the times indicated. Cytocentrifuge slides were prepared and apoptosis assessed morphologically. Results are expressed as the mean ± s.e. mean.

Pre-incubation of PMNs with CD120b mAb caused a marked inhibition (81.0 ± 6.9%, $p<0.05$, n=4) of the pro-apoptotic effect of TNFα at 6 h (% apoptosis, control 2.8 ± 0.3, TNFα 13.1 ± 2.4, anti-CD120b 2.9 ± 0.3, anti-CD120b+TNFα 4.3 ± 0.6. No inhibition was observed with the isotyped matched mAb. In contrast CD120b mAb did not abrogate the ability of TNFα to inhibit apoptosis at 20 h (% apoptosis, control 58.7 ± 1.2, TNFα 38.7 ± 3.4, anti-CD120b 51.4 ± 1.6, anti-CD120b+TNFα 32.4 ± 1.0). The specificity of the CD120b mAb was demonstrated by its inability to block TNFα induced priming of fMLP-stimulated superoxide anion generation. The ability of TNFα to accelerate apoptosis at 6 h was also lost if cells were pre-incubated for 5 min with 1 µM platelet-activating factor (PAF) (% apoptosis, control 6.0 ± 1.3, TNFα 25.6 ± 5.2, PAF 4.3 ± 1.3, PAF+TNFα 4.9 ± 0.8, $p<0.05$, n=3). This effect of PAF was not secondary to shedding or internalisation of CD120b. Statistical significance was determined using ANOVA.
These data indicate a role for CD120b in signalling the early pro-apoptotic effect of TNFα in human neutrophils. Whether this effect involves 'ligand-passing' to CD120a that contains the classic death domain sequence is currently under study.
This work was funded by the Wellcome Trust, UK.

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9P THE EFFECT OF EXPOSURE TO SIN-1 ON CYCLOOXYGENASE ACTIVITY AND EXPRESSION IN BOVINE AORTIC ENDOTHELIAL CELLS

JS Matthews, BJ Key & M Keen, Department of Pharmacology, The Medical School, University of Birmingham, Birmingham B15 2TT, UK.

We have previously shown that exposure of the bovine aortic endothelial cell line, AG4762, to nitric oxide (NO) donors results in pronounced inhibition of prostacyclin (PGI_2) release from these cells (Matthews et al., 1995). A similar phenomenon has been reported by Swierkosz et al. (1995) in macrophages treated with lipopolysaccharide. These macrophages initially produce both NO and PGI_2 due to the expression of the inducible forms of NO synthase (iNOS) and cyclooxygenase (COX-2), respectively. However, the large amounts of NO subsequently reduce PGI_2 production, via inhibition of both the activity and expression of COX-2. In this study we have investigated whether a similar mechanism underlies the reduction in PGI_2 release from AG4762 cells produced by the NO donor, 3-morpholinosydnonimine (SIN-1).

Confluent AG4762 cells (passage 23-25) were grown for 18h in the absence (saline control) or presence of 500μM SIN-1. Cells were then washed and harvested in phosphate buffered saline and pelleted cells were stored at -80°C until required. Cyclooxygenase (COX) activity was measured as described by Swierkosz et al. (1995), using radioimmunoassay to measure the conversion of arachidonic acid (30μM) to 6-keto-$PGF_{1\alpha}$ by cell homogenates. COX expression was measured by immunoblotting, using a specific antiserum against COX-1 (PG20; Biogenesis, Poole) and ECL detection.

The effect of both acute and chronic exposure to SIN-1 are shown in Table 1. For acute exposure, 500μM SIN-1 was added directly to the COX assay. In the case of chronic exposure, cells had been pretreated for 18h in the presence of 500μM SIN-1, which was then removed by washing.

Table 1. The effect of SIN-1 on COX activity, expressed as ng 6-keto-$PGF_{1\alpha}$. mg protein^{-1}.15min^{-1}. Data are mean±s.e.mean. ** significantly different from control, $P<0.05$ in a *t*-test.

control activity	+ SIN-1	% reduction
Acute exposure		
91.9±19.5, n=4	26.4± 4.7, n=4 **	71%
Chronic exposure		
94.8±16.8, n=5	82.4±10.9, n=5	13%

Hence, acute exposure to SIN-1 inhibits the conversion of arachidonic acid to 6-keto-$PGF_{1\alpha}$, consistent with inhibition of COX and/or PGI_2 synthase. In contrast, chronic exposure to SIN-1 produces only a minor effect in this system, which is not statistically significant. The apparent lack of effect of pretreatment with SIN-1 on COX activity is supported by the immunoblotting studies, in which the levels of COX-1 appear to be the same in both control and pretreated cells.

Thus while acute exposure to SIN-1 can inhibit COX or PGI_2 synthase activity, pretreatment with SIN-1 does not appear to mediate significant inhibition of COX or PGI_2 synthase, nor a decrease in the expression of COX-1 in endothelial cells. Therefore it seems unlikely that inhibition of COX underlies the decrease in PGI_2 release produced by pretreatment of these cells with SIN-1 and the mechanism by which NO donors inhibit PGI_2 release from endothelial cells remains to be determined.

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10P *IN VITRO* BINDING OF [^{14}C]-SULPHAMETHAZINE TO CHICKEN LIVER MICROSOMES

L. Parkinson, H.W. Symonds[1] and C.J. Bowmer, Department of Pharmacology, [1]Department of Animal Physiology and Nutrition, The University of Leeds, LS2 9JT.

Sulphamethazine (SMZ) is used as a feed additive to control coccidiosis in chickens. However, this may have implications for the health of treated birds as sulphonamides induce hypersensitivity reactions in mammals, possibly via covalent binding of reactive metabolites to tissue proteins (Cribb *et al.*, 1995). Hence, the aim of this study was to investigate the potential of SMZ to covalently bind to chicken liver microsomes.

[^{14}C]-SMZ (0.2 μCi) and SMZ (1 mM) were incubated at 41°C and pH 7.4 for 15 minutes with chicken liver microsomes (2 mg ml^{-1} protein) in the presence of an NADPH-generating system. Protein was precipitated with 3M trichloroacetic acid and washed four times with methanol to remove unbound SMZ. Residual radioactivity was quantified by liquid scintillation counting. Data are given as mean ± s.e. mean with the number of observations in parenthesis. Statistical significance was determined by non-paired Student's t-test.

Binding followed Michaelis-Menten kinetics where estimates of Km and Vmax were 360 ± 7 μM and 70 ± 1.4 pmol min^{-1} mg^{-1}, respectively. Table 1 shows that removal of $NADP^+$, incubation in an anaerobic atmosphere, incubation at 4°C and heat denaturation of microsomes all resulted in statistically significant decreases in binding. Moreover, binding was reduced in the presence of the cytochrome P-450 inhibitors cimetidine (1 mM) and SKF-525A (0.5 mM), and when the sulphydryl compounds glutathione (0.5 mM) or L-cysteine (0.5 mM) were added to the reaction medium.

These results suggest: 1) SMZ has the potential to undergo covalent binding to avian hepatic microsomes; 2) binding is mediated by cytochrome P-450 dependent mono-oxygenases and 3) binding may involve formation of an electrophilic intermediate since the nucleophiles glutathione and cysteine inhibited binding.

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Table 1 Covalent binding of SMZ to chicken liver microsomes

Group	nmol SMZ bound mg^{-1} microsomal protein	% Inhibition	Group	nmol SMZ bound mg^{-1} microsomal protein	% Inhibition
Control	1.37 ± 0.04 (12)	0	Control	2.02 ± 0.06 (22)	0
-$NADP^+$	0.26 ± 0.02 (6)*	81	Cimetidine (1 mM)	1.43 ± 0.05 (6)*	29
Nitrogen	0.55 ± 0.03 (5)*	60	SKF-525A (0.5 mM)	0.92 ± 0.02 (5)*	54
4 °C	0.24 ± 0.01 (6)*	83	Glutathione (0.5 mM)	0.97 ± 0.03 (6)*	52
Boiled for 15 min	0.45 ± 0.01 (6)*	68	L-Cysteine (0.5 mM)	0.41 ± 0.03 (6)*	80

* $P < 0.001$ relative to control incubation mixture.

11P THE ANTIARRHYTHMIC EFFECTS OF ISCHAEMIC MYOCARDIAL PRECONDITIONING: A ROLE FOR PROTEIN KINASE C?

P Gundkalli, A Markham & G Boachie-Ansah, School of Health Sciences, University of Sunderland, Sunderland SR1 3SD.

Ischaemic myocardial preconditioning (PC) is the phenomenon whereby prior brief periods of sublethal ischaemia markedly protect against the deleterious effects of a subsequent prolonged period of ischaemia. This protection may be manifested as reduced susceptibility to ischaemia/reperfusion-induced myocardial necrosis, contractile dysfunction or arrhythmias and has been demonstrated in all animal species studied to date (Parratt, 1995). Although the precise underlying mechanism(s) remain as yet unclear, recent evidence suggests that the PC-induced limitation of infarct size may be mediated via activation of protein kinase C (PKC), at least, in the rat and rabbit (Speechly-Dick *et al.*, 1994; Ytrehus *et al.*, 1993). We sought, therefore, to establish whether PC-induced protection against ischaemic arrhythmias in the rat also involves PKC activation, by examining whether the selective PKC activator and inhibitor, 1,2 dioctanoyl-*sn*-glycerol (DOG) and chelerythrine (CHL), respectively, could mimic or antagonise the antiarrhythmic effect of PC.

Hearts, excised from male Sprague-Dawley rats (300-350 g) under anaesthesia (sodium pentobarbitone 60 mg kg^{-1}, i.p.), were perfused (10 ml min^{-1}) at 37°C in the Langendorff mode with Krebs-Henseleit solution containing 4 mM K^+. Regional ischaemia and reperfusion was induced by tightening and loosening a snare (6/0 braided silk suture) placed around the left main coronary artery. Perfusion pressure and the electrogram were continuously monitored *via* a Grass 79D Polygraph. Following a 15 min equilibration period, hearts were randomised to one of eight protocol groups: (i) *Time matched controls*, hearts were subjected to a 30 min period of ischaemia; (ii) *PC*, hearts subjected to 3 min ischaemia and 10 min reperfusion prior to 30 min ischaemia; (iii) & (iv) *DOG-treated*, hearts perfused with 10 μM and 30 μM, respectively, for 5 min, followed 10 min later by 30 min ischaemia; (v) & (vi) *PC + CHL-treated*, hearts perfused with 2.5 μM and 10 μM, respectively, starting 5 min before the 3 min PC protocol and continuing until 5 min prior to the 30 min ischaemia; (vii) *CHL controls*, hearts received 10 μM CHL for 13 min, followed 5 min later by 30 min ischaemia; (viii) *Solvent controls*, hearts perfused with 0.1% DMSO (solvent for DOG) for 5 min, followed 10 min later by 30 min ischaemia. Mean data were compared using the Kruskal-Wallis test and Dunn's *post-hoc* test. Categorical data were analysed using Fisher' exact test.

In time-matched control hearts, a 30 min sustained regional ischaemia elicited typical time-related ventricular ectopic activity. Ischaemic myocardial preconditioning (PC) significantly reduced both the incidence and severity of ischaemic arrhythmias arising during the sustained 30 min ischaemia. Thus, the mean total number of ventricular ectopic beats (VEBs) and the total incidence of ventricular fibrillation (VF) in PC hearts (n=10) were 132±43 and 0%, compared to 1105±197 and 83% in control hearts (n=12), respectively ($P<0.05$). Pre-treatment with CHL 10 μM (but not 2.5 μM) significantly attenuated the marked antifibrillatory effect of PC (60% VF incidence, compared to 0% and 83% in PC and control hearts, respectively; $P<0.05$), but failed to modify the PC effect on VEBs. 5 min DOG perfusion in place of ischaemic PC resulted in dose-dependent reductions in VF incidence, but was without any effect on VEBs. Thus, in DOG (30 μM)-treated hearts (n=10), VF incidence was 30% compared to 83% and 80% in time-matched and solvent controls, respectively ($P<0.05$), whereas mean total VEB count was 674±147 compared to 1105±197 and 822±122 in time-matched and solvent controls, respectively ($P>0.05$). 13 and 5 min perfusion with CHL 10 μM and DMSO (0.1%), respectively, failed to modify any of the arrhythmia indices measured. DOG (10-30 μM), CHL (2.5-10 μM) or DMSO (0.1%) had no significant effect on perfusion pressure or heart rate.

Taken together, these findings suggest that the pronounced antifibrillatory effect of ischaemic myocardial PC in the rat may be mediated, at least in part, via activation of PKC.

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12P ENDOTHELIN- AND U 46619-INDUCED INOSITOL PHOSPHATE FORMATION IS REDUCED IN THE KIDNEY OF SPONTANEOUSLY HYPERTENSIVE RATS

K.Becker & O.-E.Brodde, Institute of Pharmacology, University of Halle, D-06097 Halle, Germany

In the kidney of spontaneously hypertensive rats (SHR) α_1-adrenoceptor-mediated inositol phosphate(IP) formation is desensitized (Michel et al., 1993) possibly due to a decrease in renal $G_{q/11}$-protein (Michel et al. 1994). The aim of this study was to find out whether in general renal $G_{q/11}$-coupled receptors might be desensitized in the SHR. For this purpose we assessed in slices of renal cortex of 12-16 wks old SHR and age-matched normotensive Wistar-Kyoto (WKY) rats IP-generation induced by endothelins (ET) and the thromboxan A_2 (TXA_2) mimetic U 46619. IP-generation was assessed as accumulation of total [^{3}H]IP in [^{3}H]myo-inositol labelled renal slices during a 45 min incubation at 37°C in Krebs-Henseleit solution that contained 10 mM LiCl. Data are means ± s.e.mean; statistical analysis was evaluated in non-paired two-tailed t-tests with $P < 0.05$ considered significant.

In renal slices of WKY-rats ET-1 (0.1nM - 1 μM) concentration-dependently increased IP-formation; ET-1 was about 100 times more potent than ET-3. Maximal increase (at 1μM) was 154 ± 16 % (n=6) over basal (=100%) for ET-1, 127 ± 12 % (n=6) for ET-3. However,we could not test ET-1 in concentrations > 1μM and, hence, do not know whether 1μM ET-1 causes maximal IP-formation.The ET-receptor subtype non-selective antagonist bosentan (10μM) inhibited 100nM ET-1 induced IP-formation completely. On the other hand, the ET_A-receptor antagonist BQ 123 (10μM) inhibited 100nM ET-1 induced IP-formation by 71 ± 9 % (n=5), the ET_B-receptor antagonist IRL 1038 (1μM) by 26 ± 16 % (n=5), whereas BQ+IRL inhibited ET-1 induced IP-formation completely. Thus, in rat kidney ET-1 evoked IP-formation is mediated not only by ET_A- but to a minor but sizable extent also by ET_B-receptors. The TXA_2-mimetic U 46619 (10 nM-10μM) concentration-dependently (EC_{50} = 1.4±0.2μM) increased IP-formation; maximal increase (at 10μM) was 128 ± 12 % (n=6).The TXA_2-receptor antagonist SQ 29548 (1nM-1μM) inhibited 1μM U 46619 induced IP-formation with a K_i-value of 12 ± 3nM (n=4). In renal slices of SHR, however, both U 46619 and ET (ET-1 >> ET-3) evoked IP-formation was significantly reduced; maximal increases were for 10μM U 46619 111 ± 5 % (n=6, $P < 0.05$ vs. WKY) and for 1μM ET-1 120 ± 8 % (n=6, $P < 0.05$ vs. WKY).

In conclusion: in renal slices of SHR α_1-adrenoceptor- (Michel et al., 1993), ET-receptor- and TXA_2-receptor-mediated IP-formation is reduced. Thus, it appears that in general $G_{q/11}$-coupled receptors are desensitized in the kidney of SHR with established hypertension.

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13P A_1 AND A_2 ADENOSINE RECEPTOR-MEDIATED VASODILATATION IN THE GUINEA-PIG CORONARY ARTERIAL BED

C.R. Hiley, F.E.Bottrill, C.E. Otley & P.J. Richardson, Department of Pharmacology, University of Cambridge, Cambridge CB2 1QJ

Adenosine dilates the coronary artery and this is thought to be mediated by the adenosine A_{2A} receptor (Vials & Burnstock, 1993). Other reports have suggested that adenosine may use several systems to regulate coronary vascular resistance. Merkel et al. (1992) showed that A_1 receptors may also be involved and Nakhostine & Lamontagne (1993) found adenosine caused vasodilatation in hypoxia by mechanisms involving ATP-sensitive K^+ channels (K_{ATP}) and adenylyl cyclase. Vials & Burnstock (1993) also suggested adenosine acts by releasing nitric oxide. Hence we have used selective adenosine agonists and antagonists to identify the receptors present in the guinea-pig coronary arterial bed as well as using glibenclamide and N^G-nitro-L-arginine methyl ester (L-NAME) to study the respective involvement of K_{ATP} and nitric oxide in the responses.

Hearts from male Dunkin-Hartley guinea-pigs (250-600g; Tucks) were paced at 230 beats min^{-1} with platinum electrodes placed on the left ventricle and perfused retrogradely through an aortic cannula at constant flow (at a rate to maintain coronary perfusion pressure at 55-60mmHg) with physiological salt solution (composition, mM: NaCl 115.3, KCl 4.6, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, $CaCl_2$ 2.5, glucose 11.1), equilibrated with 95% O_2/5% CO_2, and containing 0.5g l^{-1} bovine serum albumin. Perfusion pressure was measured with a transducer attached to a side-arm of the aortic cannula. A latex balloon, connected to another pressure transducer, was placed in the left ventricle. Responses were measured as changes in perfusion pressure.

Addition of the A_{2A} antagonist 8-(3-chlorostyryl)caffeine (CSC, 500 nM) to the perfusate increased vascular resistance from 5.42±0.84 to 7.32±0.81mmHg.min.ml^{-1} (n = 8) but the A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10nM) had no significant effect.

Adenosine reduced coronary perfusion pressure but the log dose/response curve was biphasic. 8-PT (3μM) moved the curve to the right, but the effect was greater (37 fold) at smaller than at larger responses (2.5 fold). Both the A_1 agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA), and the A_{2A} agonist, 2-p-(-2-carboxyethyl)phenethylamino - 5'-N-ethylcarboxamidoadenosine (CGS 21680), also reduced the coronary resistance. The respective maximal responses (20.3±0.8mmHg, n = 6 and 20.1±0.6mmHg, n = 8) were similar to those of adenosine (22.7±2.3mmHg, n = 7). The log dose/response curve for CCPA was biphasic, but that of CGS 21680 was monophasic.

CSC (500nM) antagonised the responses to adenosine, moving the curve in the lower response range to the right and increasing its slope such that it was no longer biphasic (slope: control, 0.37±0.06, n = 7; with CSC = 0.76±0.07, n = 6). In contrast, DPCPX (10nM) did not affect the size of the responses obtained in the lower dose range and increased the slope of the curve (to 0.84±0.13, n = 4) such that the responses to higher doses were potentiated, although the maximum response was unchanged. Glibenclamide (1 μM) inhibited responses to CCPA, CGS 21680 and adenosine whilst L-NAME (100μM) potentiated those to 0.3 nmol (from 7.9±1.5 to 20.5±2.7mmHg) and 300 nmol adenosine (from 12.3±1.9 to 25.0±2.7mmHg, n = 7 for both).

These results suggest that basal coronary tone is regulated primarily by A_{2A} receptors, both A_1 and A_{2A} receptors may mediate vasodilation to exogenous adenosine, stimulation of either receptor can achieve the same maximal response, these receptors may interact and part of the responses mediated by both involve K_{ATP}. Furthermore, nitric oxide synthase inhibition potentiates the responses to adenosine.

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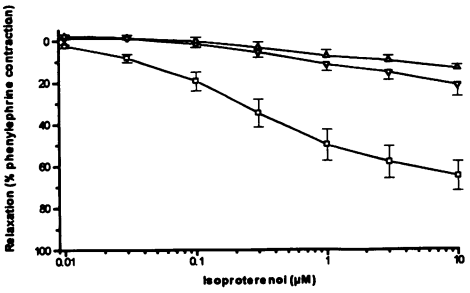
14P cGMP-ELEVATING AGENTS POTENTIATE ISOPROTERENOL EFFECTS IN RAT AORTIC SMOOTH MUSCLE: ROLE OF PDE 3

A.C. Le Monnier de Gouville, H. Coste and E. Delpy : GlaxoWellcome, 25 av du Quebec, 91951 Les Ulis France.

Increases in intracellular cAMP or cGMP content produce relaxation of vascular smooth muscle. In these cells, cAMP can be increased by stimulation of β adrenoceptors, A_2 purinoceptors, and prostacyclin receptors ; on the other hand cGMP is increased by EDRF or ANF via guanylate cyclase stimulation. The aim of our study was to investigate possible cross talk between cGMP and cAMP particularly at phosphodiesterases level.

Relaxation studies were performed in rat aortic rings precontracted with phenylephrine. Cyclic nucleotide determinations were carried out in aortic rings under conditions similar to those of the relaxation studies

In rat aortic rings with endothelium, 10μM isoproterenol (ISO) produced a 60% relaxation. In contrast, in denuded rings, 30μM ISO only produced a 20% relaxation. In rings with endothelium, pretreatment by methylene blue or L-NAME reduced relaxation to ISO to a level similar to that measured in denuded rings. In denuded rings, the relaxation to ISO (16% at 10μM) was increased to 69% and 45% respectively by pretreatement with ANF (3nM) or SNP (3nM) which do not relax on their own. Relaxation was further increased in the presence of the PDE 5 inhibitor DMPPO : (1,3-dimethyl-6-(2-propoxymethane-sulfonylamidophenyl)pyrazolo [3, 4d] -pyrimidin-4-(5H)-one), ISO only relaxed by 16% and 11% aortic rings pretreated with 1nM SNP or 30nM DMPPO respectively. However, when rings were pretreated by the combination of SNP (1nM) and DMPPO (30nM), ISO (10μM) induced 53 % relaxation. Similar results were obtained with ANF (figure 1) :



Relaxation induced by ISO in denuded rat aortic rings pretreated with ANF (1nM) ∇ , DMPPO (30nM)Δ, or the combination of ANF (1nM) plus DMPPO (30nM)□

Strikingly in denuded rings, ISO-induced relaxation was potentiated by cilostamide (a PDE 3 inhibitor) to a same extent as that measured in the presence of DMPPO plus ANF or SNP.

In rat aortic rings, ISO increased intracellular cAMP levels. This increase in cAMP was potentiated by cilostamide, rolipram (PDE 4 inhibitor) and cGMP elevating agents (ANF or SNP plus DMPPO). When cAMP levels were increased by treatment with ISO plus rolipram, cilostamide and cGMP-elevating agents were able to further increase cAMP. In contrast cGMP elevating agents and cilostamide did not potentiate each other.

In conclusion, intracellular cAMP levels and cAMP-dependent relaxation are potentiated by cGMP elevating agents. This effect is probably mediated by PDE 3 whose activity is negatively regulated by cGMP.

15P MECHANISMS FOR ENDOTHELIUM-DEPENDENT RELAXATION IN RAT ISOLATED MESENTERIC ARTERY: VARIATION WITH CONTRACTILE AGONIST

F. Plane & C.J. Garland, Department of Pharmacology, University of Bristol, Bristol, BS8 1TD.

Acetylcholine (ACh)-evoked endothelium-dependent relaxation of rat isolated mesenteric arteries appears to be mediated by both nitric oxide (NO) and NO-independent repolarization (Waldron & Garland, 1994). However, the influence of contractile mechanisms on the relative contribution of these two pathways to relaxation is unclear. In this study, the mechanisms mediating ACh-evoked relaxation of arterial segments pre-contracted with noradrenaline (NA) and the thromboxane A_2 analogue, U46619 were examined.

Male Wistar rats (250-300 g) were stunned and killed by cervical dislocation. Segments of third order mesenteric artery (D_{100} 300 ± 11 μm; n=20) were mounted in a myograph under a normalised tension (Mulvany and Halpern, 1977) for simultaneous measurement of tension and membrane potential, as previously described (Garland & McPherson, 1992). Tissues were maintained in oxygenated Krebs buffer at 37°C. All data are expressed as mean ± s.e. mean and differences between mean values were calculated using the Students' t-test.

The resting membrane potential of the muscle cells was -58.1 ± 3.9 mV (n=20 cells from 12 tissues). Application of NA (1-3 μM) stimulated depolarization and contraction of the arterial segments which were concentration-dependently reversed by ACh (0.01-5 μM). The maximum reversal of the NA-stimulated depolarization and contraction by ACh (5 μM) was 96.0 ± 2.3 % and 98.5 ± 1.5 % (n=8), respectively. Exposure to the NO-synthase (NOS) inhibitor N^G-Nitro-L-arginine methyl ester (L-NAME; 100 μM; 30 mins) alone, or in combination with N^G-Nitro-L-arginine (L-NOARG; 100 μM;30 mins), did not significantly alter ACh-evoked repolarization and relaxation. The maximum changes in membrane potential and tension evoked by ACh in the presence of both NOS inhibitors were 93.6 ± 4.6 % and 95.5 ± 2.7 %, respectively (n=4; P>0.05). Pre-incubation of arterial segments with nifedipine (0.1 μM; 10 mins) significantly depressed NA-evoked contractions and a higher concentration of NA (100 μM) was needed to induce a similar level of tone. In the presence of nifedipine, ACh-evoked relaxation was significantly inhibited (maximum relaxation; 74.8 ± 5.4 %; n=4; P<0.01) and exposure to nifedipine and the NOS inhibitors together abolished ACh-evoked relaxation. (n=4).

U46619 (0.5-1 μM) induced the same level of contraction as NA (n=10; P>0.01) but the accompanying depolarization was significantly smaller (16.6 ± 3.2 mV; n=6; P<0.01) and lagged behind the increase in tone. ACh (0.01-5 μM)-stimulated relaxation of U46619-evoked increases in tone was not significantly different from relaxation of NA-evoked contractions (maximum relaxation; 97.4 ± 2.3 %; n=10; P>0.05). However, ACh had little effect on the membrane potential of U46619-stimulated arterial segments (maximum repolarization; 3.0 ± 2.3 mV; n=6) and exposure to L-NAME (100 μM; 30 mins) significantly inhibited ACh-evoked relaxation of U46619-contracted tissues (maximal relaxation; 15.0 ± 5.2 %; n=5; P<0.01). In the presence of nifedipine, ACh-evoked relaxation of U46619-evoked contractions was unaltered (maximum relaxation; 94.9 ± 3.7 %; n=4; P<0.01).

These data demonstrate that the relative contribution of voltage-dependent and -independent mechanisms to endothelium-dependent relaxation may be determined by the processes mediating pre-contraction. ACh-evoked relaxation of nifedipine-sensitive NA-evoked increases in tone, appear to be mediated by both NO and repolarization. In contrast, in the presence of U46619, which induces tone largely via nifedipine-insensitive pathways, ACh-evoked relaxation was not accompanied by repolarization and appears to be mediated entirely by NO.

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16P ACTION OF THIAZIDES AND INHIBITORS OF CARBONIC ANHYDRASE ON TONE OF GUINEA-PIG ISOLATED SMALL MESENTERIC ARTERIES

Pickkers P, Garcha R, Schachter M, & Hughes AD. Department of Clinical Pharmacology, St Mary's Hospital Medical School, Imperial College of Science Technology & Medicine, London W21NY, UK.

We have previously shown that hydrochorothiazide (HCT) relaxes guinea-pig arteries by opening calcium-activated potassium (K_{Ca}) channels (Pickkers & Hughes, 1995). However the mechanism underlying this action of thiazides remains unclear. This study has investigated the possible role of inhibition of carbonic anhydrase (CA) in the vasorelaxant effects of thiazide diuretics.

Small arteries (internal diameter 365±15μm; n=28) were isolated from guinea-pig mesentery and studied using a myograph technique (Pickkers & Hughes, 1995). Vessels were bathed in Krebs-Henseleit physiological saline and tone was induced by 10μM noradrenaline (NA) or a high potassium physiological saline (KPSS) containing an equimolar substitution of K^+ for Na^+ (118mM). The effects of HCT, bendroflumethiazide (BFZ) a thiazide with minimal inhibitory effects on CA, acetazolamide (ACZ) an inhibitor of CA, benzolamide (BZL) a hydrophilic inhibitor of CA and ethoxzolamide (EZL) a highly lipophilic inhibitor of CA (Maren, 1992) were examined. In addition 20 min preincubation with charybdotoxin (CTX), a selective K_{Ca} channel blocker was used in some experiments to determine whether vasorelaxation was due to K_{Ca} channel activation. Vasorelaxation was calculated as % reduction of induced tone. Data are means±s.e.means. Statistical comparisons were made using a 2-sample Wilcoxon test, p<0.05 was considered significant.

BFZ (30μM) had little effect on NA-induced tone (relaxation = 16±8%; n=12) compared with 30μM hydrochlorothiazide (74±12%; n=8). CTX (100nM) inhibited responses to HCT (9±8%; n=4, p<0.05), but did not significantly affect the small relaxation in response to BFZ (7±2%; n=4, NS). ACZ, BZL and EZL relaxed NA-induced tone in a concentration-dependent manner (E_{max} = 67±12%, pD_2 = 5.7±0.1, n=4; E_{max} = 99±1%, pD_2 = 6.3±0.3, n=4; and E_{max} =99±1%, pD_2 = 7.0±0.3, n=4 respectively). These effects were blocked by 100nM CTX (n=4-7), and HCT and the inhibitors of CA failed to relax KPSS-induced tone.

HCT and other inhibitors of CA relax guinea-pig vascular smooth muscle through a mechanism involving K_{Ca} channels. This effect of HCT is not shared by BFZ and may therefore be related to its activity as an inhibitor of CA. Since this vasorelaxation was seen in response to the hydrophilic CA inhibitor, BZL, the action of these drugs may not involve an effect on intracellular CA.

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17P EVIDENCE FOR COEXISTENCE AND DESENSITISATION OF A_2 ADENOSINE RECEPTOR SUBTYPES IN NG108-15 CELLS

S.J.Mundell, S.Smith and E.Kelly
Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD

The presence and desensitization of A_2 adenosine receptors positively coupled to adenylyl cyclase in the NG108-15 neuroblastoma x glioma hybrid cell line has been reported (Keen *et al.*,1989). The purpose of this study was to further characterize the adenosine receptor subtypes present in this cell line and their susceptibility to agonist-induced desensitization. NG108-15 cells (passage 20-40) were cultured in Dulbecco's modified Eagle's medium containing 6% fetal bovine serum. Following drug treatment (if any), cells were harvested, washed and frozen at -70°C until required. Adenylyl cyclase activity was then assessed in cell homogenates using a binding protein assay (Williams *et al.*, 1993) and expressed as pmol cAMP / min / mg protein.

5'-(N-Ethylcarboxamido)-adenosine (NECA), an adenosine receptor agonist produced a concentration-dependent increase in adenylyl cyclase activity (EC_{50} 6.0±0.5μM, n=5). However the Hill Coefficients for these curves were much less than 1 (0.65±0.06, n=5), suggesting the possibility of both A_{2a} and A_{2b} adenosine receptors being present. Resolution of these two sites indicated the presence of high (EC_{50} 0.70±0.03μM, n=5) and low (EC_{50} 10.0±0.2μM, n=5) potency sites for NECA-activated adenylyl cyclase activity. CGS21680 (0.1-1000μM), a selective A_{2a} over A_{2b} adenosine receptor agonist, also displayed high (EC_{50} 0.27±0.13 μM, n=6) and low (EC_{50} 26.3±1.4 μM, n=6) potency sites for adenylyl cyclase activation. The non-selective A_2 adenosine antagonist xanthine amine congener (30μM) almost completely inhibited NECA-induced (0.1-300μM) increases in adenylyl cyclase activity. On the other hand 1,3,7-trimethyl-8-(3-chlorostyryl)xanthine (CSC; 1μM), a selective A_{2a} receptor antagonist abolished the high potency site for activation such that NECA now had a single EC_{50} of 34.4±0.9μM, n=6, the Hill coefficient being increased from 0.70±0.02 in the absence of CSC to 1.00±0.03, in the presence of CSC, n= 6.

Pretreatment of NG108-15 cells with NECA (10μM) produced time-dependent desensitization of subsequent NECA (10μM) stimulated adenylyl cyclase activity (57±4% desensitization after 30min pretreatment increasing to 80±3% after 4hrs). Pretreatment of cells with the proposed βARK inhibitor zinc (200μM; 75min) decreased the level of desensitization of NECA-stimulated adenylyl cyclase activity produced by NECA pretreatment (10μM; 60min) from 56±5% to 28±4% ($p>0.05$ Mann Whitney test). Thus there is rapid desensitization of adenosine receptor-activated adenylyl cyclase in these cells which may be mediated by a βARK-like receptor kinase. This work indicates that A_{2a} and A_{2b} adenosine receptors coexist in NG108-15 cells. It should be possible in this model system to compare the rates and mechanisms of desensitization of these two closely related adenosine receptors.

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18P DIRECT LABELLING OF $P2X_3$ AND $P2X_4$ PURINOCEPTORS USING [^{35}S]ATPγS

Miller, K.J., Michel, A.D., Buell, G., Lündstrom, K., Humphrey, P.P.A. Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Cambridge, CB2 1QJ.

At present four P2X purinoceptors designated $P2X_1$ - $P2X_4$ have been identified (see Bo *et al.*, 1995). We have shown that $P2X_1$ purinoceptors can be directly labelled using [^{35}S]ATPγS (Michel *et al.*, 1996) and in this study have examined whether [^{35}S]ATPγS can also label the $P2X_3$ and $P2X_4$ purinoceptor.

Rat recombinant P2X purinoceptors were expressed in CHO-K1 cells using Semliki forest virus vectors and membranes were prepared from these cells as described (Michel *et al.*, 1996). In binding studies membranes, competing drugs and 0.1 nM [^{35}S]ATPγS were incubated in a 50mM Tris 1mM EDTA assay buffer (pH 7.4) at 4°C for 3 hrs. Incubations were terminated by vacuum filtration. Non specific binding was defined using 10μM ATPγS. Data are the mean ± s.e. mean of 3-6 experiments.

[^{35}S]ATPγS (0.1 nM) bound reversibly to both the $P2X_3$ and $P2X_4$ purinoceptors ($P2X_3$ K_{-1} = 0.063±0.003 min^{-1}; $P2X_4$ K_{-1} = 0.024± 0.002). In saturation studies [^{35}S]ATPγS identified single populations of $P2X_3$ and $P2X_4$ purinoceptors with B_{max} values of 38 ± 9 and 52 ± 15 $pmol.mg^{-1}$ protein, respectively.

In competition studies (see Table) all the agonists possessed high affinity for the recombinant $P2X_3$ purinoceptor. Similar to data from functional studies (Chen *et al.*, 1995), 2-me-S-ATP was the most potent compound in binding studies on the $P2X_3$ purinoceptor. αβ meATP also possessed higher affinity at this "αβmeATP-sensitive" $P2X_3$ purinoceptor (Chen *et al.*, 1995) than at the $P2X_4$ or $P2X_1$ (pIC_{50}=7.1; Michel *et al.*, 1996) purinoceptors. In agreement with data from functional studies suramin possessed lower affinity for the $P2X_4$ than for $P2X_3$ or $P2X_1$ (pIC_{50}=6.6, Michel *et al.*, 1996) purinoceptors.

These data suggest that [^{35}S]ATPγS can label the $P2X_3$ and $P2X_4$ purinoceptors, in addition to labelling $P2X_1$ purinoceptors. However, since agonist affinities were higher in binding than functional studies it is possible that [^{35}S]ATPγS may label a desensitised state of the P2X purinoceptor. Furthermore, the finding that antagonists may affect binding in an allosteric rather than a competitive manner (Michel *et al.*, this meeting) suggests that the binding characteristics of the P2X purinoceptor are as complex as those of other ligand-gated cation channels.

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	$P2X_3$		$P2X_4$			$P2X_3$		$P2X_4$	
Compound	*pIC_{50}*	*Hill slope*	*pIC_{50}*	*Hill slope*	**Compound**	*pIC_{50}*	*Hill slope*	*pIC_{50}*	*Hill slope*
ATP	9.5 ± 0.14	1.0 ± 0.04	9.3 ± 0.12	0.9 ± 0.03	**Suramin**	5.7 ± 0.12	1.0 ± 0.04	3.4 ± 0.08	0.6 ± 0.02
ATPγS	9.3 ± 0.11	1.0 ± 0.02	9.2 ± 0.06	1.0 ± 0.04	**Cibacron blue**	6.0 ± 0.03	3.9 ± 0.98	5.4 ± 0.14	0.6 ± 0.02
2-me-S-ATP	9.9 ± 0.08	1.4 ± 0.19	8.7 ± 0.09	0.9 ± 0.05	**PPADS**	3.9 ± 0.12	1.3 ± 0.09	3.2 ± 0.07	0.7 ± 0.12
αβmeATP	8.4 ± 0.11	1.1 ± 0.05	7.8 ± 0.11	1.0 ± 0.02	**Dextran**	5.5 ± 0.13	0.5 ± 0.02	<3	<0.2

19P EVIDENCE FOR ALLOSTERIC EFFECTS OF ANTAGONISTS AT THE $P2X_4$ PURINOCEPTOR LABELLED USING [^{35}S]ATPγS

Michel., A.D., Miller, K.J., and Humphrey, P.P.A. Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Cambridge, CB2 1QJ.

The $P2X_4$ purinoceptor can be labelled with [^{35}S]ATPγS (Miller *et al.*, this meeting). We have now studied the effects of P2 purinoceptor antagonists on [^{35}S]ATPγS binding to the $P2X_4$ purinoceptor and have obtained data which suggest that these antagonists may allosterically regulate [^{35}S]ATPγS binding to this receptor.

Rat recombinant $P2X_4$ purinoceptors were expressed in CHO-K1 cells, using a Semliki forest virus vector, and membranes were prepared from these cells as described (Michel *et al.*, 1996). Competition binding studies were performed as described previously (Miller *et al.*, this meeting). In kinetic studies membranes and radioligand were incubated for 2 hrs before initiating dissociation by addition of 10μM ATPγS in the presence or absence of the indicated compounds. Incubations were terminated by vacuum filtration. Data are the mean ± s.e.mean of 3-4 experiments.

In competition studies binding of [^{35}S]ATPγS (0.1nM) was inhibited by cibacron blue (RB2) and suramin but the Hill slopes were less than unity (Miller *et al.*, this meeting). Furthermore, d-tubocurarine (d-TC), a P2 purinoceptor antagonist in PC12 cells (Nakazawa *et al.*, 1991) potentiated binding (162 % of control at 1 mM) with a pEC_{50} value of 4.2 ± 0.1. To determine whether these complex effects of the antagonists were due to allosteric actions we examined their affects on the dissociation of [^{35}S]ATPγS from the receptor.

Dissociation of [^{35}S]ATPγS from the $P2X_4$ purinoceptor measured following isotopic dilution was best described by a single exponential with a $t_{1/2}$ of 29.9 ± 2.9 mins. Dissociation of the radioligand measured after 25mins (50.8 ± 1.0% of pre-dissociation level) was increased in the presence of 100 μM suramin (35.3 ± 3.2% of pre-dissociation level) or 100μM RB2 (21.5 ± 5.1% of pre-dissociation level) while 100μM d-TC reduced dissociation (110.2 ± 7.7% of pre-dissociation level). Concentration-effect curves for the ability of suramin, RB2 and d-TC to affect dissociation of the radioligand were determined and yielded pEC_{50} values of 3.63±0.16, 5.79±0.10, and 4.18±0.09, respectively. Several other P2 purinoceptor antagonists, including DIDS, PPADS and pyridoxal 5'-phosphate (Humphrey *et al.*, 1995), increased [^{35}S]ATPγS dissociation at concentrations of 100μM.

The ability of P2 purinoceptor antagonists to affect dissociation of [^{35}S]ATPγS from the $P2X_4$ purinoceptor suggests that they may affect [^{35}S]ATPγS binding to this receptor in an allosteric rather than a competitive manner. Although an additional competitive effect of the antagonists at the $P2X_4$ purinoceptor cannot be ruled out it is noteworthy that P2 antagonists do not act as simple competitive antagonists in functional studies (Nakazawa *et al.*, 1991).

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20P REQUIREMENTS FOR BOTH PROTEIN KINASE C AND MITOGEN-ACTIVATED PROTEIN KINASE ACTIVITIES FOR P_2-PURINERGIC STIMULATION OF ENDOTHELIAL PROSTACYCLIN PRODUCTION

M.R. Boarder , C. Brown, & V. Patel, Dept of Cell Physiology and Pharmacology, University of Leicester, University Road, LE1 9HN.

ATP elicits prostacyclin production from cultured bovine aortic endothelial cells by acting on co-existing P_{2Y}- and P_{2U}- purinoceptors by a mechanism which has been proposed to comprise stimulation by raised cytosolic Ca^{2+} with modulation by protein kinase C (PKC). However, we have recently shown that tyrosine phosphorylation is also required (Bowden et al, 1995). Here, we present evidence that both PKC and mitogen activated protein kinase (MAPK) are required for the endothelial P_2-purinoceptor control of prostacyclin.

The presence of specific PKC isoforms in cultured bovine aortic endothelial cells was determined by Western blot using isoform specific antiserum, either on control cells or on those subject to pretreatment with 1 μM phorbol myristate acetate (PMA). Phosphorylation of MAPK on the tyrosine residue was determined by Western blotting with a phospho-MAPK specific antiserum; we have shown elsewhere that this corresponds to activation of MAPK enzymic activity in these cells (Patel et al, 1996). Prostacyclin production was estimated by assay of 6-keto $PGF_{1\alpha}$ by radioimmunoassay. Drugs used were the non-selective PKC inhibitors Ro 31-8220 and Go 6850 ((Davis et al, 1989; Martiny-Baron et al, 1993), the selective inhibitor for Ca^{2+} sensitive PKC isoforms, Go 6976 (Martiny-Baron et al, 1993) and the inhibitor of the MAPK activating enzyme (MEK) PD98059 (Pang et al, 1995).

Immunoblots showed the presence of α, ε, and ζ, but not of β, γ, δ, η and θ isoforms of PKC. Afer 6h pretreatment with PMA 90 % of PKC-α immunoreactivity was lost, while ε and ζ isoforms remained largely unchanged, and the 6-keto $PGF_{1\alpha}$ was not attenuated. Both Ro 31-8220 and Go 6850 inhibited the 6-keto $PGF_{1\alpha}$ response, while the more selective Go 6976 did not (all at 10 μM). These results show that PKC (but not PKC-α) is required for the stimulation of prostacyclin production by P_2-purinoceptors.

Agonists of P_{2Y}- and P_{2U}- purinoceptors stimulate tyrosine phosphorylation and activation of both p42 and p44 forms of MAPK (Patel et al, 1996). The ATP (300 μM) stimulated tyrosine phosphorylation of MAPK was inhibited by 10 μM PD98059. The ATP elicted 6-keto $PGF_{1\alpha}$ response was inhibited by PD98059 with a mean EC_{50} of 1.35 μM. With 10 μM PD98059 the 6-keto $PGF_{1\alpha}$ response was lost (control, 81.0 ± 2.0; 300 μ ATP, 245.9 ± 7.0*; 10 μM PD98059, 6.7 ± 2.6; PD98059 + ATP, 11.93 ± 3.5**: data are pg 6 keto-$PGF_{1\alpha}$ per 0.1 ml medium, * $P < 0.005$ compared to control and ** $P < 0.0001$ compared to ATP without PD98059). The phospholipase C response to P_2-purinoceptor stimulation was not inhibited with this concentration dependency.

These results provide strong evidence that MAPK activation is required for the control of endothelial prostacyclin responses by ATP. Taken with a previous report (Bowden et al, 1995) the study shows that the control by ATP of endothelial prostacyclin production requires PKC, tyrosine phosphorylation and MAPK.

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21P MAP KINASE ANTISENSE PHOSPHOROTHIOATE DEOXYOLIGONUCLEOTIDES INHIBIT PDGF-STIMULATED DNA SYNTHESIS IN RAT AORTIC SMOOTH MUSCLE

C. J. M. Robinson, A. McLees, G. W. Gould[1] & R. Plevin.
Department of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow. G1 1XW [1]Division of Biochemistry & Molecular Biology, Institute of Biomedical & Life Sciences, University of Glasgow, Glasgow. G12 8QQ

In previous studies we have shown that in both vascular and airways smooth muscle cell types, PDGF stimulates a sustained activation of MAP kinase and this may be obligatory for the initiation of DNA synthesis by PDGF (Malarkey *et al.*, 1995; Malarkey *et al.*, 1996). To test this hypothesis we have utilised the 17-mer antisense phosphorothioate modified deoxyoligonucleotides (ODN) directed against both the 42 and 44 kDa forms of MAP kinase (Sale *et al.*, 1995) and determined the effect upon MAP kinase expression and function in vascular smooth muscle cells.

Rat aortic smooth muscle cells (RASMC) were grown to approximately 50% confluency and preincubated with 1-10μM of either antisense (5'-GCC GCC GCC GCC GCC-AT 3') sense (5'-ATG GCG GCG GCG GCG GC-3') or non-sense sequence (5' CGC GCG CTC GCG CAC CC-3') with transfection reagent (DOTAP 1/1) for 8 h. Cells were then washed and incubated with fresh oligonucleotides in the absence of DOTAP for a further 64 h. MAP kinase expression was measured by Western blotting and activities of MAP kinase, MAP kinase kinase (MEK) and p90 ribosomal S6 kinase ($p90^{s6k}$) by *in vitro* kinase assay. DNA synthesis was measured by incorporation of [^{3}H]thymidine into cells over a 24 h period. All results are expressed as mean±s.e.mean, n=3.

Pretreatment of RASMC with antisense ODN (AS) resulted in the complete loss in the cellular expression of MAP kinase at concentrations between 5-10μM of the ODN. In contrast, no loss of MAP kinase was observed with 10μM of either sense or non-sense ODN. Expression of the homologous stress kinases, p46 JNK and p38 MAP kinase, were also not affected by antisense ODN pretreatment. MAP kinase activity stimulated by either PDGF or AII was abolished by antisense pretreatment (c.p.m.: cont=19,749±793; cont+AS=12,227±6264; AII =48,437±3591; AII+AS=6826±2815; PDGF=71,907±8023; PDGF+AS=11356±4738). In contrast, stimulation of the immediate upstream activator of MAP kinase, MEK, was not affected. Antisense pretreatment also resulted in the substantial inhibition of AII and PDGF-stimulated $p90^{s6k}$ activity, an immediate downstream target of MAP kinase (fold stimulation: PDGF=4.14±1.28; PDGF+AS=1.58±0.49; AII=4.0±0.63; AII+AS=0.91±0.64). Furthermore, PDGF-stimulated DNA synthesis was essentially abolished following antisense treatment while basal incorporation was also reduced (d.p.m.: cont=8527±2501; cont+AS=963±448; PDGF =55,818±4508; PDGF+AS =2378±209).

These results indicate that in vascular smooth muscle cells MAP kinase appears to be essential for the initiation of DNA synthesis in response to growth factors. Thus antisense deoxyoligonucleotides may represent useful tools for the treatment of diseases where accelerated smooth muscle cell growth is a feature.

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22P THE MAP KINASE KINASE INHIBITOR PD098059 PREVENTS PDGF-STIMULATED DNA SYNTHESIS IN RAT AORTIC SMOOTH MUSCLE CELLS

R. Plevin, P. H. Scott, A. McLees & G. W. Gould[1].
Department of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow. G1 1XW [1]Division of Biochemistry & Molecular Biology, Institute of Biomedical & Life Sciences, University of Glasgow, Glasgow. G12 8QQ

A major cell signalling pathway stimulated by both growth factors and G-protein receptor coupled agonists involves the activation of the mitogen-activated protein (MAP) kinases (Malarkey *et al.*, 1995). This enzyme family is believed to play a crucial role in the initiation of DNA synthesis in smooth muscle cells (Robinson *et al.*, 1996). Recently, PD098059 an inhibitor of the activation of the immediate upstream activator of MAP kinase, MAP kinase kinase (MEK) has been developed (Dudley *et al.*, 1995; Alessi *et al.*, 1995). In this study we have investigated the effects of this compound upon angiotensin II (AII) and platelet-derived growth factor (PDGF)-stimulated MAP kinase activation and DNA synthesis in vascular smooth muscle cells.

Rat aortic smooth muscle cells (RASMC) were rendered quiescent by serum deprivation for 48 h. MAP kinase activity was assayed by Western blotting or by *in vitro* kinase assay. MAP kinase kinase (MEK) activity was also assayed by *in vitro* kinase assay using wild type MAP kinase and the EGF receptor peptide as sequential substrates. DNA synthesis was estimated by measuring [^{3}H]thymidine incorporation over a 24 h period. All results are expressed as mean±s.e.mean, n=3.

In RASMC, both AII (100nM) and PDGF (50ngml^{-1}) stimulated a transient activation of MEK activity peaking at 5min and returning to near basal values by 15min. However, the initial magnitude of MEK activation was greater in response to PDGF (fold stimulation: AII=13.8±0.4; PDGF=38.5±6.3). AII-stimulated MAP kinase activity was approximately equal in magnitude to PDGF (fold stimulation: AII=5.2±1.72; PDGF=7.44±2.6), but was transient whilst the response to PDGF was sustained. Pretreatment of the cells with a maximal concentration of PD098059 (50μM PD) for 30 min reduced both AII and PDGF-stimulated MEK activity by approximately the same proportion (% inhibition: AII=73± 16; PDGF=71±14), however a greater residual activation of MEK was observed in PDGF stimulated cells. PD098059 reduced AII-stimulated MAP kinase activity at 5 min but only marginally affected the PDGF response (% inhibition: AII=61±8; PDGF=10±7). However, PD098059 substantially reduced PDGF-stimulated MEK activity at 30 min and this corresponded with a loss of MAP kinase activity (fold stimulation: cont=2.7±0.15; cont+PD=0.97±0.08). PDGF stimulated an 8-10 fold increase in DNA synthesis whilst AII was without effect. PD098059 reduced the response to PDGF by approximately 90% (dpm:cont=1376±338; cont+PD=1412±131;PDGF=13457±799;PDGF+PD=2067±60).

These studies show that PD098059 is more effective against AII-stimulated MAP kinase activity because of the relative magnitudes of MEK activation induced by the two agonists. However, despite only being effective against prolonged PDGF stimulated MAP kinase activity, PD098059 abolished DNA synthesis.

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23P ANGIOTENSIN II-STIMULATED PHOSPHORYLATION OF MITOGEN-ACTIVATED PROTEIN KINASES IN SHR- AND WKY-DERIVED VASCULAR SMOOTH MUSCLE CELLS

N. Wilkie, L.L. Ng* & M.R. Boarder. Department of Cell Physiology and Pharmacology, and *Department of Medicine and Therapeutics, University of Leicester, University Road, Leicester LE1 9HN.

We have previously reported that angiotensin II (AII) and phorbol myristate acetate (PMA) stimulate mitogen-activated protein kinases (MAPKs) in vascular smooth muscle cells. This stimulated MAPK activity was elevated in cells derived from spontaneously hypertensive rats (SHR) compared to cells derived from normotensive control animals, WKY (Wilkie *et al*, 1995). MAPK activation requires dual tyrosine and threonine phosphorylation by an activating enzyme called MEK (MAPK kinase). In this report we look at the possibility of measuring MAPK phosphorylation using an antiserum raised against the tyrosine phosphorylated form of $p44^{mapk}$.

SHR and WKY cells were cultured from 12 week old rats and used between passages 8 - 14. Cells were stimulated with 100nM AII or PMA for 5 min. The MAPK activities of the cell extracts were then compared using a kinase assay, which measured the ^{32}P incorporation into a nonapeptide substrate that was partially selective for MAPK. MEK activity directed at both p44 and p42 forms of MAPK was estimated by Western blot using an antibody raised against tyrosine 204 phosphorylated MAPK, followed by densitometric scanning of the autoradiograph.

Protein equalised SHR and WKY cells extracted from culture were shown to contain equal abundance of both $p42^{mapk}$ and $p44^{mapk}$. Stimulated MAPK activity, however, was found to be greater in the SHR cells (as measured using the assay). Results show mean ± s.e.m., cpm / μg protein, n = 4. Unstimulated WKY = 340 ± 46; unstimulated SHR = 501 ± 111; WKY + AII = 436 ± 79; SHR + AII = 1177 ± 236 (SHR / WKY comparison : $p < 0.05$, students T test); WKY + PMA = 476 ± 78; SHR + PMA = 1082 ± 234 ($p < 0.05$). These results correlate closely with those obtained using the Western blotting technique involving the phospho-MAPK antibody. Immunoblots were scanned for each of the 2 molecular weight bands and data expressed as optical density units / μg protein, mean ± s.e.m., n = 5. With SHR derived cells, for $p42^{mapk}$, control = 0.023 ± 0.010, AII stimulated = 1.37 ± 0.56, and for $p44^{mapk}$, control = 0.007 ± 0.004, AII stimulated = 0.41 ± 0.27. When the results from WKY were expressed as a % of those from SHR derived cells, the analysis showed that AII stimulated $p42^{mapk}$ phosphorylation in WKY cells = 45.9 ± 9.3% and PMA stimulated = 51.0 ± 7.7% of that in SHR cells. For $p44^{mapk}$, the WKY stimulation was, for AII stimulated = 53.7 ± 9.5% and for PMA stimulated = 54.1 ± 8.7% of that seen in SHR cells.

These results show that by using the phospho-MAPK antibody it is possible to assay the agonist stimulation of tyrosine phosphorylation of p42 and p44 forms of MAPK separately, providing an assay for the AII stimulation of MEK. The results demonstrate for the first time that stimulation of MEK activity is higher in SHR than in WKY derived cells. This is consistent with earlier results using the peptide kinase assay, namely that MAPK activity is higher in SHR than in WKY derived cells in response to both AII and PMA stimulation, despite similar levels of abundance of MAPK protein. Here we show that this is the result of enhanced MEK activity in the SHR cells

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24P AGONIST-STIMULATED DIACYLGLYCEROL ACCUMULATION AND PROTEIN KINASE C ISOFORM TRANSLOCATION IN CHO CELLS EXPRESSING RECOMBINANT MUSCARINIC RECEPTORS

J.A. Stuart & S.R. Nahorski, Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN.

Muscarinic receptor subtypes M_1 and M_3 link preferentially to phosphoinositidase C (PIC) via $G_{q/11}$, and activation leads to the generation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which releases intracellular Ca^{2+}, and diacylglycerol (DAG), which activates various forms of protein kinase C (PKC) (Caulfield, 1993). Agonist-stimulated PIC and PKC activations were examined in a Chinese hamster ovary (CHO-k1) cell line expressing recombinant human M_1 or M_3 muscarinic receptors.

Confluent CHO cell monolayers were washed with Krebs-Henseleit buffer (KHB) and incubated at 37°C for 20 min before additions of methacholine (MCh). Incubations were terminated by the addition of ice-cold methanol and samples were processed for the determination of DAG and $Ins(1,4,5)P_3$. DAG mass was measured by radioenzymatic conversion to [^{32}P] phosphatidic acid using [^{32}P]ATP and DAG kinase. 1 mM MCh addition to CHO-M_3 cells caused a time-dependent accumulation of DAG with two distinct phases. The first phase was rapid (2.07 ± 0.13 (basal), rising to a peak of 3.81 ± 0.01 nmol/mg protein after 10 sec) and transient (declining to basal levels within 1min), the second was larger and more sustained (4.72 ± 0.32 nmol / mg protein after 30 min, all n=3). $Ins(1,4,5)P_3$, measured by mass assay (Challiss *et al.*, 1988), accumulated with a rapid, transient phase (basal [$Ins(1,4,5)P_3$] =13.7 pmol / mg protein rising to 185.0 ± 22.1 pmol / mg protein after 10 sec stimulation) which declined to a lower, more sustained phase (60.0 ± 9.4 pmol / mg protein after 5 min, all n=3). Addition of 1 mM MCh to CHO- M_1 cells caused similar time-dependent accumulations of DAG and $Ins(1,4,5)P_3$.

Translocation of PKC immunoreactivity was used as a measure of PKC activation. Cells were lysed in a buffer containing protease inhibitors and Ca^{2+} chelators. Unbroken cells and nuclear material were removed from lysates by centrifugation (500 x *g*, 5 min) and cytosolic fractions were separated from particulate fractions by further centrifugation (30,000 x *g* for 20 min). Cell fractions were separated on a 10 % SDS-polyacrylamide gel, transferred to nitrocellulose and incubated with monoclonal antibodies against various isoforms of PKC.

Under basal conditions, CHO cell lysates contained at least five different PKC isozymes (α, ε, γ, ι, and ζ). PKCα was predominantly associated with the cytosolic fraction, PKCε with the particulate, and PKCι and ζ were bimodally distributed. Incubation of CHO-M_1 cells with 1 mM MCh induced a transient translocation of PKCα immunoreactivity from the cytosolic fraction to the particulate fraction (detected within 1 min, almost total loss from the cytosol after ~5 min). Phorbol ester (1 μM PDBu) induced a more rapid, and greater, translocation of PKCα than MCh (total loss from the cytosol within 1 min), which was sustained for at least 30 min. Both PDBu and MCh induced a greater association of PKCε with the particulate fraction. The isozyme PKCζ did not translocate in response to either stimuli.

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25P DESENSITISATION OF SOMATOSTATIN-INDUCED INTRACELLULAR CALCIUM INCREASES FOLLOWING HUMAN RECOMBINANT sst_5 RECEPTOR ACTIVATION

G.F. Wilkinson, W. Feniuk and P.P.A. Humphrey. Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Cambridge CB2 1QJ.

Activation of human recombinant somatostatin (SRIF) sst_5 receptors increases intracellular calcium ion concentration ($[Ca^{2+}]_i$) and stimulates the formation of inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) (Akbar *et al.*, 1994). The aim of this study was to determine the mechanisms involved and to compare them with the changes in $[Ca^{2+}]_i$ and $Ins(1,4,5)P_3$ produced by UTP.

$Ins(1,4,5)P_3$ mass was measured in CHO-K1 cells stably transfected with the human recombinant SRIF sst_5 receptor, using a radioreceptor assay (Challiss *et al.*, 1988), while changes in $[Ca^{2+}]_i$ were determined by fluorescence imaging of small populations of cells (6-12) loaded with fura-2AM (4μM).

SRIF produced concentration-dependent increases in $[Ca^{2+}]_i$ (pEC_{50} 7.02 ± 0.06) from resting levels of 39.7 ± 3.6 nM to 347.0 ± 67.0 nM at 10^{-6}M. Maximal increases in $[Ca^{2+}]_i$ in response to UTP (10^{-4}M) were 775 ± 172 nM. Peak increases were reached approximately 15 to 20s after agonist addition. SRIF (10^{-6}M) also stimulated the formation of $Ins(1,4,5)P_3$ from a resting concentration of 10.3 ± 1.2 pmoles/well to 22.3 ± 3.2 pmoles/well after 10s. $Ins(1,4,5)P_3$ levels fell after this time but remained significantly ($p < 0.05$) elevated after 60s. UTP (10^{-4}M) stimulated increases in $Ins(1,4,5)P_3$ formation (23.6 ± 2.1 pmoles/well at 10s), with a time course similar to that for SRIF.

Incubation of cells for 18 hours with pertussis toxin (PTx) (0.1 - 100 ng ml^{-1}) resulted in a rightward shift and a reduction in the maximum of the $[Ca^{2+}]_i$ concentration-effect curve to SRIF. After incubation with 100 ng ml^{-1} PTx, SRIF induced increases in $[Ca^{2+}]_i$ were abolished, whereas responses to UTP (10^{-4}M) were unaffected (772.0 ± 118.0 nM, in the presence of 100 ng ml^{-1} PTx).

In the absence of extracellular Ca^{2+} (+ 1mM EGTA) peak responses to SRIF and UTP were unchanged. Thapsigargin (10μM) pretreatment (10 min) abolished $[Ca^{2+}]_i$ increases in response to SRIF (10^{-6}M) and UTP (10^{-4}M). Preincubation with SRIF (10^{-8} - 10^{-6}M) for 5 min resulted in a concentration-dependent desensitisation of subsequent $[Ca^{2+}]_i$ responses to SRIF (10^{-6} M) (96 ± 5% reduction after preincubation with 10^{-6}M SRIF) but not to UTP. However, if UTP was administered first, subsequent responses to SRIF were not observed. In the absence of extracellular Ca^{2+}, preincubation with SRIF reduced responses to UTP by 54.5 ± 5.0%, while prior exposure to UTP abolished effects to SRIF.

In conclusion, both SRIF and UTP stimulate the formation of $Ins(1,4,5)P_3$ and increases in $[Ca^{2+}]_i$. However, these responses to SRIF and UTP appear to be mediated via distinct G-proteins. The desensitisation of responses to SRIF following prior exposure to UTP in the absence of extracellular Ca^{2+} suggests that desensitisation is in part due to an effect on the Ca^{2+} pool. However, the observation that prior exposure to SRIF desensitised responses to SRIF, but not UTP, suggests that other mechanisms are also involved, possibly at the level of the sst_5 receptor/pertussis toxin-sensitive G-protein complex.

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26P SOMATOSTATIN RECEPTORS IN Neuro2A NEUROBLASTOMA CELLS: PHARMACOLOGICAL CHARACTERISATION AND INTERNALISATION OF [^{125}I]-BIM-23027, A SOMATOSTATIN ANALOGUE

J.A. Koenig[1,2], R. Kaur[2], J.M. Edwardson[1] and P.P.A. Humphrey[2]. 1) Department of Pharmacology, 2) Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Cambridge CB2 1QJ.

Agonist stimulation of G-protein-coupled receptors typically results in internalisation of the receptor and, in some cases, of the agonist as well. In the case of somatostatin receptors, there have been conflicting reports as to whether somatostatin internalisation occurs following its action at its receptor (Hofland *et al.*, 1995; Sullivan and Schonbrunn, 1986). We have examined the characteristics of somatostatin receptors in Neuro2A neuroblastoma cells.

Ligand binding studies, using either [^{125}I]-SRIF-14 or [^{125}I]-BIM-23027, were initially carried out on cell membranes prepared in 10 mM Hepes, pH 7.4, 11 mM $MgCl_2$, 1mM EDTA, with 10 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor and 0.2 mg/ml bacitracin. The binding of 0.05 nM [^{125}I]-SRIF-14 could be totally inhibited by BIM-23027, suggesting that the somatostatin receptors in these cells were of the sst_2 subtype (Raynor *et al.*, 1993). The affinity estimates (pIC_{50} ± s.e.m., n=3) for a number of a number of ligands, including SRIF-14 (9.91 ± 0.04), SRIF-28 (9.69 ± 0.02), L362855 (9.60 ± 0.04) and BIM-23056 (7.09 ± 0.05) against [^{125}I]-BIM-23027 binding were consistent with this conclusion (Holloway *et al.*, 1995).

The binding of [^{125}I]-BIM-23027 was then examined in intact Neuro2A cells grown on 24-well plates. In order to determine the proportion of ligand bound either at the surface or internalised within the cell, [^{125}I]-BIM-23027 (0.2 nM) was incubated with cells for 30 min at 37°C. The cells were then washed for varying times with cold buffer at 4°C either pH 7 or pH 5 to prevent further internalisation or recycling of the ligand. Control cells were washed rapidly (less than 30 s wash time) with pH 7 buffer. The amount of ligand remaining reached a plateau of 80 ± 4 % (n=3) at pH 7 or 66 ± 6% (n=3) at pH 5. These results indicate that most of the ligand remaining associated with the cells had been internalised. In subsequent experiments, a 10-min wash with pH 5 buffer was used routinely to quantitate internalised ligand. If cellular ATP was depleted by pre-treatment with antimycin and deoxyglucose, the amount of ligand internalised accounted for only 34 ± 4% of control. This suggested that, in common with most receptor-mediated endocytic mechanisms, the internalisation of [^{125}I]-BIM-23027 was ATP-dependent. Its internalisation was also time-dependent, being detectable at 2 min and reaching a maximum after 30 min.

Removal of high-affinity agonist binding by pertussis toxin (0.5μg/ml) treatment decreased the amount of ligand internalised to 60 ± 9 % of control (n=4). Treatment of cells with hyperosmolar sucrose (0.5 M), which is thought to prevent internalisation via clathrin-coated pits, reduced the amount of ligand internalised to 3 ± 3 % of control. In contrast, binding of [^{125}I]-BIM-23027 to membrane homogenates was inhibited only slightly by the presence of 0.5 M sucrose (67 ± 5 % of control, n=3). The internalisation of [^{125}I]-BIM-23027 could be inhibited in a concentration-dependent manner by SRIF14 (pIC_{50} = 7.8 ± 0.1, n=3).

We have demonstrated that Neuro2A cells contain sst_2 somatostatin receptors and that somatostatin agonists are indeed internalised in a receptor-dependent manner.

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27P IS THE C-TERMINUS OF THE RECOMBINANT δ-OPIOID RECEPTOR IMPORTANT FOR ADENYLYL CYCLASE COUPLING?

R.A. Hirst, L.A. Devi[1] & D.G. Lambert. University Dept. of Anaesthesia, Leicester Royal Infirmary, Leicester. LE1 5WW.
[1]Dept. of Pharmacology, NY University Medical Center, 550 First Avenue, New York, NY10016.

The recent cloning of the mouse δ-opioid receptor (δOR) from NG108-15 hybrid cells revealed a structure consistent with its identity as a G-protein coupled receptor (Evans et al., 1992). When transfected into CHO cells, the δOR displayed a pharmacological profile consistent with its identity as a δOR (Reisine, 1995). In order to understand the role of the C-terminus in functional coupling of the δOR, we have utilised three CHO cell lines expressing complete and truncated δOR (Cvejic et al, 1996), these are designated δOR (complete), δOR_{15} (final 15 amino acids deleted from the C-terminus) and δOR_{37} (final 37 amino acids deleted from the C-terminus). The relative ability of [D-Ala^2,D-Leu^5]enkephalin (DADLE) and [D-Pen^2,D-Pen^5] enkephalin (DPDPE) to inhibit forskolin stimulated adenylyl cyclase was examined and compared to NG108-15 cells.

CHOδOR cells were maintained in supplemented Hams F12 medium. cAMP was measured in suspensions of whole cells in Krebs/HEPES buffer pH7.4 at 37°C. The incubation cocktail (300μl) contained DADLE and DPDPE (10^{-5}-10^{-11}M), IBMX (1mM) and forskolin (1μM) in various combinations. After 15 minutes reactions were terminated and cAMP extracted and measured by a radio-receptor assay as described previously (Brown et al., 1971).

DPDPE and DADLE inhibited adenylyl cyclase in NG108-15 cells with IC_{50} values of 1.01 and 0.53nM respectively. These values were not significantly different from those obtained using δOR cells. However, there was a small but statistically significant increase in the IC_{50} for DPDPE (but not DADLE) in δOR_{15} and δOR_{37} (Table 1). The results presented here for δOR_{37} are in agreement with those of Cvejic et al (1996). These small differences in IC_{50} indicate that the C-terminus of δOR is unlikely to be important in the acute adenylyl cyclase coupling.

Table 1. Inhibition of forskolin stimulated cAMP formation by DPDPE and DADLE in CHOδOR cells.

Cell Type	DPDPE		DADLE	
	IC_{50} (nM)	I_{max} (%)	IC_{50} (nM)	I_{max} (%)
NG108-15	1.01±0.20	65.3±2.5	0.53±0.07	66.6±4.0
δOR	1.93±0.32	51.7±3.2	0.99±0.29	44.3±3.8
δOR_{15}	6.33±1.40*	49.1±5.6	1.54±0.51	53.0±1.2
δOR_{37}	8.72±1.75*	52.1±4.1	2.60±0.83	51.3±4.8

IC_{50}, half maximum inhibition. I_{max}, maximum inhibition. Data are mean±s.e. mean (n=4-7). *p<0.05 compared with δOR.. B_{max} values for [^{3}H]diprenorphine in NG108-15, δOR, δOR_{15} and δOR_{37} were 280, 452, 561 and 163fmol/mg protein.

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28P IS THE C-TERMINUS OF THE RECOMBINANT δ-OPIOID RECEPTOR IMPORTANT FOR PHOSPHOLIPASE C COUPLING?

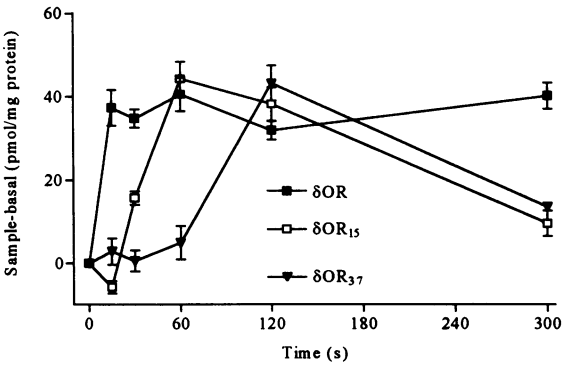
D. Smart, L.A. Devi[1] & D.G. Lambert. University Dept. of Anaesthesia, Leicester Royal Infirmary, Leicester. LE1 5WW. (Supported by The Wellcome Trust).
[1]Dept. of Pharmacology, NY University Medical Center, 550 First Avenue, New York, NY10016.

The endogenous δ-opioid receptor (δOR) in NG108-15 cells (Smart & Lambert 1996) and the cloned δOR expressed in Ltk$^-$ fibroblasts (Tsu et al. 1995) couples to phospholipase C (PLC), as does the cloned μOR in CHO cells (Lambert et al. 1996). In addition, we find that the C-terminus of δOR does not play a significant role in adenylyl cyclase coupling (Hirst et.al., 1996). Therefore, we have examined the role of the C-terminus in δOR-PLC coupling in CHO cells.

CHO cells expressing similar levels of either δOR, or δOR with the C-terminal 15 (δOR_{15}) or 37 (δOR_{37}) amino acids deleted (Cvejic et al. 1996, Hirst et.al, 1996) were maintained in supplemented Hams F12 medium. $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ were measured in whole cell suspensions at 37°C in Krebs/HEPES buffer, pH 7.4 as described previously (Smart & Lambert 1996). Data are mean ±s.e.mean.

Basal $Ins(1,4,5)P_3$ formation in CHO cells expressing δOR, δOR_{15} or δOR_{37} were 32±3, 24±3 and 26±2pmol/mg protein respectively. [D-Pen^2,D-Pen^5] enkephalin (DPDPE, 1μM) caused a naloxone (1μM)-reversible (data not shown) stimulation of $Ins(1,4,5)P_3$ formation in CHO cells expressing δOR, δOR_{15} or δOR_{37} (Fig. 1), as well as increasing $[Ca^{2+}]_i$ (85±7 to 323±2nM, n=5), in δOR expressing cells. The peak DPDPE (0.1nM-10μM)-induced $Ins(1,4,5)P_3$ response was dose-dependent for all three clones, with EC_{50} values of 55±4(n=7), 61±3(n=6) and 48±3(n=5)nM for δOR, δOR_{15} and δOR_{37} respectively.

Fig. 1. DPDPE (1μM)-induced $Ins(1,4,5)P_3$ formation



These data clearly demonstrate that the δOR couples to PLC in CHO cells, consistent with other studies (Tsu et al., 1995, Smart & Lambert 1996). Although the EC_{50} values remained relatively constant the time courses differed, suggesting that the C-terminus may have a role in δOR-PLC coupling in CHO cells.

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29P THE PUTATIVE A_{1L} RECEPTOR (AR): A DISTINCT PHARMACOLOGICAL STATE OF THE α_{1A}-ADRENOCEPTOR?

APDW Ford, DV Daniels, DJ Chang, MR Diaz, JR Gever, JR Jasper, JD Lesnick & DE Clarke. Neurobiology Unit, Roche Bioscience, 3401 Hillview Avenue, Palo Alto, CA 94304, USA.

Three α_1-ARs (α_{1A}, α_{1B} and α_{1D}) have been fully classified (Hieble *et al.*, 1995). A fourth α_1-AR (putative α_{1L}-AR) has been defined functionally and has been proposed to mediate contraction of human prostate (Muramatsu *et al.*, 1993; Ford *et al.*, 1996). Its relationship to the three fully characterized α_1-ARs is not known.

In the present study, binding affinities were estimated against [^{3}H]prazosin in membrane homogenates of Chinese hamster ovary (CHO-K1) cells stably expressing the human α_{1A}-AR (see Blue *et al.*, 1995). These affinity estimates were compared with those obtained functionally in identical cells measuring inhibition of noradrenaline-stimulated accumulation of [^{3}H]inositol phosphates (InsPs; see Alexander *et al.*, 1989), and studying contractions of human prostatic tissue (Ford *et al.*, 1996).

Binding studies revealed a pharmacological profile typical for the classically defined α_{1A}-AR (Table 1), which mirrored that described in perfused kidney of rat (an α_{1A}-AR functional assay; Blue *et al.*, 1995). Accordingly, prazosin, RS-17053 (Ford *et al.*, 1996), WB 4101, 5-methylurapidil, REC 15/2739 (Hieble *et al.*, 1995) and *S*-niguldipine all displayed subnanomolar affinity.

A different profile of affinity was obtained in InsPs studies using intact α_{1A}-AR-CHO cells (Table 1). Prazosin, WB 4101, 5-methylurapidil, RS-17053 and *S*-niguldipine showed 10 to 40-fold lower affinity than in membrane binding. Affinity estimates were not 'frameshifted', however, as tamsulosin, indoramin and REC 15/2739 yielded similar, high affinity estimates in binding and InsPs assays. Furthermore, results with human α_{1B}- and α_{1D}-ARs expressed in CHO-K1 cells gave identical affinity estimates for prazosin, tamsulosin and RS-17053 in both assays (not shown). A concordance of affinity estimates from the InsPs studies in α_{1A}-AR-CHO was found with estimates from contractile studies in human prostate (putative α_{1L}-AR; Ford *et al.*, 1996).

Table 1. Affinity estimates at cloned and native α_{1A}-AR, and α_{1L}-AR in human prostate (means ± s.e. mean, n≥3)

ANTAGONIST	α_{1A}-CHO binding pK_i	α_{1A} rat kidney[a] pA_2	α_{1A}-CHO InsPs pK_b	α_{1L} prostate[b] pA_2
Prazosin	9.9 ±.0	9.5	8.7 ±.1	8.7 ±.1
RS-17053[b]	9.2 ±.1	9.8	8.3 ±.1	7.3 ±.2
WB 4101[c]	9.8 ±.1	10.3	8.8 ±.1	8.9 ±.1
5-Me-urapidil	9.2 ±.1	9.2	8.1 ±.1	8.2 ±.1
S-Niguldipine	9.9 ±.2	10.5	8.2 ±.3	7.5 ±.5
Tamsulosin	10.4 ±.2	10.0	10.2 ±.1	10.4 ±.1
REC 15/2739[c]	9.7 ±.2	*no data*	9.3 ±.1	9.2 ±.2
Indoramin	8.4 ±.1	*no data*	8.4 ±.1	8.5 ±.2

([a]Blue *et al.*, 1995; [b]Ford *et al.*, 1996; [c]see Hieble *et al.*,1995)

Data from the InsPs studies show that the cloned α_{1A}-AR displays pharmacological recognition properties similar, although not identical, to the putative α_{1L}-AR. Why this profile differs from that obtained in membrane binding, and whether it explains the α_{1L}-AR pharmacology observed in many native tissues requires further investigation.

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30P PURIFICATION AND CHARACTERISATION OF CENTRAL PORCINE 5-HT_3 RECEPTORS

S. Fletcher & N.M. Barnes. Dept of Pharmacology, University of Birmingham, Edgbaston, Birmingham, B15 2TT U.K.

In the present studies we report the preliminary characterisation of the 5-HT_3 (5-hydroxytryptamine$_3$) receptor purified from pig brain.

Porcine 5-HT_3 receptors were solubilised as described previously (Fletcher & Barnes 1995) and purified using a GR119566X affinity column (25 mL) as described previously (Boess et al., 1992). Briefly solubilised receptor was applied to the affinity column at a rate of 20 ml/hour for at least 12 hours. The column was subsequently subjected to the following washes: solubilisation buffer (25 mM Tris, 2 mM EDTA, 100 µM PMSF, 10 µg/mL bacitracin, 10 µg/mL sodium azide, 0.4 % Triton X-100, pH 7.4) containing 500 mM NaCl (wash buffer 1, 10 volumes), and solubilisation buffer containing 250 mM NaCl (wash buffer 2, 30 volumes). 5-HT_3 receptor bound to the affinity column was eluted with 5 volumes of 100 µM granisetron in wash buffer 2, in 3 aliquots. The eluents were concentrated before the protein was precipitated using acetone and subsequently solubilised in either reducing or non-reducing sodium dodecyl sulphate (SDS) buffer (2% SDS, 50 mM Tris, 3% sucrose, 0.02% bromophenol blue, containing 5% 2-β-mercaptoethanol for the reducing buffer, pH 7.0). The purified receptor preparation was then boiled for 3 min, before an aliquot (15 µl) was subject to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking gel, 10% separating gel). Gels were stained using a silver stain procedure (Silver Stain Plus, Bio-Rad). For binding studies with the affinity purified 5-HT_3 receptor, the eluting granisetron was removed using gel permeation chromatography. Radioligand binding was performed essentially as described previously (Fletcher & Barnes 1995) using [^{3}H]-(S)-zacopide (2 nM).

SDS-PAGE of the protein fraction eluted from the affinity column in reducing buffer resulted in silver stained bands at apparent molecular masses of 37, 46, 53, 61, 66 and 70 kDa, which are in the same range as reported for NG108-15 cells (Boess et al., 1992). The corresponding sample in non-reducing buffer failed to enter the separating gel, indicating a molecular mass for the receptor complex of > 200 kDa. In a dot-blot procedure, the purified receptor gave a positive reaction with an antiserum specific for the 5-HT_3 receptor (Turton et al., 1993). Preliminary studies suggest that the pharmacological profile of 5-HT_3 receptor sites purified from pig cerebral cortex is similar to that in both crude homogenates and solubilised 5-HT_3 receptor sites from pig brain. For example, zacopride, ondansetron and 5-HT competed for [^{3}H]-(S)-zacopride binding to purified receptor preparations giving similar IC_{50} values to those obtained with crude homogenate and solubilised receptor preparations; 3.9, 7.8 and 2936 nM respectively (n=1), compared to values of 1.4±0.2, 22.2±2.5 and 938±47 nM obtained in crude homogenates, and 24.7±3.4 and 668±202 nM for ondansetron and 5-HT respectively obtained with 5-HT_3 receptor sites solubilised from pig brain (mean±SEM, n=3-6). Methiothepin (1µM), 5-carboxamido-tryptamine (1µM), paroxetine (1µM) and GR113808A (100 nM, Grossman et al., 1993) did not influence the binding of [^{3}H]-(S)-zacopride to the purified receptor. No specific binding was detected in any of the washes prior to granisetron-induced elution of the 5-HT_3 receptor from the GR119566X affinity column.

The present study provides preliminary evidence for the purification of the porcine central 5-HT_3 receptor.

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31P A COMPARISON OF 5-HT-STIMULATED INOSITOL 1,4,5-TRISPHOSPHATE PRODUCTION MEDIATED BY HUMAN 5-HT_{2A} AND 5-HT_{2C} RECEPTORS EXPRESSED IN SH-SY5Y CELLS

S.J. Briddon, R.A. Leslie and J.M. Elliott. Department of Clinical Pharmacology, University of Oxford, Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE, U.K.

We have previously shown that the human 5-HT_{2C} receptor expressed in the human neuroblastoma cell line SH-SY5Y undergoes agonist-induced desensitisation at the level of second messenger production (Briddon *et al.*, 1995). Here we report characterisation and comparison of 5-HT-stimulated changes in inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) mass in this cell line and in SH-SY5Y cells transfected with the human 5-HT_{2A} receptor, and describe its alteration after receptor desensitisation.

SH-SY5Y/5-HT_{2C} cells (B_{max} = 500 fmol/mg protein) and SH-SY5Y/5-HT_{2A} cells (B_{max} = 450 fmol/mg protein) were grown on 24-well tissue culture plates in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 5% dialysed foetal calf serum and 1% glutamine. After overnight incubation in serum-free DMEM, cells were transferred to Kreb's buffer with 10mM HEPES for 30 min. Where appropriate, cells were exposed to 5-HT during equilibration in buffer followed by three rapid washes prior to stimulation. Cells were stimulated with agonist and the reaction terminated with ice-cold 1M trichloroacetic acid. $Ins(1,4,5)P_3$ was solvent extracted using Freon/Trioctylamine (1:1) and neutralised with 25mM $NaHCO_3$. $Ins(1,4,5)P_3$ levels were determined by the method of Challis *et al.* (1990). Results were corrected for a parallel incubation with buffer and two protein samples were assayed per plate (Peterson, 1977).

5-HT caused a dose-dependent increase in cellular $Ins(1,4,5)P_3$ levels in both clones with pEC_{50} values of 8.02±0.13 and 7.42±0.11 (n=3, mean±s.e.mean) for SH-SY5Y/5-HT_{2C} and SH-SY5Y/5-HT_{2A} cells, respectively, measured at peak response times. Maximal stimulation was seen with 1μM and 10μM 5-HT, respectively. No stimulation of $Ins(1,4,5)P_3$ production was seen in response to 100μM 5-HT for up to 300s in non-transfected cells. Basal levels of $Ins(1,4,5)P_3$ were significantly higher in SH-SY5Y/5-HT_{2A} cells compared to SH-SY5Y/5-HT_{2C} cells (27.9± 6.3 and 7.9±1.1 pmol/mg protein, respectively, n=5, $p<0.05$, unpaired Student's t-test). Stimulation of cells with maximally effective concentrations of 5-HT for 0-300s caused a sharp initial rise in $Ins(1,4,5)P_3$ levels, which then fell to basal in both cases. For the 5-HT_{2C} receptor, the peak response (54.8±7.4 fmol/mg protein, n=5) occurred at 5s, whilst for the 5-HT_{2A} receptor the peak response was significantly greater (122.3±16.7 pmol/mg protein, n=5, $p<0.01$ vs 5-HT_{2C}) and occurred after 20s stimulation. Prior exposure of cells to 5-HT for 15 min caused a significant decrease in the magnitude of the peak response in SH-SY5Y/5-HT_{2C} cells (54.0±7.2 vs 21.7±4.2pmol/mg protein for control and 1μM 5-HT samples, respectively, n=3, $p<0.05$) but no change in the peak response in SH-SY5Y/5-HT_{2A} cells (119.7±22.6 vs 120.6±7.0 pmol/mg protein, control v 10μM 5-HT, n=3). Pre-treatment did not change the basal levels of $Ins(1,4,5)P_3$ in either cell line.

We conclude that 5-HT induces a rapid and transient increase in $Ins(1,4,5)P_3$ levels in SH-SY5Y/5-HT_{2A} and SH-SY5Y/5-HT_{2C} cells, with significant differences apparent in the magnitude and time course of the responses mediated by the two receptors. Furthermore, desensitisation induced by prolonged exposure to 5-HT is evident for the 5-HT_{2C}, but not the 5-HT_{2A} receptor.

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S.J.B. is a Wellcome Prize Student.

32P REGULATION OF POLYPHOSPHOINOSITIDE AVAILABILITY FOR MUSCARINIC CHOLINOCEPTOR SIGNALLING IN SH-SY5Y NEUROBLASTOMA CELLS

G.B. Willars, S.R. Nahorski & R.A.J. Challiss Dept of Cell Physiology & Pharmacology, University of Leicester, University Rd., Leicester, LE1 9HN

Agonist occupation of a wide variety of plasma membrane receptors activates phosphoinositidase C (PIC). This enhances hydrolysis of $PtdIns(4,5)P_2$, liberating both $Ins(1,4,5)P_3$ and diacylglycerol. $PtdIns(4,5)P_2$ is resynthesized via sequential phosphorylation of PtdIns and PtdIns(4)P by PtdIns 4-kinase and PtdIns(4)P 5-kinase. Little is known, however, of the regulation of $PtdIns(4,5)P_2$ synthesis or how this influences signalling. We have studied this in the human neuroblastoma, SH-SY5Y, using wortmannin (WT), as a WT-sensitive PtdIns 4-kinase has been reported to be critical for maintenance of agonist-sensitive $PtdIns(4,5)P_2$ pools (Nakanishi *et al.*, 1994).

Experiments (at 37°C) were on cells with phosphoinositide pools equilibrium labelled (48h) with [^{3}H]inositol. Recycling of [^{3}H]inositol was blocked during experiments with 10mM inositol. Phospholipids were extracted, deacylated and water soluble glycerophosphoinositols separated by anion-exchange chromatography (Jenkinson *et al.*, 1994). EC_{50}s and IC_{50}s are $-\log_{10}$ mean±sem ($n \geq 3$) with comparison by Student's t-test.

Under basal conditions ^{3}[H]PtdIns, [^{3}H]PtdIns(4)P and [^{3}H]$PtdIns(4,5)P_2$ represented 94.3%, 2.9% and 2.8% of phosphoinositides respectively. Carbachol (1mM) caused a marked and rapid [^{3}H]$InsP_X$ accumulation by 1min (2.5-fold) which was sustained for at least 10min. There were equally rapid and sustained reductions in [^{3}H]PtdIns(4)P and [^{3}H]$PtdIns(4,5)P_2$ (by 61% and 58% at 1min) but a only a minor fall in PtdIns (2%). Carbachol depleted [^{3}H]PtdIns(4)P more potently than [^{3}H]$PtdIns(4,5)P_2$ (EC_{50}s 5.82±0.15 and 5.25±0.11, $p<0.02$) suggesting maintenance of $PtdIns(4,5)P_2$ at the expense of PtdIns(4)P during sub-maximal stimulation. After atropine (10μM) block of the carbachol response, restoration of [^{3}H]$PtdIns(4,5)P_2$ was more rapid than that of [^{3}H]PtdIns(4)P (81% vs. 51% of basal after 100s). WT (10min) dose-dependently reduced basal [^{3}H]PtdIns(4)P to a greater extent and more potently than [^{3}H]$PtdIns(4,5)P_2$ (IC_{50}s 6.23±0.04 and 5.82±0.03, $p<0.01$) again suggesting differential activation of PtdIns 4- and PtdIns(4)P 5-kinases to maintain basal [^{3}H]$InsP_X$ turnover. During carbachol challenge, WT enhanced depletions of [^{3}H]PtdIns(4)P and [^{3}H]$PtdIns(4,5)P_2$ to 19% and 15% of basal levels. These lipid pools were unable to support stimulated [^{3}H]$InsP_X$ generation which fell to basal suggesting >80% of polyphosphoinositides are accessible to PIC. Maximal WT effects required >3μM for 10min.

These data demonstrate regulatory features of phospholipid synthesis which maintain levels of $PtdIns(4,5)P_2$ at the expense of PtdIns(4)P and suggest differential activation PtdIns 4- and PtdIns(4)P 5-kinases. They are also consistent with a WT-sensitive PtdIns 4-kinase essential for maintenance of an agonist-sensitive pool of $PtdIns(4,5)P_2$ but provide no evidence for agonist-mediated breakdown of PtdIns.

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33P HISTAMINE AND SUBSTANCE P DECREASE INTRACELLULAR CALCIUM IN THAPSIGARGIN-TREATED HUMAN U373 MG ASTROCYTOMA CELLS

K.W. Young, W.J. Gibson[1], R.D. Pinnock & J.M. Young[1], Parke-Davis NRC, Cambridge University Forvie Site, Robinson Way, Cambridge CB2 2QB and [1]Department of Pharmacology, Univ. of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ.

Agonists coupled to phospholipase C-β (PLC-β) stimulate the production of diacylglycerol and inositol 1,4,5-trisphosphate. The former activates protein kinase C (PKC), which can play a role in the regulation of the response, while the latter releases Ca^{2+} from intracellular stores. After the initial peak release the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) must be returned towards resting levels. We have investigated the role of histamine and substance P in the feedback control of $[Ca^{2+}]_i$ in U373 MG astrocytoma cells in which $[Ca^{2+}]_i$ has been artificially raised by the store Ca^{2+}/ATPase pump inhibitor thapsigargin.

U373 MG cells were cultured and dissociated as described previously (Arias-Montaño *et al.*, 1994). [^{3}H]-Inositol phosphate ([^{3}H]-IP) and $[Ca^{2+}]_i$ measurements were carried out in a HEPES-buffered medium (in mM: NaCl 120, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.6, D-glucose 11 and HEPES 25), adjusted to pH 7.4 with NaOH and gassed with 100% O_2. [^{3}H]-IP accumulation was measured over a 30 min period in the presence of 30 mM Li^+ as described previously (Arias-Montaño *et al.*, 1994). For $[Ca^{2+}]_i$ measurements, made at room temperature (*circa* 22°C), U373 MG cells were grown to confluency on coverslips and incubated in HEPES medium containing 2 μM fura-2 AM and 1 mg.ml^{-1} BSA. The ratio of fluorescent emission monitored at 510 nm, after excitation at 340 and 380 nm, was converted to $[Ca^{2+}]_i$.

Histamine stimulated a concentration-dependent increase in [^{3}H]-IP accumulation in U373 MG cells with a best-fit EC_{50} value of 19.1 ± 5 μM (3). The mean accumulation in response to 100 μM histamine was 3.9 ± 0.1 fold of basal (3). Histamine-induced increases in $[Ca^{2+}]_i$ were transient, consisting of an initial peak response which rapidly returned to pre-stimulated levels and were often followed by further small transient increases. The EC_{50} for the initial peak increase, 4.6 ± 2.2 μM (6), was significantly less than that for the histamine-induced [^{3}H]-IP_1 response. The mean resting $[Ca^{2+}]_i$ was 23 ± 2 nM. Pretreatment of cells with 5 μM thapsigargin, followed by readdition of 1.8 mM Ca^{2+} to the perfusion buffer produced a near steady-state level of $[Ca^{2+}]_i$ 97 ± 5 nM (23) above resting levels. Histamine (100 μM, 100 s application) caused the slow decline in the thapsigargin-induced plateau phase to increase from 0.010 ± 0.006 to 1.08 ± 0.09 nM s^{-1} (3). The effect was concentration-dependent (EC_{50} 0.8 ± 0.2 μM) and usually reversible on washout. A second application of 100 μM histamine 400 s after the initial application stimulated a 0.85 ± 0.02 nM s^{-1} (3) decrease in $[Ca^{2+}]_i$. The effect of histamine was blocked by 1 μM mepyramine (4). A 15 min preincubation with staurosporine (1 μM) reduced the depressant effect of 100 μM histamine on $[Ca^{2+}]_i$ by 61 ± 7% (4). Substance P (10 nM), presumably acting via NK_1 receptors (Heuillet *et al.*, 1993), also inhibited the thapsigargin-induced plateau phase, suggesting that the effect may be common to agonists which stimulate PLC-β.

The rate of quench of fura-2 fluorescence (excited at 360 nm) by 750 μM Mn^{2+} in HEPES medium to which no Ca^{2+} had been added approximated well to a monoexponential and the rate constant was increased from 4.25 ± 0.03 x 10^{-3} s^{-1} to 5.36 ± 0.02 x 10^{-3} s^{-1} (6) after pretreatment of the cells with 5 μM thapsigargin. Histamine (100 μM), added after thapsigargin, did not reduce the rate constant for Mn^{2+} quench, suggesting that the decrease in steady-state $[Ca^{2+}]_i$ induced by histamine is not due to decreased Ca^{2+} entry. On the present evidence it seems likely that histamine stimulates Ca^{2+} extrusion from U373 MG cells.

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34P CONSTITUTIVE ACTIVITY OF RECOMBINANT TYPE 1α METABOTROPIC GLUTAMATE RECEPTORS AND INVERSE AGONIST ACTIVITY OF PHENYLGLYCINE ANALOGUES

A.M. Carruthers, R.A.J. Challiss and S.R. Nahorski, Department of Cell Physiology & Pharmacology, University of Leicester, University Road, Leicester, LE1 9HN

We have reported (Carruthers *et al.*, 1996) that pertussis toxin (PTX) pre-treatment of baby hamster kidney cells expressing the recombinant type 1α metabotropic glutamate receptor (BHK-mGluR1α) results in a profound increase in basal [^{3}H]-inositol monophosphate ($InsP_1$) accumulation. The magnitude of this effect (10-15 fold) is approximately twice that evoked by L-glutamate (Glu ; 1 mM) in control cells not exposed to PTX. Glu addition to PTX-treated cells only evokes a modest further increase in $InsP_1$ accumulation, an observation consistent with the apparent partial PTX sensitivity of mGluR1α reported previously (Aramori & Nakanishi, 1992; Thomsen *et al.*,1993). Here, we have investigated the mechanisms underlying the enhanced basal activity of PTX-treated BHK-mGluR1α cells.

BHK-mGluR1α cells (passage 3-40) were maintained in DMEM containing 5% dialysed foetal calf serum, 2 mM glutamine, 50 μg ml-1 gentamicin, 0.5 mg ml^{-1} G418 and 1μM methotrexate. For determination of $InsP_1$, cells were labelled with [^{3}H]-inositol (1μCi ml^{-1}) for 48 h. Cells were washed in oxygenated Krebs-Henseleit buffer (KHB) and preincubated with KHB supplemented with 10 mM LiCl for 15min. Additions of mGluR receptor antagonists or the Glu transporter inhibitor were made 15 min before agonist. Cells were incubated in the presence of agonist for a further 30 min at 37°C and incubations terminated with trichloroacetic acid. $InsP_1$ was measured in a neutral cell extract. Where indicated, cell cultures were pre-incubated with PTX (100ng ml^{-1}) for 22-24 h.

Glu induced a dose-dependent increase in $InsP_1$ accumulation in untreated cells (basal, 365 ± 19; +Glu (300 μM), 2873 ± 284 d.p.m./mg protein (n=5); EC_{50} 19 μM). PTX pre-treatment increased basal InsP accumulation by 12.8 ± 0.9 fold-over-control levels and was further dose-dependently increased by Glu (basal, 4672 ± 253; +Glu (300 μM), 9530 ± 1182 d.p.m./mg protein (n=3); EC_{50} 12 μM). Incubation of cells with the Glu transporter inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC; Thomsen *et al.*,1994) had no significant effect on $InsP_1$ accumulation in either untreated or PTX-treated cells up to 1mM (n=5). Incubation of PTX-treated, but not control, cells with the mGluR antagonists (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) and (S)-4-carboxy-3-hydroxyphenylglycine (4C3HPG) dose-dependently inhibited basal $InsP_1$ accumulation, whilst (-)-α-methyl-4-carboxy-phenylglycine (1 mM) had no effect. (+)MCPG (1 mM) suppressed the basal response by 85 ± 8% (IC_{50} 360 μM (n=5)), 4C3HPG (1 mM) by 95 ± 2 % (IC_{50} 70 μM (n=5)).

These data are consistent with a 'constitutive' activity of recombinant mGluR1α following PTX treatment of BHK cells. Under these conditions (+)MCPG and 4C3HPG do not act as 'neutral' competitive antagonists, but display profound inverse agonist activity. Furthermore, these results indicate that mGluR1α expressed in BHK cells preferentially couple to G_q proteins in the absence of agonist and when competing G_i proteins are inactivated by PTX.

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35P CHARACTERIZATION OF THE INTERACTION OF DARIFENACIN AT MUSCARINIC RECEPTOR SUBTYPES *IN VITRO*

R.M. Eglen, M.T. Pulido-Rios, A.P. Webber, E. Leung and S.S. Hegde. Institute of Pharmacology, Neurobiology Unit, Roche Bioscience, 3401 Hillview Avenue, Palo Alto, CA 94303, USA.

Darifenacin is a novel muscarinic receptor antagonist selective for the muscarinic M_3 receptor. In radioligand binding studies, affinities (pKi) of 7.4, 7.4, 8.4, 8.0 and 8.0 were observed at recombinant human m1, m2, m3, m4 and m5 receptors, respectively (Nunn *et al.*, 1996). In functional studies, darifenacin exhibited pA_2 values of 7.8, 7.3 and 8.7 at putative M_1, M_2 and M_3 receptors in rabbit vas deferens, guinea-pig atria and urinary bladder, respectively (Newgreen & Naylor, 1996). Preliminary clinical data suggest that darifenacin (2.5-10 mg *bid* for 7-10 days) has utility in the treatment of urge incontinence. Specifically, an improvement in incontinent episodes (28 / 30 patients) was seen. Dry mouth side-effects were observed in 3 out of 25 patients (Swami & Abrams, 1995). The aim of the present study was to characterize the interaction of darifenacin at several muscarinic receptors in several functional studies.

Methods. All experiments were conducted according to methods described previously (Watson *et al.*, 1995). Rings of saphenous vein were isolated from male dogs, while atria (left, paced at 0.5 Hz, supramaximal voltage), oesophageal muscularis mucosae (OMM), ileum and trachea were isolated from male Dunkin Hartley guinea-pigs. In all studies (+)*cis* dioxolane was used as the agonist and darifenacin was equilibrated with the tissues for 60 min, prior to constructing the concentration-effect curves. The values quoted are pA_2 values determined using the method of Arunlakshana and Schild (1959).

Results. Darifenacin was selective toward the M_3 receptor in guinea-pig ileum, with respect to the atrial M_2 receptor (150 fold) or saphenous vein M_1 receptor (15 fold). Moreover the compound did not discriminate between M_3 receptors in the tissues studied. In this respect, darifenacin differed from the structurally related zamifenacin (Watson *et al.*, 1995) or *para*-fluorohexahydrosiladifenidol (Eglen *et al.*, 1990) which exhibit lower affinities at M_3 receptors in trachea and urinary bladder, respectively, than in ileum or OMM .

Table 1. pA_2 values for darifenacin at various muscarinic receptor subtypes.

Preparation		pA_2	Schild slope
canine saphenous vein	(M_1)	7.9 ± 0.1	0.8 ± 0.1
guinea-pig atria (left)	(M_2)	6.9 ± 0.1	0.8 ± 0.3
guinea-pig OMM	(M_3)	9.5 ± 0.1	0.9 ± 0.1
guinea-pig ileum	(M_3)	9.1 ± 0.1	0.9 ± 0.1
guinea-pig trachea	(M_3)	9.3 ± 0.1	1.1 ± 0.1

Values are mean ± s.e. mean, n=6-16.

Conclusion. These data are consistent with darifenacin acting as a selective high affinity M_3 receptor antagonist *in vitro* and support the data by Wallis et al., (1995). Moreover, they suggest selectivity for the M_3 receptor greater than that estimated from radioligand binding studies (Nunn et al., 1996). This disparity emphasizes the importance of conducting functional studies, in addition to radioligand binding studies, when assessing the selectivity of novel antagonists

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36P MUSCARINIC RECEPTOR COUPLING IN THE HIPPOCAMPUS OF OLD RATS

Catherine Dacquet, Chantal Lahaye, Anne Renouard, Jean Lepagnol & Michael Spedding, Institut de Recherches SERVIER, 125 Chemin de Ronde, 78290 Croissy-sur-Seine, France.

Changes in muscarinic receptor coupling have been reported with ageing and in Alzheimer's disease (see Hernandez-Hernandez et al., 1995, for refs). We investigated the effect of GppNHp on the specific binding of [^{3}H]-oxotremorine-M in hippocampal membranes from young (3 months) and old (22 months) Fisher rats. Male Fisher rats were decapitated and the hippocampus separated from other brain regions over ice. Membranes were prepared by homogenization in ice-cold 50 mM Tris HCl (pH 7.4 at 25°C) with a polytron PT10 tissue disruptor. The homogenate was centrifuged at 48 000 g for 15 min at 4°C. The pellet was resuspended in the same buffer and washed by resuspension and centrifugation. The final pellet was resuspended in Tris HCl buffer at an approximate protein level of 5 mg ml^{-1} and stored at -80°C until required. Binding assays were initiated by the addition of membrane protein (100-200 µg) to 50 mM Tris HCl (pH 7.4) containing 0.65 nM [^{3}H]-oxotremorine-M and various concentrations of GppNHp (10^{-11} M to 3.10^{-4}M), NaCl (0.01 mM to 300 mM) or test drugs (10^{-11}M to 10^{-5}M) (500 µl final assay volume). Non specific binding was determined in the presence of 1 µM atropine. Reactions were incubated for 60 min at 25°C and bound ligand were separated from free by vacuum filtration through Whatman GF/B filters presoaked for 30 min in assay buffer containing 0.1% polyethylenimine. The filters were washed with 3x5 ml assay buffer and bound ligand was estimated by liquid scintillation spectrometry.

GppNHp concentration-dependently decreased the [^{3}H]-oxotremorine-M binding and a significant difference was observed between the young and old rats (IC_{50} values were respectively equal to 36.7 µM and 103 µM). The maximum [^{3}H]-oxotremorine-M specific binding inhibition by GppNHp (300 µM) was the same with the young and the old rats (-55 ± 5 %). There was no difference in increasing the NaCl concentration on the sensitivity of [^{3}H]-oxotremorine-M binding to GppNHp either in the presence or absence of $MgCl_2$ (10 mM), in membranes from young or old rats. However, when hippocampal membranes were pretreated with $MgCl_2$ (10 mM), which induced the low affinity receptor state by promoting the decoupling between the receptor and the G protein, these IC_{50} values were significantly shifted to values expressing a higher sensitivity of [^{3}H]-oxotremorine-M specific binding to GppNHp. In the presence of $MgCl_2$, IC_{50} values were 4.54 µM (young rats) and 0.85 µM (old rats); the effect of $MgCl_2$ was significantly ($p<0.05$) more pronounced in the old rats than in the young rats (ratio between the GppNHp IC_{50} was respectively equal to 8 and 121). In the presence of $MgCl_2$ (10 mM) the threshold for the effects of GppNHp was significantly ($p<0.05$) higher for young rats (about 30 nM) than old rats (0.1 nM). At a GppNHp concentration equal to 30 nM no significant inhibition was observed in young rats (-11 %) whereas 36 % of specific binding was lost in the old rats. These results suggest that during ageing the sensitivity of the agonists could be changed without affecting that of the antagonist. Moreover, in the old Fisher rat hippocampal membranes, the oxotremorine-M specific binding is more sensitive to the decoupling agent GppNHp in the presence of $MgCl_2$ than in membranes from the young rats. These subtle changes in hippocampal muscarinic receptor - G protein coupling may be important for memory dysfunction in ageing.

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37P EFFECTS OF REPEATED TREATMENT WITH L-DOPA, BROMOCRIPTINE AND ROPINIROLE IN DRUG NAIVE MPTP-TREATED COMMON MARMOSETS

R.K.B. Pearce, T. Banerji, P. Jenner & C.D. Marsden, Neurodegenerative Diseases Centre, Pharmacology Group, Biomedical Sciences Division, King's College, Manresa Road, London SW3 6LX

Chronic administration of L-DOPA to patients with Parkinson's disease (PD) induces dyskinesia. In contrast, the D-2 agonist bromocriptine does not appear to induce significant dyskinsia when administered to drug naive patients with PD or to non-human primates treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), although other D-2 agonists such as quinpirole and (+)-PHNO will induce dyskinesia (Bédard *et al.*, 1986 & 1993). We now compare the ability of chronic treatment with L-DOPA and the D-2 agonists ropinirole and bromocriptine to induce dyskinesia in common marmosets with MPTP-induced motor deficits not previously exposed to L-DOPA or other drugs.

Common marmosets (*Callithrix jacchus*, n=16) were treated acutely with MPTP to induce a chronic stable parkinsonian state. After a recovery period of 10 weeks the animals were divided into treatment groups of 4 animals: control; L-DOPA (12.5 mg/kg po plus carbidopa 12.5 mg/kg po daily); bromocriptine (1.0 mg/kg sc reduced to 0.5 mg/kg sc daily after 7 days) or ropinirole (0.3 mg/kg po increased to 0.5 mg/kg po daily after 7 days). The drugs were administered for a total of 30 days, with the doses of bromocriptine and ropinirole titrated after one week to elicit comparable motor responses. Locomotor activity was assessed weekly in computer-linked activity cages equipped with infrared diod monitors. Disability and dyskinesia scoring was also undertaken as previously described (Pearce *et al.*, 1995).

All drug treatments were comparably effective in alleviating MPTP-induced akinesia and motor disability as reflected in significantly improved locomotor activity and disability scores, but L-DOPA rapidly induced moderate to severe levels of dyskinesia while only mild dyskinesia was associated with chronic ropinirole and bromocriptine administration (Figure 1).

Our results confirm that MPTP-lesioned primates treated with L-DOPA, but not bromocriptine, develop marked dyskinesia. In addition, the selective D-2 agonist ropinirole, like bromocriptine, evokes less dyskinesia than L-DOPA when given *de novo* to MPTP-lesioned common marmosets. These findings suggest that certain D-2 agonists may result in less dyskinesia in drug naive subjects with nigrostriatal damage, although the specific role of the D-2 receptor in producing this syndrome remains unclear.

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Bédard P.J., Gomez-Mancilla B., Blanchette P. *et al.* (1993) *Adv. Neurol.* **60,** 113-118.
Pearce R.K.B., Jackson M., Smith L. *et al.* (1995) *Mov. Disord.* **10,** 731-740.

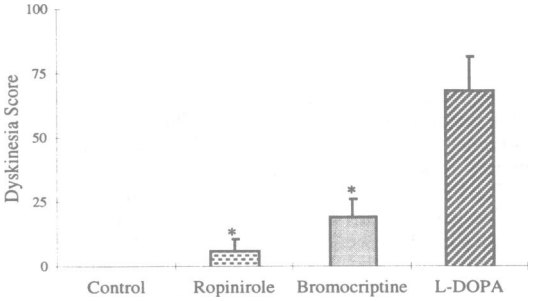

Figure 1. Cumulative mean daily dyskinesia scores in MPTP-treated common marmosets over 30 days receiving L-DOPA (12.5 mg/kg daily plus carbodopa 12.5 mg/kg po), ropinirole (0.3 mg/kg X 7 days then 0.5 mg/kg po daily) or bromocriptine (1.0 mg/kg X 7 days then 0.5 mg/kg daily sc) against controls (n=4 in each group). * $p<0.05$ vs L-DOPA by Kruskal-Wallis test.

38P EFFECTS OF ACUTE NICOTINE ADMINISTRATION ON PARKINSONIAN DISABILITY AND DYSKINESIA IN MPTP-TREATED COMMON MARMOSETS

T. Banerji, R.K.B. Pearce, N.B. Desai, M.J. Jackson, P. Jenner & C.D. Marsden, Neurodegenerative Diseases Research Centre, Pharmacology Group, King's College, London SW3 6LX, UK.

Muscarinic antagonists are effective in reversing some symptoms of Parkinson's disease (PD). However, despite evidence that nicotine may reverse motor deficits (Court & Perry, 1994) and the presence of nicotinic receptors in substantia nigra and striatum which increase striatal dopamine release (James & Nordberg, 1995), there has been no comprehensive evaluation of nicotine in experimental models of PD. The common marmoset shows parkinsonian motor disability following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure and develops dyskinesias during chronic L-DOPA dosing (Pearce *et al.*, 1995). We now report the effects of acute nicotine administration on motor disability and dyskinesia in this model.

Common marmosets (*Callithrix jacchus*) (n=12) were divided into 3 groups : drug naive animals; MPTP-treated; MPTP-treated and L-DOPA-primed for 26 days to induce dyskinesias. Nicotine was administered at doses of 0.1, 0.3 and 0.5 mg/kg s.c. consecutively. The L-DOPA-primed group also received L-DOPA (12.5 mg/kg p.o.) plus carbidopa (12.5 mg/kg p.o.) with nicotine (0.1 - 0.5 mg/kg s.c.). Activity levels were monitored in locomotor cages. Disability was scored (0 = normal to 10 = severely parkinsonian) and dyskinesias were rated as previously described (Pearce *et al.*, 1995).

Nicotine had no effect on basal activity or parkinsonian disability in normal or MPTP-treated animals whereas L-DOPA reversed MPTP-induced akinesia. The combination of nicotine and L-DOPA resulted in no significant difference in total motor activity but a marked delay in the onset of activity induced by L-DOPA (Fig. 1). In L-DOPA-primed animals, nicotine reduced parkinsonian disability but there was no additive effect of coadministering L-DOPA and nicotine. Also in this group, nicotine induced only baseline levels of dyskinesia. L-DOPA administration induced marked dyskinesia and there was no significant difference in the type or intensity of dyskinesia when nicotine was coadministered with L-DOPA.

These findings show reduced disability scores in L-DOPA-primed animals after nicotine but no significant positive effect of acute nicotine administration on dyskinesia or locomotor activtiy in this model. However, the delay of onset of L-DOPA's actions by nicotine suggests either an impairment of L-DOPA absorption or an inhibitory effect of nicotine on the action of L-DOPA in brain.

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James J.R. & Nordberg A. (1995) *Behaviour Genetics* 25: 149-159.
Pearce R.K.B., Jackson M.J., Smith L.A. *et al.* (1995) *Mov. Disord.* 10:731-740.

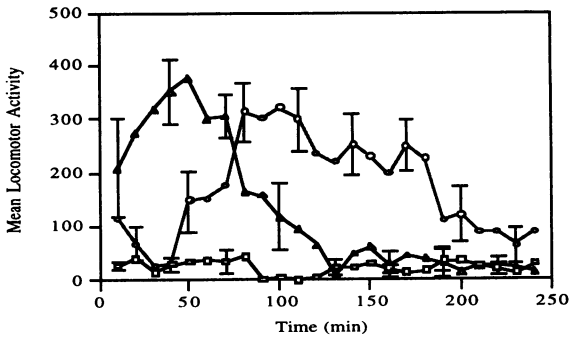

Fig. 1.
Mean locomotor activity in MPTP-treated, L-DOPA-primed marmosets at baseline (—□—), after L-DOPA (12.5 mg/kg p.o.; —△—) and after L-DOPA plus nicotine (0.3 mg/kg s.c.; —○—).

39P VARIATION IN ETHANOL CONSUMPTION AND PLASMA ETHANOL LEVELS IN MICE DURING CHRONIC TREATMENT

M-F. Shih, P. Jelic & P.V. Taberner, Department of Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD

A number of long term ethanol treatment schedules have been employed to investigate the adaptive changes in response to chronic alcohol consumption. These usually involve adding ethanol either to the drinking water or including ethanol in a liquid diet (Lieber & DeCarli, 1989). Because the blood alcohol level at any particular moment will depend upon the drinking or feeding pattern of the animals, the average daily dose of ethanol consumed (usually expressed as g/kg body weight) will not necessarily provide an accurate indication of the concentration of ethanol to which the tissues are exposed during chronic treatment. We have therefore compared blood ethanol levels during different chronic treatments in two mouse strains to see how the tissue concentration varies with time.

Groups of 8 adult male CBA or TO mice (25-30 g) were given either solid diet and 20% (w/v) ethanol (CET) *ad libitum* as sole drinking fluid for 4 weeks or 7% ethanol in a liquid diet (ELD) plus water *ad libitum* for 5 days. The latter treatment produces higher blood levels than the drinking schedule and is consequently less well tolerated, necessitating the shorter duration. The mice were housed with a 12 h dark period from 21.00h. The liquid diet (250 ml) was provided at 17.00h. Blood samples (300 µl) were collected from individual mice at 4-6 h intervals over 24h. Ethanol (25% w/v in saline) was given acutely by i.p. injection. Ethanol levels were assayed in triplicate using a standard NAD-coupled enzyme assay. Differences between groups (means ± s.e.mean) were analysed by Student's t-test.

During the CET schedule in TO mice there was a very marked and consistent diurnal variation, with the peak (35.4 ± 8.8 mM) occurring at 24.00h and the lowest levels (10.5 ± 3.9 mM) at 19.00h. CBA mice on the same schedule showed a similar peak (34.3 ± 8.2 mM at 24.00h), but the lowest level (0.8 ± 0.5 mM) was significantly less ($p < 0.01$) than in the TO mice and occurred at 14.00h. The ethanol level during the ELD schedule in CBA mice peaked at 19.00h (105 ± 7.7 mM) and remained high until 05.00h with the lowest level (1.2 ± 0.3 mM) at 14.00h. This suggests that the mice consumed the ELD very promptly from the time it was provided. In the same mice the mean daily alcohol consumption (g/kg body weight) was 15.7 ± 1.8 on CET and 20.0 ± 0.9 on ELD. For comparison, acute ethanol at doses of 0.1 and 2.5 g/kg produced peak plasma levels of 1.7 ± 0.8 and 37.5 ± 6.7 mM respectively at 60 min post-injection.

The results confirm earlier findings which demonstrate that the peak ethanol levels occur during the periods of maximum behavioural activity of the animals (Deimling & Schnell, 1980). Although the daily dose of ethanol with the ELD was only 30% higher than with the drinking schedule, there was a highly significant threefold increase in the peak plasma level. We conclude that measuring average daily ethanol consumption is not sufficient to indicate the likely tissue levels of ethanol and that there are different circadian patterns of consumption depending on the method of delivery of alcohol and also the strain of animal used. The daily high peak ethanol levels during the ELD may contribute to the chronic toxicity observed on this schedule (Lieber & DeCarli, 1989).

P Jelic is supported by an MRC studentship

Deimling, M.J. & Schnell, R.C. (1980) *J.Pharmac.Exp.Ther.* **213**: 1-8
Lieber, C.S. & DeCarli, L.M. (1989) *Alcohol & Alcoholism* **24**: 197-211

40P CO-ADMINISTRATION OF THE CALCIUM CHANNEL ANTAGONIST, NIMODIPINE, DECREASES ENVIRONMENTAL-INDEPENDENT (CONTEXT NON-SPECIFIC) TOLERANCE TO ETHANOL

Smith, J.W.*, Wilson, J.* and Little, H.J., Department of Psychology, Science Laboratories, South Road, Durham DH1 3LE and *Pharmacology Department, The Medical School, Bristol, BS8 1TD

Environmental variables may influence tolerance to drug effects. Administration of ethanol by liquid diet produces tolerance to the ataxic actions of ethanol without providing environmental cues (context non-specific tolerance). This. unlike the tolerance seen when environmental cues accompany the administration of ethanol (context-specific tolerance) is not affected by concurrent administration of NMDA antagonists (Szabo et al., 1994). Dihydropyridine calcium channel antagonists decrease the development of context specific tolerance when given concurrently with injections of ethanol (Dolin and Little, 1989). The present study was designed firstly, to determine the time of maximum tolerance after administration of ethanol by liquid diet and secondly, the effect of a dihydropyridine calcium channel antagonist, nimodipine, on context non-specific tolerance

Groups of 8-10 male Wistar rats (75-100g) were given ethanol by liquid diet for 9 days; the ethanol intake was 14-17 g/kg/day over the last 7 days of the treatment. On the final day, separate groups of rats were withdrawn from the diet and the ataxic effects of ethanol tested, beginning at 3h, 6h and 9h from withdrawal.

During the tests, all the rats were given 2 g/kg ethanol. i.p., then placed on the rotarod every 15 min for 105 min. The time each animals was able to stay on the rotating rod (6 r.p.m.) was measured; a cutoff time of 180s was applied. From these results 6h withdrawal was chosen as showing optimal tolerance.

In the second experiment, the same ethanol treatment was used, and the effects of nimodipine, 50 mg/kg (suspended in tween 80, 0.05%) given i.p. for the last seven days of the ethanol treatment were studied. Separate groups of rats were given either (a) control diet + vehicle (b) control diet + nimodipine (c) ethanol diet + vehicle or (d) ethanol diet + nimodipine. The amount of tolerance developed to the ataxic actions of ethanol was tested, beginning 6h after withdrawal from the liquid diet.

Co-administration of nimodipine significantly decreased the contect-nonspecific tolerance to ethanol. This is in contrast to the effects of an NMDA antagonist, which affected only the context-specific component of tolerance (Szabo et al, 1994).

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Szabo, G. et al. (1994) *Psychopharmacol.*, 113, 511 - 517

Times (s, mean ± s.e.m.), at 15 - 45 min after acute administration of ethanol, for which the animals were able to stay on the rotarod
*P < 0.05, comparison with controls receiving vehicle; † P < 0.05, comparison with ethanol plus vehicle (Mann-Whtitney U test)

Table 1

Treatment	15 min	30 min	45 min
Con. 3h	24.3 ± 22.3	25.5 ± 22.1	24.8± 22.2
Eth. 3h	27.0 ± 22.0	38.5 ± 38.5	46.6 ± 29.1
Con. 6h	24.3 ± 22.2	38.5 ± 25.4	46.6 ± 29.1
Eth. 6h	95.1 ± 30.5*	100.1 ± 30.3*	136.9 ± 28.2*
Con. 9h	15.1 ± 13.3	43.3 ± 25.5	54.7 ± 32.4
Eth. 9h	75.9 ± 28.4*	115.0 ± 31.7*	138.0 ± 24.0*

Table 2

Treatment	15 min	30 min	45 min
Con.+veh	2.6 ± 0.7	4.5 ± 0.6	17.6 ± 3.7
Con. + nim	2.3 ± 0.5	3.8 ± 0.5	44.9 ± 20.4
Eth. + veh	93.3 ± 8.3*	136.3 ± 2.2*	180 ± 0*
Eth. + nim	20.1 ± 11.7†	53.8 ± 18.5†	149.6 ± 28.7†

41P COMPARISON OF THE EFFECTS OF CALCIUM CHANNEL ANTAGONISTS ON HIPPOCAMPAL CALCIUM CURRENTS WITH THEIR ACTIONS ON ETHANOL WITHDRAWAL HYPEREXCITABILITY

Molleman, A. and Little, H.J.*, Pharmacology Department, Medical School, University Walk, Bristol BS8 1TD, *Department of Psychology, Science Laboratories, South Road, Durham DH1 3LE

The ethanol withdrawal syndrome is characterised by tremor, anxiety, and convulsions, and the density of dihydropyridine binding sites (thought to correspond to L-type calcium channels) is increased by chronic ethanol treatment. Isolated hippocampal slices prepared after chronic ethanol treatment in vivo show hyperexcitability. The calcium channel antagonists, nitrendipine and isradipine, completely prevented behavioural and electrophysiological signs of withdrawal, but other calcium blockers had different effects. Another dihydropyridine, felodipine, gave no protection in vivo or in vitro (Watson et al., 1994; Bailey et al., 1996) while the benzothiazepine, diltiazem, increased the severity of behavioural signs (Watson & Little, 1994) and did not prevent hippocampal hyperexcitability (Bailey et al., 1996). The present study compares the effects of these compounds on calcium currents in isolated hippocampal slices, using barium as the charge carrier.

Male TO mice, 25-30 g, were used for hippocampal slice preparation. Blind whole cell patch clamping was used to record the currents. The holding potential was -60 mV and two pulse protocols, one in 15 steps of 10 mV, from -80 mV, and the second a ramp from +40 mV to -60 mV were applied, 5 min after each solution change. The bathing medium was buffered with HEPES and barium choride, 2 mM, replaced the 2 mM calcium chloride at the beginning of the testing period. Intracellular potassium was replaced by caesium ions. Tetrodotoxin, 300 nM was present throughout, and the calcium antagonists were added to the bathing medium, in increasing concentrations, each for 5 min. Statistical analysis was by paired Student's t test, N = 5 for isradipine and felodipine and 6 for diltiazem (one cell and one drug per slice, one slice per mouse).

Average control steady state barium current was maximally 2.7 ± 0.4 nA. At 1 μM, neither isradipine or felodipine, had any effect on the currents. Isradipine at 4 μM produced a significant decrease in the currents ($P < 0.05$), and 10 μM felodipine caused a small, but significant decrease ($P < 0.05$). Both concentrations of diltiazem, 20 μM and 100 μM significantly decreased the calcium currents ($P < 0.05$ and $0.01<P<0.02$, respectively), although the effect of 20 μM was very small. Cadmium chloride, 20 μM, completely blocked the currents.

The results indicate that the effects of the calcium channel antagonists on the barium currents in hippocampal pyramidal cells did not parallel their effects on ethanol withdrawal hyperexcitability, either in vivo or in isolated hippocampal slices. This suggests either that the effects of the compounds on the withdrawal hyperexcitablity are not due to actions on calcium currents or that chronic ethanol treatment alters the drug sensitivity as well as the density of calcium channels.

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Watson,W.P. & Little, H.J.1994 Psychopharm., 114, 321-328

Table 1. Amplitude of barium currents, expressed as percentages of control values, mean + s.e.m.
* $P < 0.05$, † $0.01 < P < 0.02$, compared with control values recorded prior to addition of drug

Isradipine 1μM	95.6 ± 2.4	Felodipine 1 μM	97.6 ± 2.2	Diltiazem 20 μM	94.5 ± 2.0 *
Isradipine 4 μM	80.6 ± 2.9 *	Felodipine 10 μM	87.2 ± 4.3 *	Diltiazem 100 μM	69.2 ± 3.4 †

42P HIGH EXTRACELLULAR CONCENTRATIONS OF GLUTAMATE AND SEIZURE ACTIVITY

T.P. Obrenovitch, J. Urenjak[1] & E. Zilkha, Department of Neurological Surgery, Institute of Neurology, Queen Square, London WC1N 3BG and [1]Dept of Discovery Biology, Pfizer Central Research, Sandwich CT13 9NG

As seizures in experimental models can be induced by the activation and suppressed by the inhibition of glutamate receptors, it is often proposed that high extracellular glutamate levels subsequent to excessive presynaptic release and/or altered glutamate uptake are epileptogenic (Walker *et al.*, 1995). The purpose of this study was to ascertain the link between seizure activity and high extracellular glutamate.

Microdialysis electrodes (Obrenovitch *et al.*, 1994) were implanted in the dorsal hippocampus of adult, male Sprague-Dawley rats anaesthetized with halothane (approximately 1.2 % in 1:1 O_2:N_2O during seizures). Recording of EEG and field potential through the dialysis membrane allowed us to confirm that epileptic activity was present at the sampling site. Detection of any putative rise in extracellular glutamate during seizures was optimized by coupling microdialysis to on-line enzymatic analysis of glutamate. In the first series (n = 6), epileptic seizures were induced by injection of picrotoxin (12 mg kg^{-1} i.v.). Normal ACSF was perfused throughout in 3 rats; in the others the concentration of K^+ in the perfusion medium was increased to 10 mM to compensate for any possible dampening of seizure-increased extracellular K^+ by microdialysis (Obrenovitch *et al.*, 1995). In the second series (n = 12), endogenous extracellular glutamate was elevated by perfusion of increasing concentration of the glutamate-uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-PDC) through the probe, and the EEG examined for any sign of epileptic activity.

No increase in dialysate glutamate was detected during picrotoxin-induced seizures, even when the K^+ concentration in the perfusion medium was raised above extracellular levels measured previously during paroxysmal activity. Sustained inhibition of glutamate uptake by L-*trans*-PDC increased markedly extracellular glutamate (24-fold the basal concentration of 0.61 ± 0.03 μM with 10 mM L-*trans*-PDC, n = 12). Despite this marked effect, no obvious EEG changes were detected by visual examination of the EEG with an oscilloscope during the experiment. Analysis of the average amplitude of the EEG in the frequency windows 0.25-6 Hz and 6-21 Hz showed only a slight increase in the 6-21 Hz frequency window (106 ± 1.1 %, 108 ± 3 %, and 107 ± 3 %; with 2.5, 5, and 10 mM L-*trans*-PDC; n = 12, $p < 0.05$ by Student's paired t test). There were no corresponding changes in the low frequencies. The magnitude of these EEG alterations did not suggest excessive neuronal excitation.

These findings indicate that seizures are not necessarily accompanied by increased extracellular glutamate, and that increased glutamatergic excitation in epilepsy may result from other abnormalities such as increased density of glutamate receptors, enhanced activation subsequent to reduced modulation, or sprouting of glutamatergic synapses. Efficient glutamate uptake probably contributes to the weak epileptogenicity and neurotoxicity of glutamate *in vivo*, but other factors may be more important.

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Obrenovitch, T.P. *et al.* (1995) *J. Neurochem.* 64, 1884-1888.
Walker, M.C. *et al.* (1995) *J. Neurochem.* 65, 1003-1011.

43P DEPRESSION OF GLUTAMATE INPUT TO MIDBRAIN DOPAMINE NEURONES IN RAT BRAIN SLICES BY METABOTROPIC GLUTAMATE RECEPTOR AGONISTS

M.A.Wigmore & M.G. Lacey, Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, BIRMINGHAM B15 2TT.

Dopamine release from the terminal projections of midbrain dopamine neurones is thought to exert a critical influence on the control of voluntary movement, locomotor drive, motivation, reward and cognitive function. A major excitatory drive to dopamine neurones arises from the glutamate-containing neurones of the subthalamic nucleus (STN). Here we report that agonists of metabotropic glutamate receptors (mGluRs; see Roberts (1995) for review) can depress glutamate-mediated synaptic transmission to dopamine neurones, probably by a presynaptic mechanism.

Parasagittal slices (400 μm) of rat ventral midbrain containing the STN and substantia nigra (SN) were continuously superperfused at 1-2 ml/min with a standard bicarbonate-buffered medium at 32-33 °C, to which drugs could be added in known concentrations. Intracellular recordings were made from presumed dopamine neurones with microelectrodes (50 - 130 MΩ resistance) filled with 2M KCl. A bipolar stimulating electrode was placed 300 - 600 μm rostral to the SN near or in the STN and single stimuli (100 μs) were used to evoke synaptic potentials. Membrane potential was held at around -80 mV by constant current injection. Fast depolarising synaptic potentials (EPSPs; 4-10 mV in amplitude) were studied in the presence of the $GABA_A$ receptor antagonist picrotoxin (50 μM). These EPSPs were blocked by tetrodotoxin (TTX; 1 μM) or a low Ca^{2+} (0.24 mM) - containing medium, and also the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM).

The mGluR group III selective agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4; 0.3 - 30 μM) depressed the EPSP in a dose-dependent manner in all 32 cells examined, to a maximum of 41.5% of control, with EC_{50} of 1.6 μM with a maximal effect at 30 μM. L-AP4 was without effect on cell membrane potential at these concentrations. This action of L-AP4 (3 μM) was reversed by 96.1 $\pm$ 4.6 % (3 cells) by the group III - preferring antagonist (RS)-α-methyl-4-phosphonophenylglycine (MPPG; 250 μM). The mGluR group I and II - preferring agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid [(1S,3R)-ACPD; 30 μM) also depressed the EPSC (by 38 $\pm$ 4 %, 6 cells), sometimes accompanied by a membrane depolarisation (which was offset by current injection).

These results indicate that presynaptic group III mGluRs can depress excitatory glutamate-mediated synaptic transmission between the STN and midbrain dopamine neurones. An additional depression via mGluRs of either the group I or II class is also indicated.

Roberts, P.J. (1995) *Neuropharmacol.* **34**, 813-819

MAW is a Wellcome Prize Student.

44P METABOTROPIC GLUTAMATE RECEPTORS IN RAT CA1 NEURONES: RECEPTOR SUBTYPE AND INTRACELLULAR MEDIATORS

Ibbotson, T. & Boden, P. Parke-Davis Neuroscience Research Centre, Addenbrookes Hospital Site, Cambridge, CB2 2QB.

Recent studies have revealed that activation of metabotropic glutamate receptors in CA1 neurones of the rat hippocampus causes a large inward current carried through N-type calcium channels (Ibbotson *et al.*, 1995). Further experiments were performed to determine the subtype of mGluR involved and the intracellular mediators through which this response is generated. Whole-cell voltage-clamp recordings were made from slices (300μm) of male rat mid-brain (animals 5-7wks old) containing the hippocampal CA1 region. For recording, slices were perfused with an artificial cerebrospinal fluid (aCSF) at 37°C and were treated with tetrodotoxin (1μM; 4min). The pipette solution contained (in mM) K-gluconate 130, KCl 10, EGTA 10, $CaCl_2$ 1, HEPES 20, Mg-ATP 1 & Mg-GTP 0.5. Neurones were voltage-clamped at -70mV and current / voltage (I/V) relationships were measured by voltage ramps (-100mV to +10mV, 3.7mV sec^{-1}). All data are expressed as mean $\pm$ s.e.mean.

1S, 3R-aminocyclopentane dicarboxylic acid (1S, 3R-ACPD; 50μM) caused a large inward current between -40mV and -20mV with a peak inward current of 883 $\pm$ 76pA (n=11). In certain neurones the response to 1S, 3R-ACPD was compared with the selective mGluR1 / 5 agonist, 3,5-dihydroxyphenylglycine (DHPG; Schoepp *et al.*, 1994). In these neurones, 1S,3R-ACPD (50μM) and DHPG (50μM) both activated an inward current with mean peak magnitudes of 1180 $\pm$ 90pA and 980 $\pm$ 88pA respectively (n=4). Further experiments were then conducted to examine whether release of intracellular calcium was required for the generation of this response. Both caffeine and ryanodine are known to interfere with the release of calcium from discrete stores (Ehrlich *et al.*, 1995) and so the ability of these agents to inhibit this calcium current was examined. In the presence of caffeine (1mM), the large 1S, 3R-ACPD-induced inward current was reduced compared to initial control values (control, 675 $\pm$ 108pA; +caffeine, 110 $\pm$ 63pA, n=4). Conversely, ryanodine (10μM) had no effect on the magnitude of the calcium current compared to control responses (control, 758 $\pm$ 85; +ryanodine, 735 $\pm$ 114pA, n=3). Finally, the involvement of a GTP-binding protein was evaluated by including the non-hydrolysable analogue GTP-γ-S (0.5mM) in the recording electrode. Application of 1S, 3R-ACPD (50μM) to 3 neurones caused an inward current at -70mV (101 $\pm$ 23pA) which was maintained upon withdrawal of the agonist. I/V analysis of these responses revealed that this initial application of 1S,3R-ACPD also generated the inward, calcium current (450 $\pm$ 135pA, n=3). Further application of either 1S,3R-ACPD (50μM) or DHPG (50μM) had no effect either on the holding current at -70mV or on the voltage ramps. In one further neurone containing GTP-γ-S, application of 1S, 3R-ACPD or DHPG had no effect

These data indicate that activation of metabotropic glutamate receptors by 1S,3R-ACPD or DHPG in the CA1 region of the rat hippocampus activates a high-threshold calcium current. The ability of DHPG to generate this response indicates that either mGluR1 or mGluR5 is the subtype of receptor involved. This is further supported by the finding that caffeine but not ryanodine was able to inhibit this response, again indicating the involvement of group 1 mGluR's which are linked to phosphoinositide hydrolysis.

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45P EFFECT OF EXTRACELLULAR ACIDOSIS ON *N*-METHYL-D-ASPARTATE (NMDA)-EVOKED RESPONSES *IN VIVO*

J. Urenjak[1], E. Zilkha, M. Gotoh & T.P. Obrenovitch, Dept of Neurological Surgery, Institute of Neurology, Queen Square, London WC1N 3BG and [1]Dept of Discovery Biology, Pfizer Central Research, Sandwich CT13 9NG

Exposure of cultured neurons to acidic media inhibits NMDA-evoked responses (Traynelis & Cull-Candy, 1990). The aim of this study was to examine how various challenges to brain acid-base regulation altered NMDA-evoked depolarisation, *in vivo*.

Field (d.c.) potential was recorded with microdialysis electrodes implanted in the striatum of adult, male rats anaesthetised with halothane (Obrenovitch *et al.*, 1994). Repeated depolarizations were evoked by perfusion of 200 μM NMDA through the probe for 2 min, each followed by 20 min of recovery. The first NMDA-stimulus, performed under physiological conditions (perfusion medium composition in mM, NaCl 125, KCl 2.5, $MgCl_2$ 1.18, $CaCl_2$ 1.26; pH 7.3), was used as the individual control. Three groups were considered, each with its corresponding control (normal conditions throughout); (i) hypercapnia (ventilation with 7.5 and 15 % CO_2 for 15 min); (ii) perfusion of acidic media (10 mM PO_4 buffered media, pH 6.5 and 5.8); and (iii) 20-min perfusion of 50 mM NH_4^+, to cause transient intracellular alkalinization subsequent to influx and protonation of NH_3, followed by marked acidosis when NH_4^+ perfusion was terminated. NMDA was applied 10 min after hypercapnia or perfusion of acidic medium, and 5 min after termination of NH_4^+ perfusion. Results (mean ± s.e.mean) were compared by Student's *t*-test.

Perfusion of 200 μM NMDA evoked consistent depolarizations (initial response in all animals was 5.4 ± .21 mV, n = 28),

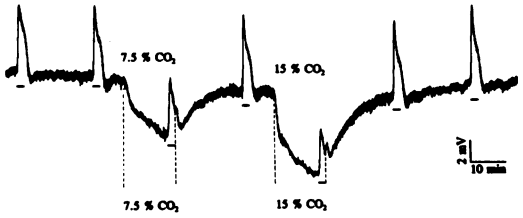

Figure 1. Representative inhibition of NMDA responses by hypercapnia. NMDA (200 μM) was perfused for 2 min through a microdialysis probe implanted in the rat striatum (-).

with a slight tendency to increase progressively throughout control experiments. Hypercapnia reduced the amplitude of NMDA responses (74 ± 4.0 and 64 ± 1.7 % for 7.5 and 15 % CO_2, respectively; $P < 0.005$, $n = 5$), but this effect was rapidly reversed (Figure 1). Extracellular acidosis subsequent to termination of NH_4^+ perfusion also reduced NMDA responses (56 ± 2.6 %; $P < 0.001$, $n = 6$) and this effect persisted for > 1 h. Direct perfusion of acidic media did not alter NMDA responses, but this may only illustrate the large capacity of the brain tissue to buffer H^+. These *in vivo* data confirm that extracellular acidosis inhibits NMDA responses. Whether this effect is potentially neuroprotective in ischaemia (Kaku *et al.*, 1993) is debatable, because ischaemia combines acidosis and alteration of membrane potential (i.e. reduction of NMDA-receptor Mg^{2+} block).

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46P GLUTAMATERGIC DEFICITS IN THE FRONTAL CORTEX IN AIDS

A.M. Sardar, P.H. Hutson* and G.P. Reynolds, Department of Biomedical Science, The University of Sheffield, Sheffield, S10 2TN U.K., *Merck Sharpe and Dohme Research Laboratories, Terlings Park, Harlow, Essex CM20 2QR. U.K.

Human immunodeficiency virus (HIV) infection of the central nervous system is associated with neuronal loss, and this deficit may be responsible for the dementia occurring in AIDS patients (Navia et al, 1986). Glutamate receptor-mediated neurotoxicity has been implicated in the pathogenesis of a variety of CNS diseases and has been proposed to account for these HIV-related neurological deficits.

We measured glutamate concentrations by HPLC analysis (Pearson et al, 1991) and NMDA receptor density by saturation analysis of specific (^{3}H)L-689,560 binding to the glycine site (Reynolds et al, 1994), in brain tissue taken post-mortem from the frontal cortex of AIDS patients and age-matched controls. Glutamate concentrations were decreased in 20 AIDS patients (0.68±0.05μg/g tissue) compared to 5 control subjects (1.53±0.21μg/g tissue, p<0.001, by t-test). This deficit may reflect a loss of glutamatergic neurones in the disease process.

Receptor binding showed a significant decrease in Bmax values for the AIDS group when compared to control values, whilst Kd values were unchanged (table 1). This effect did not apparently differentiate demented and non-demented cases. Reduced NMDA receptor density may reflect a loss of the neurones on which these receptors are sited. Such a finding is consistent with an NMDA receptor mediated excitotoxic mechanism of neuronal death. Potential causative agents include the kynurenine-derived compounds quinolinic acid and 3-hydroxykynurenine both of which are increased in AIDS patients (Heyes et al, 1989; Sardar et al, 1995). Whilst the precise mechanism remains to be identified, the reduction in NMDA receptor density reported here is likely to be a reflection of HIV-associated neuronal damage.

Table 1. Binding with (^{3}H)L-689,560 to the NMDA receptor in the frontal cortex of AIDS patients.

	Bmax (fmol/mg tissue)	Kd (nM)
Control (n=7)	56.8±8.3	6.6±1.1
AIDS (n=17)	37.9±4.3*	5.7±0.7
Non-Dementia (n=8)	39.9±8.0	5.0±0.8
Dementia (n=9)	36.0±4.5*	6.1±1.0

Results are expressed as mean ± S.E.M. *p < 0.05 (t-test).

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47P FURTHER CHARACTERIZATION OF THE 5-HT RECEPTOR MEDIATING SPINAL MONOSYNAPTIC REFLEX DEPRESSION IN THE NEONATE RAT

N.A. Manuel* and D.I. Wallis, Physiology Unit, MOMED, UWCC, P.O. Box 911, Cardiff CF1 3US.

The spinal monosynaptic reflex (MSR) is subject to modulation by 5-hydroxytryptamine (5-HT) and 5-HT receptor agonists. Antagonist potencies against various 5-HT receptor agonists has led us to propose that a receptor similar to the 5-$HT_{1D\alpha}$ subtype is involved (Manuel et al., 1995). The present experiments attempt to locate the receptor using paired pulse testing and further define its nature.

Hemisected spinal cords from 4-8 day old Wistar rats were maintained *in vitro*. A lumbar (L3-L5) dorsal root was supramaximally stimulated (0.1ms, 0.067Hz) and the reflex recorded in the segmental ventral root via extracellular suction electrodes. For paired pulse testing, 2 pulses S1 and S2 were delivered with a 50ms interval between stimuli. Pairs of pulses were presented every 30 s.

Paired pulse testing was used to investigate the site of action of 5-HT (30μM, n=3) and the 5-$HT1_D$ agonist sumatriptan (15nM, n=3). Both these agents increased the S2:S1 ratio, suggesting an action at presynaptic sites (Yomono et al., 1992). Baclofen is known to act presynaptically in the spinal cord and also increased the S2:S1 ratio (0.3μM, n=3). Conversely, the glutamate antagonist CNQX did not increase the S2:S1 ratio, depressing both S1 and S2 similarly, consistent with a post synaptic action.

Sumatriptan, potently inhibited the MSR, EC_{50} 8nM [3-21nM], geometric mean [95% C.L.]. Isamoltane at 0.5μM should block 5-HT_{1B} but not 5-HT_{1D} receptors (Davidson & Stamford, 1995). In its presence the concentration response (CR) curve was unaffected, EC_{50} 7nM [2-23nM], n=5. Ritanserin (1μM) caused a dextral shift in the CR curve to sumatriptan, pA_2 7.48, consistent with its reported affinity for 5-$HT_{1D\alpha}$ sites (Weinshank et al., 1991). GR55562, a novel, selective antagonist of 5-HT_{1D} receptors (Connor et al., 1995) was a non-competitive antagonist, sumatriptan EC_{50} 13nM [10-17nM], sumatriptan + 3μM GR55562 EC_{50} 45nM [28-71nM], n=5. We confirm that 5-HT_3 and 5-HT_4 receptors do not account for 5-HT-induced depressions of the MSR, 5-HT EC_{50} 12μM [6-23μM], 5-HT + 1μM ondansetron EC_{50} 13μM [6-29μM] n=3. The selective 5-HT_4 antagonist GR113808 (Grossman et al., 1993) also had no effect, 5-HT EC_{50} 13μM [9-20μM], 5-HT + GR113808 EC_{50} 11μM [6-22μM], n=3.

The signal transduction pathway of 5-HT_1 receptors is thought to be mediated via a decrease in the second messenger cAMP. However, application of 10μM forskolin, an adenylate cyclase stimulator, had no effect on MSR depressions induced by 10μM 5-HT, n=3 or 15 nM sumatriptan, n=1. Similarly, 10μM MDL12330A, an inhibitor of adenylate cyclase (Correia-de-Sá & Ribeiro, 1994), had no effect on 5-HT-(10, 15, 30μM) or baclofen-(0.3μM) induced depressions of the MSR, all n=1.

These data further support the proposal that presynaptic 5-$HT_{1D\alpha}$ receptors mediate depression of the MSR by 5-HT receptor agonists, although applied 5-HT acts via different, uncharacterized receptor subtype(s) (see Manuel et al., 1995).

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48P EFFECT OF 14-DAY TREATMENT WITH NERVE GROWTH FACTOR (NGF) ON THE RELEASE OF SUBSTANCE P-LIKE IMMUNOREACTIVITY FROM RAT ISOLATED SPINAL CORD

M. Malcangio, N.E. Garrett, N.G. Bowery[1] & D.R. Tomlinson, Dept of Pharmacology, Queen Mary and Westfield College, Mile End Road, London, E1 4NS and [1] Dept of Pharmacology, The Medical School, Edgbaston, Birmingham B15 2TT

Nerve growth factor (NGF) is the prototype of a family of closely related polypeptide growth factors. Systemically injected NGF is retrogradely transported by sensory neurones. It has been previously shown that compensation for deficient trophic support in diabetic rats by NGF, prevented the loss of substance P (SP) in dorsal root ganglia (DRG) (Diemel *et al.*, 1994). This effect has been ascribed to NGF-induced stimulation of SP expression in DRG (Lindsay & Harmar, 1989).

This study determined whether 2 week-treatment with NGF could influence the basal outflow, electrically-evoked release and total content of SP from normal rat spinal cord slices *in vitro*. For this purpose rats were injected with human recombinant NGF (1 mg/kg s.c.; Genentech) three times a week for 2 weeks. Control rats were injected with saline. The last dose was injected 24 h before killing rats to remove 1 cm of sciatic nerve and the lumbar enlargement of the spinal cord. The sciatic nerve segments were frozen and then processed to extract SP in 1 ml boiling glacial acetic acid for 10 min. Samples were then homogenized, centrifuged and loaded through SEP-PAK C_{18} reverse phase silica gel cartridges to elute SP. SP-like immunoreactivity (SP-LI) content was determined by radioimmunoassay (RIA) (sensitivity 1 fmol/tube). The lumbar enlargement of the spinal cord was cut longitudinally with a Vibratome to obtain dorsal horn slices (300 μm) with dorsal roots attached (Malcangio & Bowery, 1993). Slices were mounted in the central compartment of a three-compartment chamber and perfused (1 ml/min, room temperature) with Krebs' solution containing protease inhibitors. Dorsal roots (two per side) were laid across two pairs of bipolar electrodes and immersed in mineral oil in the lateral compartments. 8ml-perfusates were collected as follows: 3 for basal outflow, 1 for stimulation (15 ± 2.2 mA for 8 mins) 3 for recovery and then desalinated by loading them through SEP-PAK C_{18} cartridges. At the end of each experiment SP-LI was extracted from spinal cord slices in acetic acid. SP-LI was determined by RIA.

Basal release of SP-LI from control spinal cords was 10.0±2.0 fmol/8 ml fraction (mean ± s.e. mean n=8). This increased by 12.8±1.9 fmol/8ml fraction over basal during electrical stimulation of the dorsal roots. Treatment with NGF for 2 weeks significantly increased the basal outflow of SP-LI to 20.5±1.7 fmol/8ml fraction (n=10 $P < 0.01$) and electrically-evoked release by 21.9±3.6 fmol/8 ml fraction over basal ($P < 0.05$). However the total SP-LI content of the cord was not significantly modified by NGF (80.0±13.6 and 115.2±29.9 fmol/mg tissue control and NGF, respectively) whereas the content of the sciatic nerve segments from NGF-treated rats was significantly increased by 55.0±16.4% over control (40.3±3.6 fmol/cm nerve).

Thus, chronic administration of NGF enhanced the basal and evoked release of SP-LI from rat spinal cord but not total content.

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49P EVIDENCE THAT PROSTAGLANDINS MAY MODULATE WIND-UP IN A SPINAL NOCICEPTIVE REFLEX IN ANAESTHETISED RATS

H.L. Willingale & B.D. Grubb (introduced by R.A.J. Challiss), Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN.

Stimulation of the sural nerve at an intensity sufficient to activate C-fibres elicits a nociceptive hindlimb withdrawal reflex. Repetitive stimulation of C-fibres at low frequency (<1.0 Hz) can enhance the magnitude of this reflex thereby inducing signs of spinal hyperexcitability (wind-up). Recent experiments (Malmberg & Yaksh, 1992; Neugebauer *et al*, 1994) have suggested a central site of action of non-steroidal anti-inflammatory drugs (NSAIDs) in spinal nociceptive pathways. In these experiments we have examined the effect of NSAIDs on the wind-up of the spinal nociceptive reflex in anaesthetised rats.

Adult male Wistar rats were anaesthetised with sodium thiobutabarbital (120-150 mg/kg, i.p., RBI) and tracheal, arterial and venous catheters were inserted. Blood pressure was maintained within normal limits (95-120 mmHg) and anaesthesia at a level sufficient to abolish reflex responses to corneal stimulation and noxious hindpaw pinch. A dorsal incision was made through the skin of the lower limb to reveal the underlying muscles. The right sural nerve was dissected close to the ankle joint, cut and placed on a pair of silver wire electrodes. C-fibre intensity stimuli were applied in trains of 20 (0.2-0.9 Hz) to evoke wind-up. A pair of electrodes was inserted into the biceps femoris muscle to record muscle activity. The number of action potentials elicited in the muscle fibre units by each stimulus was counted using a computerised data capture system. Intrathecal catheters were inserted in 5 rats through a partial laminectomy to allow intrathecal administration of drugs, given in a volume of 50 µl. P values were obtained using a 2-way anova to assess the effect of drug treatment on wind-up. The values given for the magnitude of the effect of drugs on the reflex were obtained by comparing the responses to the last stimulus.

C-fibre-intensity stimuli produced a delayed reflex response which exhibited significant wind-up upon repetitive stimulation ($p<0.001$). The size of the reflex increased throughout the train of 20 stimuli. Indomethacin (0.1-5 mg/kg, i.v., n=6) and the selective cyclooxygenase-2 (cox-2) inhibitor, SC 58125 (1-[(4-methylsulfonyl)phenyl]-3-trifluoro-methyl-5-(4-fluorophenyl)pyrazole; 1-10 mg/kg, i.v., n=5, gift from Glaxo Group Research), dose-dependently reduced the wind-up of this reflex ($p<0.001$). Indomethacin and SC 58125 reduced the size of the reflex by 55.9% and 73.5% respectively. The baseline reflex elicited by the first stimulus in each train was unaffected by these treatments except following the administration of 10 mg/kg SC 58125. In order to ascertain whether NSAIDs had a spinal site of action we applied indomethacin through the intrathecal catheter. Indomethacin (10-100 µM, i.t., n=5) dose-dependently reduced wind-up of the reflex ($p< 0.001$) by 38.0% without significantly affecting the magnitude of the baseline reflex ($p>0.05$).

These results suggest that NSAIDs have a spinal site of action which may involve inhibition of cox-2 and therefore prostaglandin synthesis. This provides evidence for a role of prostaglandins in neuronal hyperexcitability in the spinal cord of normal rats.

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50P AN ELECTROPHYISOLOGICAL STUDY OF 6-AZAWILLARDIINE DERIVATIVES ON MAMMALIAN SPINAL NEURONES

J.M. Pinkney, P. Ahluwalia, W.P. Kingston, P C-K. Pook,* D.E. Jane and *J.C. Watkins. School of Health Sciences, University of Sunderland, Sunderland, SR1 3SD. *Department of Pharmacology, University of Bristol, Bristol, BS8 1TD.

The potent agonist activity of (S)-willardiine and its 5-substituted analogues at the AMPA/kainate receptor, has been suggested to be due to the ionisation of the uracil ring (Patneau *et al*, 1992). The introduction of a nitrogen atom at position 6 of the uracil ring, to produce a 6-azawillardiine, has been reported to decrease the pKa and so increase ionisation (Jonas and Gut, 1961). A series of 6-azawillardiine analogues have been synthesised and ligand binding studies showed that they display an increased affinity for the AMPA receptor compared to their corresponding willardiine analogue (Hawkins *et al*, 1995).

The present study further investigates the affinity of this range of compounds on the isolated hemisected spinal cord of 1-5 day old female Sprague Dawley rats, in a medium containing 0.1µM tetrodotoxin. Recordings were made from a ventral root of motoneurones to excitatory amino acid agonists. Compounds (0.05µM-1mM, administered for 2 min) were tested for (a) their ability to cause depolarisation of motoneurones and (b) their selectivity for the AMPA/kainate group of receptors.

The (S) forms of the following 5-substituted-6-azawillardiine analogues depolarised motoneurones with equieffective molar concentrations (relative to (S)-AMPA=1) of 6-azawillardiine, 3.99 ± 0.15 (n=4);5-Chloro-, 0.07 ± 0.003 (n=7); 5-Bromo-, 0.08 ± 0.01 (n=6); 5-Iodo-, 0.15 ± 0.01 (n=6); 5-methyl-, 1.90 ± 0.13 (n=7), and also (R)-6-azawillardiine 3645.10 ± 126.79 (n=4) and (R,S)-5-iso-, 4.26 ± 0.38 (n=8). The depolarisations were sensitive to the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoine-2,3-dione (CNQX, 10µM), but not to the NMDA antagonists D-2-amino-5-phosphonopentanoate (D-AP5, 25µM) and $MgSO_4$ (2mM) suggesting that the compounds are selective for the AMPA/kainate group of receptors.

The azawillardiines display the same order of potency as that observed for the corresponding willardiines (Jane *et al*, 1991) and all are more potent than the willardiine analogues. This study provides further evidence for the importance of ionisation of the uracil ring in conferring potent agonist activity at the AMPA/kainate receptor and also indicates that the 6-azawillardiines may have an important role as a pharmacological tool in characterising these receptors.

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51P TACHYKININ RESPONSES IN DIFFERENT TYPES OF NEURONES OF GUINEA-PIG SUBSTANTIA NIGRA PARS COMPACTA *IN VITRO*

E. Nalivaiko, J.C. Michaud, P. Soubrié, G. Le Fur & P.Feltz[1], Department of Neuropsychiatry, Sanofi Recherche 371 rue de Professeur Blayac, 34184 Montpellier, France and [1]Laboratory of General Physiology, Louis Pasteur University, 21 avenue René Descartes, 67084 Strasbourg, France

Recently, a suggestion was made by Whitty *et al.* (1995) about the selective expression of different tachykinin receptors in different neuronal populations in the substantia nigra pars compacta (SNc), based on the results obtained by the mRNA hybridisation method. We have tested this hypothesis *in vitro* by studying the effects of selective tachykinin agonists and antagonists on the electrical activity of nigral neurones. Coronal slices containing SNc were prepared from the brains of 150-200 g male Hartley guinea pigs, as previously described (Nedergaard & Greenfield, 1992). A total of 79 neurones located in SNc were intracellularly recorded at 33-34°C with glass microelectrodes in a current clamp mode. Three types of neurones were electrophysiologically identified. Type I neurones (71% of total recorded cells) possessed properties which were typical of dopaminergic (DA) neurones while type II cells (11%) corresponded to non-DA, probably GABA-ergic interneurones (Yung *et al.*, 1991); cells of type III (16%) were similar to the neuronal groupe of unidentified biochemical nature, described in rostral SN (Nedergaard & Greenfield, 1992). In 23/26 tested type I neurones, the selective NK_3 receptor agonist senktide concentration-dependently increased the spontaneous firing rate (average maximal response (mean ± s.e.mean) from 1.7 ± 0.2 to 3.4 ± 0.7 Hz at the concentration of 30 nM; EC_{50} = 14.7 nM; n=6) while the selective NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$] Substance P and the NK_2 receptor agonist [Nle^{10}] NeurokininA (4-10) were without any effect (both up to 100 nM). Excitatory responses to senktide (30 nM) were reduced by 87.3±1.1% by the selective NK_3 antagonist SR 142801 (100 nM, 1 h; n=4), but affected neither by the selective NK-2 antagonist SR 48968 nor by the selective NK_1 antagonist SR 140333 (both at 100 nM, 1 h; n=3). Responses to senktide persisted in the low-Ca^{++} medium (n=3). As for the type II neurones, 6/9 tested cells were excited with [Sar^9,$Met(O_2)^{11}$]Substance P in a concentration-dependent manner (average response was from 14.3 ± 2.1 to 18 ± 3.2 Hz at the concentration of 100 nM; EC_{50}=41.2 nM; n=5); no effect of [Nle^{10}]Neurokinin A (4-10) was observed up to the concentration of 100 nM, while senktide (30-100 nM) excited only one type II cell. Responses to [Sar^9,$Met(O_2)^{11}$]Substance P (100 nM) were decreased by 75.7±4.9% by SR 140333 (100 nM, 1 h; n=4), but affected neither by SR 48968 nor by SR 142801 (both at 100 nM, 1 h; n=3). Concerning the type III neurones, no effect was observed in the 7 cells tested with the three NK agonists (all at 100 nM). Our results correlate well with the data concerning the localisation of tachykinin receptor mRNA in SNc (Whitty *et al.*, 1995), and confirm the selective presence of NK3 receptors on type I DA neurones and of NK1 receptors on type II non-DA cells, which may be GABA-ergic neurones.

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52P INHIBITION OF K^+-EVOKED [^{3}H]NORADRENALINE RELEASE AND Ca^{2+} CURRENTS BY ANGIOTENSIN II IN SH-SY5Y CELLS TRANSFECTED WITH THE RAT ANGIOTENSIN (AT_{1A}) RECEPTOR

P.F.T. Vaughan, R.L. McDonald & C. Peers, Institute for Cardiovascular Research, Leeds University, Leeds LS2 9JT.

Stimulation of muscarinic receptors evokes release of [^{3}H]noradrenaline ([^{3}H]NA) from human neuroblastoma (SH-SY5Y) cells (Murphy *et al.*, 1991), and also inhibits K^+-evoked [^{3}H]NA release via inhibition of voltage-gated Ca^{2+} channels (McDonald *et al.*, 1994). We have recently shown that angiotensin II (AII) also stimulates [^{3}H]NA release from SH-SY5Y cells stably expressing the recombinant rat AT_{1A} receptor (McDonald *et al.*, 1995a). Here, we have investigated whether AII modulates K^+-evoked [^{3}H]NA release and Ca^{2+} channel currents in SH-SY5Y cells.

Exposure of SH-SY5Y cells transfected with the rat AT_{1A} receptor to 100mM K^+ at 37°C evoked [^{3}H]NA release, measured as previously described (Murphy *et al.*, 1991). Mean release (with basal values subtracted) was 11.8±0.7% (mean ± s.e.mean) of total cellular ^{3}H content (n=6). Pre-treatment of cells for 4 min with AII (1-50nM) reduced K^+-evoked [^{3}H]NA release in a concentration-dependent manner (IC_{50} 2nM), with maximal inhibition of 67.3±4.8% (n=6) seen at a concentration of 10nM. Since K^+-evoked [^{3}H]NA release is dependent on Ca^{2+} influx through voltage-gated L- and N-type Ca^{2+} channels (McDonald *et al.*, 1994), we also investigated whether AII could inhibit such channels using whole-cell (conventional and perforated-patch) patch-clamp recordings. To enhance current amplitudes, 10mM Ba^{2+} was used as charge carrier (21-24°C; see McDonald *et al.*, 1995b, for further details). Currents were evoked by 200ms step depolarizations to 0mV applied at 0.2Hz from a holding potential of -80mV. Bath application of AII (3-300nM) reversibly inhibited currents. For example, at 100nM currents were inhibited by 59.4±5.5% (n=10 cells, $p<0.03$, paired Student's t-test). At concentrations > 10nM, responses to AII were often transient i.e. current amplitudes decreased then recovered partially during continued AII exposure. Ca^{2+} current inhibition was abolished by 2μM losartan (n=3) and in untransfected cells AII was without effect (n=4).

Our results indicate that recombinant rat AT_{1A} receptors couple functionally to the inhibition of Ca^{2+} channels, an effect which may account for the ability of AII to inhibit K^+-evoked [^{3}H]NA release in these cells.

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